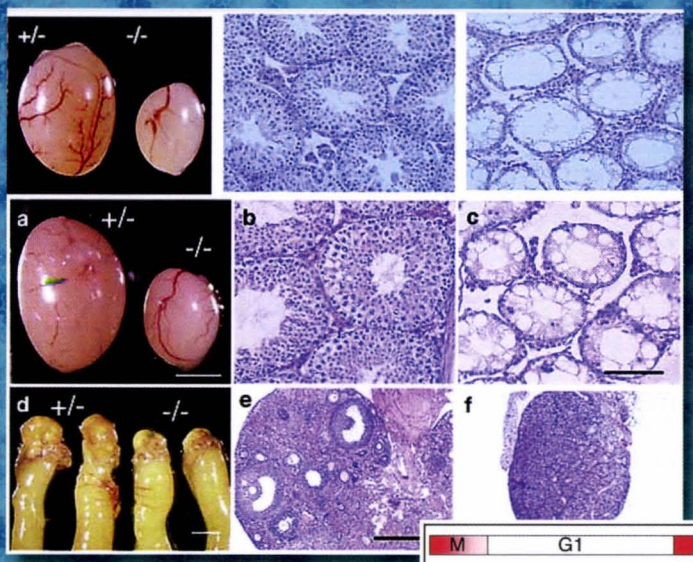
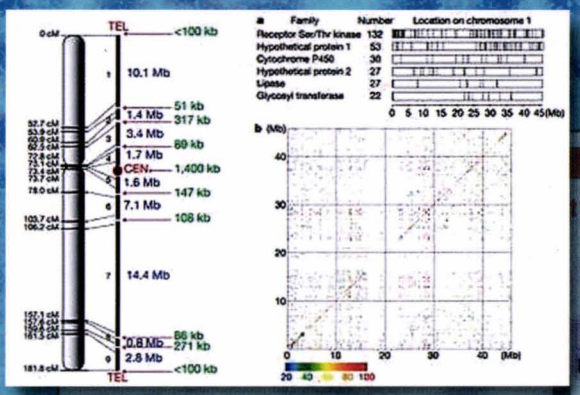
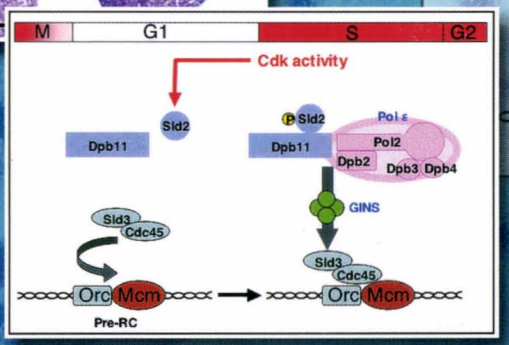
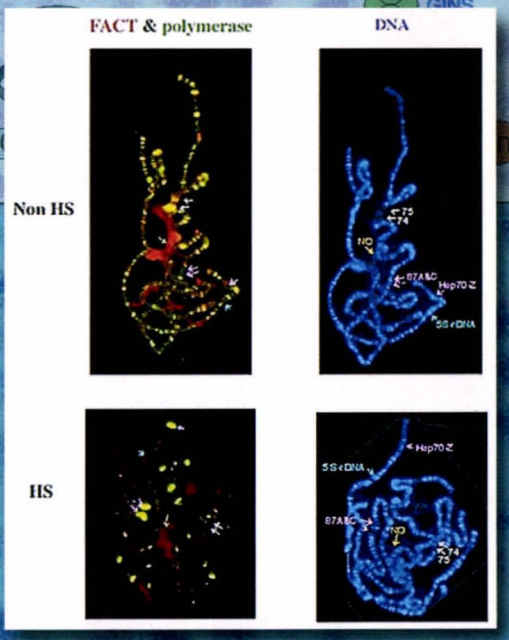
ANNUAL REPORT

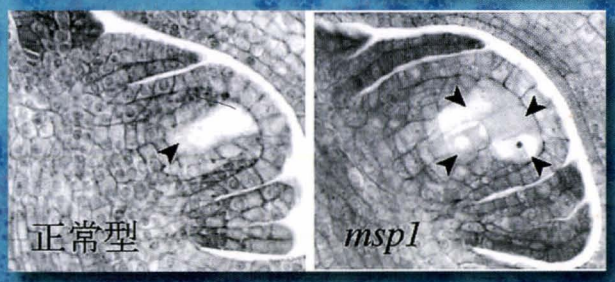
No.54
2003
ISSN 0077-4995







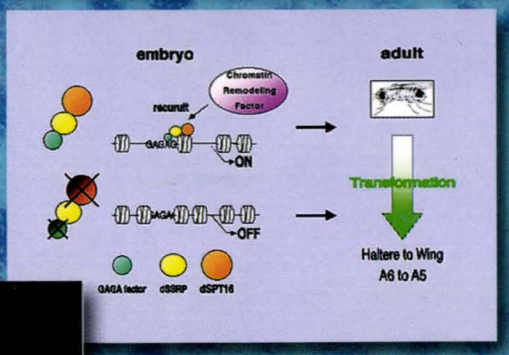




CG methylation
(by MET1)

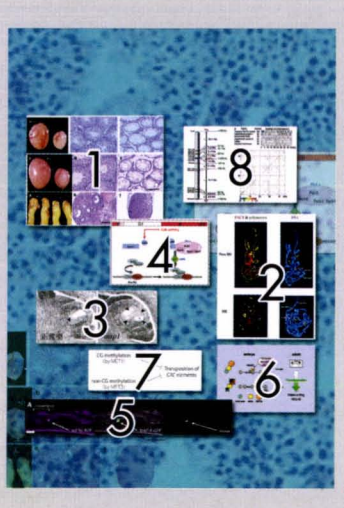
non-CG methylation
(by MET3)

Transposition of
CAC elements



Research Organization of Information and Systems
NATIONAL INSTITUTE OF GENETICS
http://www.nig.ac.jp

Highlights of 2003 (http://www.nig.ac.jp/hot/index.html)



1. **Conserved Role of nanos Proteins in Germ Cell Development.**
   Tsuda, M., Sasaoka, Y., Kiso, M.,Abe, K.,Haraguchi, S., Kobayashi, S. and Saga, Y.
   Science *301*, 1239-1241, 2003.

2. **Tracking FACT and the RNA polymerase II elongation complex through chromatin *in vivo*.**
   Saunders, A., Werner, J., Andrulis, E. D., Nakayama, T., Hirose, S., Reinberg, D. and Lis, J. T.
   Science *301*, 1094-1096, 2003.

3. **The *MSP1* gene is necessary to restrict the number of cells entering into male and female sporogenesis and to initiate anther wall formation in rice.**
   Nonomura, K., Miyoshi, K., Eiguchi, M., Suzuki, T., Miyao, A., Hirochika, H. and Kurata, N.
   Plant Cell *15*, 1728-1739, 2003.

4. **GINS, a novel multi-protein complex required for chromosomal DNA replication in budding yeast.**
   Takayama, Y., Kamimura, Y., Okawa, M., Muramatsu, S., Sugino, A. and Araki, H.
   Genes & Dev., *17*,1153-1165, 2003.
   A novel ring-like complex of Xenopus proteins essential for the initiation of DNA replication.
   Kubota, Y., Takase, Y., Komori, Y., Hashimoto, Y., Arata, T., Kamimura, Y., Araki, H. and Takasawa, H.
   Genes & Dev., *17*, 1141-1152, 2003.

5. **SDF-9, a protein tyrosine phosphatase-like molecule, regulates the L3/dauer developmental decision through hormonal signaling in *C. elegans*.**
   Ohkura, K., Suzuki, N., Ishihara, T. and Katsura, I.
   Development, *130*, 3237-3248, 2003.

6. ***Drosophila* FACT contributes to Hox gene expression through physical and functional interaction with GAGA factor.**
   Shimojima, T., Okada, M., Nakayama, T., Ueda, H., Okawa, K., Iwamatsu, A., Handa, H. and Hirose, S.
   Genes & Dev., *17*, 1605-1616, 2003.

7. **Role of CG and non-CG methylation in immobilization of transposons in *Arabidopsis*.**
   Kato, M., Miura, A., Bender, J., Jacobsen, S.E. and Kakutani, T.
   Curr. Biol., *13*, 421-426, 2003.

8. **The genome sequence and structure of rice chromosome 1.**
   Sasaki, T., Matsumoto, T., Yamamoto, K., Sakata, K., Baba, T., Katayose, Y., Wu, J., Niimura, Y., Cheng, Z., Nagamura, Y., Antonio, B. A., Kanamori, H., Hosokawa, S., Masukawa, M., Arikawa, K., Chiden, Y., Hayashi, M., Okamoto, M., Ando, T., Aoki, H., Arita, K., Hamada, M., Harada, C., Hijishita, S., Honda, M., Ichikawa, Y., Idonuma, A., Iijima, M., Ikeda, M., Ikeno, M., Ito, S., Ito, T., Ito, Y., Ito, Y., Iwabuchi, A., Kamiya, K., Karasawa, W., Katagiri, S., Kikuta, A., Kobayashi, N., Kono, I., Machita, K., Maehara, T., Mizuno, H., Mizubayashi, T., Mukai, Y., Nagasaki, H., Nakashima, M., Nakama, Y., Nakamichi, Y., Nakamura, M., Namiki, N., Negishi, M., Ohta, I., Ono, N., Saji, S., Sakai, K., Shibata, M., Shimokawa, T., Shomura, A., Song, J., Takazaki, Y., Terasawa, K., Tsuji, K., Waki, K., Yamagata, H., Yamane, H., Yoshiki, S., Yoshihara, R., Yukawa, K., Zhong, H., Iwama, H., Endo, T., Ito, H., Hahn J. H.,, Kim, H.-I., Eun, M.-Y., Yano, M., Jiang, J. and Gojobori, T.
   Nature, *420*, 312 – 316, 2002.

CONTENTS

Introduction

The paradigm of genetics is changing rapidly through the increasing rate of genome sequencings. It was symbolized by the completion of human genome project, to which our DDBJ (DNA Databank of Japan) contributed as one of the three database centers of the world. Our sequencing center is also producing genome data of various model organisms, including that of chordate. The ever-increasing accumulation of such genome-wide knowledge about many biological species, combined with the rapid progress in computer and information sciences, is revolutionizing the study of genetics. We are supporting DDBJ and its related research activities, as well as its educational programs in bioinformatics. Another power of NIG is in the research activities using many model organisms; they include phage, bacteria, yeast, nematode, Drosophila, zebra fish, medaka, mouse and man. Rice and Arabidopsis research groups are also very active. Less popular but increasingly important organisms studied here include planaria, hydra and chordate. The combination of such a variety of scientific research will provide us an additional impetus in the post-genome sequence era. In this sense, NIG has an advantage in the "scientific turmoil". NIG's mottoes are, "From Molecules to Individuals", "From Development to Evolution" and "From Experiment to Theory".

Japanese Diet passed a law to make a reform of national universities and institutes that belong to Ministry of Education, Culture, Science, Sports and Technology (MEXT). NIG is going to collaborate with other three institutes to form Research Organization of Information and Systems (ROIS) from April 2004. The unique functions of our institutes as inter-university collaboration system will remain as usual, but we would like to add a new function to create new scientific paradigm by the collaboration with institutes specialized for informatics. We believe that such a reorganization, although it was originally started as a part of national financial reform plan, will eventually prove to be the right decision to promote and contribute to the current revolution in life sciences.

Yoshiki Hotta, Director-General

STAFF (as of December 31, 2003)

Director-General
HOTTA, Yoshiki, D. Med.

Vice-Director
KOHARA, Yuji, D. Sc.

Members

1. Department of Molecular Genetics

SHIMAMOTO, Nobuo, D. Sc., Head of the Department

Division of Molecular Genetics

FUKAGAWA, Tatsuo, D.Sc., Associate Professor

Division of Mutagenesis

YAMAO, Fumiaki, D. Sc., Professor

Molecular Mechanism Laboratory

MITSUZAWA, Hiroshi, D. Sc.
SEINO, Hiroaki, D. Sc.

Division of Nucleic Acid Chemistry

AIMOTO, Saburo, D. Sc., Adjunct Professor
KATAYAMA, Tsutomu, D. Sc., Adjunct Professor

2. Department of Cell Genetics

ARAKI, Hiroyuki, D. Sc., Head of the Department

Division of Cytogenetics

YOSHIMORI, Tamotsu, D. Med., Professor
UMEBAYASHI, Kyohei, D. Ag.

Division of Microbial Genetics

ARAKI, Hiroyuki, D. Sc., Professor
YASUDA, Seiichi, D. Sc., Associate Professor
KAMIMURA, Yoichiro, D. Med.
TANAKA, Seiji, D. Sc.

Division of Cytoplasmic Genetics

YOSHIKAWA, Takeo, D. Med., Adjunct Professor
YANAGIDA, Mitsuhiro, D. Sc., Professor (Adjunct)

3. Department of Developmental Genetics

HIROSE, Susumu, D. Sc., Head of the Department

Division of Developmental Genetics

HIROMI, Yasushi, D. Sc., Professor
FUJISAWA, Toshitaka, Ph. D., Associate Professor
SHIMIZU, Hiroshi, D. Eng.
OKABE, Masataka, M. D., Ph. D.
ASAOKA, Miho, D. Sc.

Division of Gene Expression

HIROSE, Susumu, D. Sc., Professor
UEDA, Hitoshi, D. Ag., Associate Professor
YAMADA, Masa-aki, D. Ag.

NISHIOKA, Kenichi, D. Med.

Division of Molecular and Developmental Biology

KAWAKAMI, Koichi, D. Sc., Associate Professor

KISHIMOTO, Yasuyuki, D. Sc.

Division of Physiological Genetics

KOSEKI, Haruhiko, D. Med., Professor (Adjunct)

GOTOH, Yukiko, Associate Professor(Adjunct)

4. Department of Population Genetics

IKEMURA, Toshimichi, D. Sc., Head of the Department

Division of Population Genetics

SAITOU, Naruya, Ph. D., Professor

TAKANO, Toshiyuki, D. Sc., Associate Professor

SUMIYAMA, Kenta, D. Sc.

TAKAHASHI, Aya, D. Ag.

Division of Evolutionary Gentics

IKEMURA, Toshimichi, D. Sc., Professor

Division of Theoretical Genetics

FUJIYAMA, Asao, D. Sc., Professor(Adjunct)

TAKAGI, Toshihisa, D. Eng., Professor (Adjunct)

5. Department of Integrated Genetics

SASAKI, Hiroyuki, D. Med., Head of the Department

Division of Human Genetics

SASAKI, Hiroyuki, D. Med., Professor

SADO, Takashi, D. Sc.

HATA, Kenichiro, D. Med.

Division of Agricultural Genetics

KAKUTANI, Tetsuji, D. Sc., Associate Professor

SHIBAHARA, Kei-ichi, M. D., Ph. D., Associate Professor

KINOSHITA, Tetsu, D. Sc.

OGAWA, Yuya, Ph. D.

Division of Brain Fanction

HIRATA, Tatsumi, D. Med., Associate Professor

KAWASAKI, Takahiko, D. Sc.

Division of Applied Genetics

HIROCHIKA, Hirohiko, D. Sc., Adjunct Professor

SHIOTA, Kunio, D. Ag., Professor(Adjunct)

6. Genetic Strains Research Center

SHIROISHI, Toshihiko, D. Sc., Head of the Center

Mammalian Genetics Laboratory

SHIROISHI, Toshihiko, D. Sc., Professor

TAMURA, Masaru, D. Sc.

Mammalian Development Laboratory

SAGA, Yumiko, D. Sc., Professor

KOKUBO, Hiroki, D. Sc.

MITSUI, Kaoru, D. Med.

Mouse Genomics Resource Laboratory
KOIDE, Tsuyoshi, D. Med., Associate Professor
Model Fish Genomics Resource Laboratory
Plant Genetics Laboratory
KURATA, Nori, D. Ag., Professor
ITO, Yukihiro, D. Ag.
Microbial Genetics Laboratory
NISHIMURA, Akiko, D. Ag., Professor
Invertebrate Genetics Laboratory
UEDA, Ryu, D. Sc., Professor
TAKAHASHI, Kuniaki, D. Sc.
Laboratory for Frontier Research
ISSHIKI, Takako, D. Sc., Associate Professor
KUSANO, Ayumi, D. Sc.

7. Center for Genetic Resource Information
NISHIMURA, Akiko, D. Ag., Head of the Center
Genetic Informatics Laboratory
YAMAZAKI, Yukiko, D. Sc., Associate Professor
FUJITA, Masaya, D. Eng.
Genome Biology Laboratory
KOHARA, Yuji, D. Sc., Professor
ANDACHI, Yoshiki, D. Sc.
Publicty and Intellectual Property Unit
TOMIKAWA, Munehiro, D. Pharm, Professor

8. Structural Biology Center
KATSURA, Isao, D. Sc., Head of the Center
Biological Macromolecules Laboratory
TOKUNAGA, Makio, D. Sc., Professor
SHIINA, Nobuyuki, D. Sc.
Molecular Biomechanism Laboratory
SHIMAMOTO, Nobuo, D. Sc., Professor
NAKAYAMA, Hideki, D. Eng.
Multicelluar Organization Laboratory
KATSURA, Isao, D. Sc., Professor
ISHIHARA, Takeshi, D. Sc.
KIMURA, Koutarou, D. Ag.
Biomolecular Structure Laboratory
SHIRAKIHARA, Yasuo, D. Sc., Associate Professor
Gene Network Laboratory

9. Center for Information Biology and DNA Data Bank of Japan
GOJOBORI, Takashi, D. Sc., Head of the Center
Laboratory for DNA Data Analysis
GOJOBORI, Takashi, D. Sc., Professor
IKEO, Kazuho, D. Sc., Associate Professor
SUZUKI, Yoshiyuki, M. D., Ph. D.

Laboratory for Gene-Product Informatics
NISHIKAWA, Ken, D. Sc., Professor
FUKUCHI, Satoshi, D. Sc.
KINJO, Akira, D. Sc.
Laboratory for Gene Function Research
TATENO, Yoshio, Ph. D., D. Sc., Professor
Laboratory for the Research and Development of Biological Databases
SUGAWARA, Hideaki, D. Eng., Professor
MIYAZAKI, Satoru, D. Sc., Associate Professor
Gene-Expression Analysis
OKUBO, Kousaku, M. D., Ph. D., Professor
ITOH, Koichi, M. D., Ph. D.

10. Radioisotope Center
NIKI, Hironori, D. Med., Associate Professor Head of the Center
OGATA, Yasuyuki, D. Pharm. Sci.

11. Experimental Farm
KURATA, Nori, D. Ag., Head of the Farm
NONOMURA, Ken-ichi, D. Ag.

12. Technical Section
ISHII, Yuriko, Chief of the Section

13. Department of Administration
ISHIKAWA, Kenji, Head of the Department
ISHIDA, Yuzo, Chief of the General Affairs Section
KAWAGUCHI, Kenji, Chief of the Finance Section

COUNCIL (as of December 31, 2003)

Chairman

SUGIMURA, Takashi; President Emeritus, National Cancer Center

Vice-chairman

OSAWA, Shozo; Professor Emeritus, The University of Nagoya

Members (Alphabetical order)

HIROBE, Masaaki; President,University of Shizuoka
ISHII, Shiro; Vice-Derector, Japan Society for the Promotion of Science, Research Center for Science System
IWATSUKI, Kunio; Professor, University of the Air
KATSUKI, Motoya; Director-General, National Institute for Basic Biology
KODAIRA, Keiichi; President, The Graduate University for Advanced Studies
KURODA, Reiko; Professor, Graduate School of Arts and Science, The University of Tokyo
MATSUBARA, Ken-ichi; President, DNA Chip Research Inc.
MATSUO, Minoru; President, Nagoya University
MIURA, Kin-ichiro; President, Proteios Research Inc.
OKADA, Masukichi; Vice-Director, International Institute for Advanced Studies
OSAKI, Hitoshi; Director-General, Center for National University Finance
OTSUKA, Eiko; Fellow, National Institute of Advanced Industrial Science and Technology
SASAKI, Kazuo; President, Okazaki National Research Institutes
SHINJI, Isoya; President, Tokyo University of Agiculture
TOYOSHIMA, Kumao; Director-General, SNP Reserch Center, The Institute of Physical and Chemical Research
TSUNEWAKI, Koichiro; President, Fukui Prefectural University
YAMANOUCHI, Kazuya; Senior Scientific Staff, Nippon Institute for Biological Science

ADVISORY COMMITTEE (as of December 31, 2003)

Chairman

KOHARA, Yuji; Professor, National Insutitute of Genetics

Vice-chairman

SEKIGUCHI, Mutsuo; Director, Biomolecular Engineering Research Institute

Outside Members (Alphabetical order)

GO, Michiko; Dean, Faculty of Bioscience, Nagahama Institute of Bio-Science and Technology
HANAOKA, Fumio; Professor, Graduate School of Frontier Biosciences, Osaka University
ISONO, Katsumi; Director, National Institute of Technology and Evaluation
ITO, Koreaki: Professor, Institute for Virus Research, Kyoto University
MATUURA, Etsuko; Professor, Faculty of Science, Ochanomizu University
OGAWA, Tomoko; Vice-President, Iwate College of Nursing
SASAZUKI, Takehiko; Director-General, International Medical Center of Japan Research Institute
SHINOZAKI, Kazuo; Chief Scientist, RIKEN Tsukuba Institute
TAJIMA, Fumio; Professor, Graduate School of Science, The University of Tokyo

Inside Menbers (Alphabetical order)

ARAKI, Hiroyuki; Professor, National Insutitute of Genetics
GOJOBORI, Takashi; Professor, National Insutitute of Genetics
HIROMI, Yasushi; Professor, National Insutitute of Genetics
HIROSE, Susumu; Professor, National Insutitute of Genetics
IKEMURA, Toshimichi; Professor, National Insutitute of Genetics
KATSURA, Isao; Professor, National Insutitute of Genetics
NISHIKAWA, Ken; Professor, National Institute of Genetics
SASAKI, Hiroyuki; Professor, National Insutitute of Genetics
SHIMAMOTO, Nobuo; Professor, National Insutitute of Genetics
SHIROISHI, Toshihiko; Professor, National Insutitute of Genetics

A. DEPARTMENT OF MOLECULAR GENETICS

A-a. Division of Molecular Genetics Tatsuo Fukagawa Group

RESEARCH ACTIVITIES

(1) Assembly of kinetochores in higher vertebrate cells

Yoshikazu Mikami, Tetsuya Hori, Mitsuko Yoshikawa, Atsushi Fukushima and Tatsuo Fukagawa

The centromere plays a fundamental role in accurate chromosome segregation during mitosis and meiosis in eukaryotes. Its functions include sister chromatid adhesion and separation, microtubule attachment, chromosome movement and mitotic checkpoint control. Although chromosome segregation errors cause genetic diseases including some cancers, the mechanism by which centromeres interact with microtubules of the spindle apparatus during cell division is not fully understood.

To understand the function of the centromere, we were led to develop a genetic analysis method that utilizes the hyper-recombinogenic chicken B lymphocyte cell line DT40. The high level of homologous recombination in DT40 cells permit efficient targeted disruption of genes of interest. We also generated human artificial mini-chromosomes using the telomere-directed breakage method in DT40 cells. We have combined human artificial mini-chromosomes and DT40 cells to study chromosome segregation. We have improved this system and have created several cell lines with conditional knockouts of several centromere proteins to investigate the molecular mechanism of centromere assembly and function. In this year we focused on analysis of CENP-H (Fukagawa et al., EMBO J., 2001; Nishihashi et al., Dev. Cell, 2002). We identified functional domain of CENP-H using a CENP-H-deficient cells. Yeast two-hybrid analysis with the CENP-H functional domain revealed that CENP-H interacts with Hec1, which transiently associate with centromeres during mitosis. Hec1 also interact with Nuf2. We carried out several analyses concerning interaction between CENP-H and the Nuf2-Hec1 complex. We then propose that the Nuf2-Hec1 complex may act to connect between the outer and inner kinetochores.

(2) Molecular analysis of the Nuf2-Hec1 complex that transiently localizes to centromere during mitosis

Tetsuya Hori, Atsuto Uenoyama, Kazuko Suzuki and Tatsuo Fukagawa

Nuf2 and Hec1 are evolutionarily conserved centromere proteins. To clarify the functions of these proteins in vertebrate cells, we characterized them in chicken DT40 cells. We generated GFP fusion constructs of Nuf2 and Hec1 to examine in detail localization of these proteins during the cell cycle. We found that Nuf2 is associated with Hec1 throughout the cell cycle and that this complex is localized to the centrosomes during G1 and S phases and then moves through the nuclear membrane to the centromere in G2 phase. During mitosis, this complex is localized to the centromere. We also created conditional loss-of-function mutants of Nuf2 and Hec1. In both mutants, the cell cycle arrested at prometaphase, suggesting that the Nuf2-Hec1 complex is essential for mitotic progression. The inner centromere proteins CENP-A, -C, and -H and checkpoint protein BubR1 were localized to chromosomes in the mutant cells arrested at prometaphase, but Mad2 localization was abolished. Furthermore, photobleaching experiments revealed that the Nuf2-Hec1 complex is associated stably with the centromere and that interaction of this complex with the centrosome is dynamic. We also took the proteomics approach to identify proteins that interact with the Nuf2-Hec1 complex. We identified several components that localizes centrosome and centromere and are now characterizing these components.

(3) Functional roles of the RNAi machinery in verterbrate centromeres

Masahiro Nogami, Tomoko Motohashi, Mitsuko Yoshikawa and Tatsuo Fukagawa

RNAi-mediated silencing of gene expression occurs

when double-stranded RNAs (dsRNAs) are cleaved by Dicer into 21- to 23-nt small interfering RNAs (siRNAs). These siRNAs guide a multicomponent nuclease, RNA-induced silencing complex (RISC), to degrade specific mRNAs. Although the Dicer-mediated gene-silencing is evolutionarily conserved system, the biological functions of the RNAi machinery are not fully understood. Genetic strategies have been used to examine the biological functions of the RNAi machinery in *C. elegans, Arabidopsis, Drosophila* and fungi. There are reports that the RNAi machinery is related to chromosome segregation in fission yeast. However, it is unclear whether the RNAi machinery is associated with chromosome segregation in vertebrate cells. To examine the biological function of the RNAi-related pathway in vertebrate cells, we generated a conditional loss-of-function mutant of Dicer in a chicken-human hybrid DT40 cell line that contains human chromosome 21. Loss of Dicer leads to cell death with accumulation of abnormal mitotic cells that show premature sister chromatid separation. Aberrant accumulation of transcripts from α satellite sequences, which consist of human centromeric repeat DNAs, was detected in Dicer-deficient cells. Cytological analysis revealed abnormalties in localization of heterochromatin proteins, but core kinetochore proteins such as CENP-A and -C were normal. We conclude that Dicer-related RNAi machinery is involved in formation of the heterochromatin structure in higher vertebrate cells.

Publications

Papers

1. Hori, T., Haraguchi, T., Hiraoka, Y., Kimura, H. and Fukagawa, T. (2003). Dynamic behavior of Nuf2-Hec1 complex that localizes to the centrosome and centromere and is essential for mitotic progression in vertebrate cells. J. Cell Sci. **116**, 3347-3362.

2. Regnier, V., Novelli, J., Fukagawa, T., Vagnarelli, P. and Brown, W. (2003). Characterization of chicken CENP-A and comparative sequence analysis of vertebrate centromere-specific histone H3-like proteins. Gene **316**, 39-46.

EDUCATION

1. Dr. Fukagawa gave several lectures at The Graduate University for Advanced Studies, Hayama (in Japanese).

2. Dr. Fukagawa was invited a seminar on "Centromere Assembly" at Kyushu University, Fukuoka, August, 2003 (in Japanese).

A-b. Division of Mutagenesis Fumiaki Yamao Group

RESEARCH ACTIVITIES

(1) Ubiquitin Pathway Regulating Recombination and DNA Damage Repair in Fission Yeast.

Joon-Hyun Park, Toyoaki Natsume and Fumiaki Yamao

In appropriate or unregulated homologous recombination can have deleterious effects and lead to genomic instability. In diploid eukaryotes, homologous recombination is essential during meiosis, but it is suppressed during mitosis by an as yet unknown mechanism. We identified and characterized Ubc7, one of the 14 ubiquitin-conjugating enzymes in *Schizosaccharomyces pombe*. Mutants defective in Ubc7 have enhanced mitotic recombination between tandem-repeated segments at *mat1* or *ade6*. The increased mitotic recombination was observed only in *ubc7*$^-$ cells which are competent for DSB-induced homologous recombination. Furthermore, Ubc7 mutants are hyper-resistant to UV radiation and MMS-induced DNA damage in cells carrying wild type Rhp51. Genetic recombination is a two-step reaction in eukaryotes; the initiation step establishes competence for homologous recombination and DNA exchange, while the second step mediates the recombination itself. This study demonstrates that Ubc7 negatively regulates the latter step during mitosis in *S. pombe*.

Publications

Papers

Seino, H., T. Kishi,. H. Nishitani. and F. Yamao. (2003). Two Ubiquitin-Conjugating Enzymes, UbcP1/Ubc4 and UbcP4/Ubc11, Have Distinct Functions for Ubiquitination of Mitotic Cyclin. Mol Cell Biol **23**, 3497-505

EDUCATION

1. Dr. Yamao was invited to give a seminar on "Ubiquitin Regulates Recombination and DNA Damage Repair" at Virus Institute of Kyoto University, June 2003 (in Japanese).
2. Dr. Yamao gave a lecture at Civil Education Course of Numazu City, July 2003 (in Japanese)
3. Dr. Yamao gave a lecture at Department of Veterinary Medicine, Yamaguchi University, December 2003 (in Japanese).

A-c. Molecular Mechanism Laboratory Hiroshi Mitsuzawa Group

RESEARCH ACTIVITIES

(1) Identification of proteins that interact with the Rpb7 subunit of RNA polymerase II

Hiroshi Mitsuzawa

RNA polymerase II (pol II) in eukaryotes, the enzyme responsible for synthesis of all mRNAs and some of small nucleolar and small nuclear RNAs, comprises twelve subunits, Rpb1 to Rpb12. Two of them, Rpb4 and Rpb7, form a heterodimer dissociable from pol II core. The Rpb4-Rpb7 heterodimer is dispensable for RNA synthesis but required for transcription initiation. In the budding yeast *Saccharomyces cerevisiae*, deletion mutants of Rpb4 are viable under optimal growth conditions, and growth defects of the mutants are suppressed by overexpression of Rpb7. In addition, the high level of Rpb7 allows its interaction with the pol II core in the absence of Rpb4. These results suggest that Rpb7 is the critical component of the Rpb4-Rpb7 heterodimer.

To gain insight into the function of the Rpb7 subunit of pol II, we performed a two-hybrid screen for Rpb7-interacting proteins using the fission yeast *Schizosaccharomyces pombe* as a model organism.[1] The screen identified a protein (named Seb1 for seven binding) that is 28% identical to *S. cerevisiae* Nrd1, an RNA-binding protein implicated in 3'-end formation of small nucleolar and small nuclear RNAs transcribed by pol II. Conservation of functional domains between Seb1 and Nrd1 suggests that *S. pombe* Seb1 is involved in processing of pol II transcripts as in the case of *S. cerevisiae* Nrd1. Further support for the functional conservation came from the observation that Nrd1 interacted with Rpb7. We also found, through site-directed mutagenesis, that the Glu-166 and/or Asp-167 of *S. pombe* Rpb7, residues near the C-terminus of the 172-amino acid protein, were important for its interaction with Seb1.

X-ray crystal structure of the 12-subunit pol II (the 10-subunit core plus Rpb4-Rpb7) was reported in 2003 by two groups. In the pol II structure, Rpb4-Rpb7 is located in the vicinity of the linker to the CTD, with which RNA processing factors are known to associate. Because the CTD is likely to be mobile, the interaction of Seb1 with Rpb7 as well as the CTD may function to anchor the putative processing factor to the pol II apparatus, thereby processing machinery on the CTD becomes close proximity to nascent RNA transcript. In summary, our results, together with the recent structural data, suggest that the Rpb7 subunit of pol II has a role in coupling RNA processing to transcription in addition to in initiating transcription.

Publications

Papers

Mitsuzawa, H., Kanda, E. and Ishihama, A. (2003). Rpb7 subunit of RNA polymerase II interacts with an RNA-binding protein involved in processing of transcripts. Nucleic Acids Res. **31**, 4696-4701.

A-c. Molecular Mechanism Laboratory Hiroaki Seino Group

RESEARCH ACTIVITIES

(1) Ubiquitin-conjugating enzymes involved in polyubiquitination of mitotic cyclin

Hiroaki Seino

Cell cycle events are regulated by sequential activation and inactivation of Cdk kinases. Mitotic exit is accomplished by the inactivation of mitotic Cdk kinase, which is mainly achieved by degradation of cyclins. The ubiquitin-proteasome system is involved in this process, requiring APC/C (anaphase-promoting complex/cyclosome) as a ubiquitin ligase. In Xenopus and clam oocytes, the ubiquitin-conjugating enzymes

that function with APC/C have been identified as two proteins, UBC4 and UBCx/E2-C. Previously we reported that the fission yeast ubiquitin-conjugating enzyme UbcP4/Ubc11, a homologue of UBCx/E2-C, is required for mitotic transition. Here we show that the other fission yeast ubiquitin-conjugating enzyme, UbcP1/Ubc4, which is homologous to UBC4, is also required for mitotic transition in the same manner as UbcP4/Ubc11. Both ubiquitin-conjugating enzymes are essential for cell division and directly required for the degradation of mitotic cyclin Cdc13. They function nonredundantly in the ubiquitination of Cdc13 because a defect in ubcP1/ubc4$^+$ cannot be suppressed by high expression of UbcP4/Ubc11 and a defect in ubcP4/ubc11$^+$ cannot be suppressed by high expression of UbcP1/Ubc4. In vivo analysis of the ubiquitinated state of Cdc13 shows that the ubiquitin chains on Cdc13 were short in ubcP1/ubc4 mutant cells while ubiquitinated Cdc13 was totally reduced in ubcP4/ubc11 mutant cells. Taken together, these results indicate that the two ubiquitin-conjugating enzymes play distinct and essential roles in the degradation of mitotic cyclin Cdc13, with the UbcP4/Ubc11-pathway initiating ubiquitination of Cdc13 and the UbcP1/Ubc4-pathway elongating the short ubiquitin chains on Cdc13[1].

Publications

Papers

Seino, H., Kishi, T., Nishitani, H. and Yamao, F. (2003) Two Ubiquitin-Conjugating Enzymes, UbcP1/Ubc4 and UbcP4/Ubc11, Have Distinct Functions for Ubiquitination of Mitotic Cyclin. Mol Cell Biol **23**, 3497-3505.

EDUCATION

Dr. H. Seino was invited to gave a seminar on "The regulatory mechanism of cell cycle by ubiquitination" at the National Institute for Basic Biology, Okazaki, June, 2003 (in Japanese)

A-d. Division of Nucleic Acid Chemistry Saburo Aimoto Group

RESEARCH ACTIVITIES

(1) Development of a strategy that enables to use the thioester method and the native chemical ligation method for the synthesis of a single protein

Saburo Aimoto (Institute for Protein Research, Osaka University)

A thioester method and the native chemical ligation method are useful for polypeptide synthesis. Though both methods use peptide thioesters as building blocks, no route was known to use the thioester method after the peptide bond formation reaction by the native chemical ligation method. In the thioester method, the SH groups of the cysteine residues have to be protected by a group that is stable in the presence of silver ions as an activator of thioester groups. On the other hand, the native chemical ligation method produces SH groups at the condensation site for chemoselective ligation is carried out at-X-Cys- site using the cysteine residue at the N-terminal of the C-terminal building block. In order to overcome this problem, we searched a protectiong group that is easily introduced to free SH groups in polypeptide under mild conditions and stable in the presence of silver ions. The protecting group we found was a thiosulfonate group. This group was quite easily introduced to SH groups in polypeptide only by mixing with $Na_2S_4O_6$. The resulting S-SSO_3^- was stable toward silver ions. Hence this protecting group was ideal for SH protection produced by the native chemical ligation. In conclusion, the use of SSO_3^- make it possible to employ the native chemical ligation method and thioester method for the synthesis of a single polypep-tide.[3]

(2) Development of a photoremovable ligation auxiliary for an extended native chemical ligation method

Saburo Aimoto (Institute for Protein Research, Osaka University)

Most of synthetic strategies in peptide chemistry use acid for the removal of protecting groups to obtain free peptides. This procedure is true in extended native

chemical ligation strategies, too. The ligation auxiliary groups are removed by acid treatment at the final stage of synthesis. The acids frequently used for this purpose are, for example, trifluoroacetic acid or anhydrous hydrogen fluoride. This constitutes the restriction in synthesis of peptides with acid−labile modifications and also in the development of synthetic methodologies in which locally folded peptide segments are used as building blocks. To develop a procedure to remove auxiliary groups under mild conditions, we designed a photoremovable ligation auxiliary group, 2-mercapto-1-(2-nitrophenyl)ethyl group, based on the frame work developed Dawson et. al. (J. Offer, P. E. Dawson, *Org Lett.*, 2, 23-6 (2000)). A model synthetic experiment using two decapeptides as building blocks showed that ligation proceeded and the photoremoval of the ligation auxiliary group proceeded under the irradiation of 365 nm light. Each reaction step is now under optimization.[2)]

(3) Synthetic studies of G protein-coupled receptor, opioid receptor like-1 (ORL-1)

Saburo Aimoto (Institute for Protein Research, Osaka University)

About 30% of the human genome encodes membrane proteins. Among them G protein-coupled receptor family is the largest family of membrane receptor proteins and is the target of most pharmaceuticals. Much of the information concerning the structure and function of these membrane proteins, however, remains to be uncovered because of the difficulties associated with biochemical sample preparation. As an alternative approach to obtaining membrane proteins, chemical synthesis represents a viable candidate. ORL-1 is a receptor of an opioid peptide, nociceptin. The strategy that is employed for the synthesis of the C-terminal region of ORL-1 is the combination of the native chemical ligation method and the thioester method. The first coupling between ORL-1(288-328) and ORL-1(329-370) was carried out in SDS solution by the native chemical ligation method. The coupling reaction has been optimized to give a desired product. The second coupling was carried out by the thioester method to give ORL-1(288-270). We are continuously searching the route to accomplish the total synthesis of ORL-1.

(4) Design of the inhibitors to human T-cell leukemia virus type-1 protease

Saburo Aimoto (Institute for Protein Research, Osaka University)

Human T-cell leukemia virus type 1 (HTLV-1), a retrovirus associated with a number of human diseases, was the first human retrovirus isolated from patients with adult T-cell leukemia/ lymphoma by Gallo et al. An HTLV-1 gene codes an asparatic protease (PR), which processes its own polyproteins. As the result of a series of cis processing, a set of proteins is produced, which are necessary for viral replication. Thus, HTLV-1 PR plays a key role in the duplication of HTLV-1 in a manner analogous to the human immunodeficiency virus type-1 protease in acquired immunodeficiency syndrome. In the design potent protease inhibitors for this virus, the knowledge of the characteristics of HTLV-1 PR itself and its substrate specificities is critical. Then, we synthesized HTLV-1 PR and examined its substrate specificities. Based on the obtained date we designed HTLV-1 protease inhibitors containing hydroxyethylamine dipeptide isostere.[1)]

Publications

Papers

1. Akaji, K., Teruya, K. and Aimoto, S. (2003). Solid-phase synthesis of HTLV-1 protease inhibitors containing hydroxyethylamine dipeptide isostere. J. Org. Chem., **68**, 4755-4763.
2. Kawakami, T. and Aimoto, S. (2003). A photoremovable ligation auxiliary for use in polypeptide synthesis. Tetrahedron Lett., **44**, 6059-6061.
3. Sato, T. and Aimoto, S. (2003). Use of thiosulfonate for the protection of thiol groups in peptide ligation by the thioester method. Tetrahedron Lett. **44**, 8085-8087.

Reviews

4. 相本三郎(2003). タンパク質化学合成の現状と将来, 21世紀の化学の潮流を探るNo.23, 全合成, 日本化学会.

Books

5. 相本三郎(2003). タンパク質化学合成の現状と将来, 先端科学シリーズV. 日本化学会編. (東京：丸善), pp.321-325.

EDUCATION

1. Dr. S. Aimoto was invited to give a lecture at Seoul National University, Korea, November, 2003.
2. Dr. S. Aimoto was invited to give a seminar on "Perspective on chemical synthesis of protein" at the annual meeting of the Japanese Chemical Society, Tokyo, April, 2003 (in Japanese).
3. Dr. Aimoto, S. was invited to give a seminar on "The history of chemical synthesis of proteins" at a forum of the Japanese Peptide Society, Okayama, November, 2003 (in Japanese).

SOCIAL CONTRIBUTIONS AND OTHERS

1. Dr. S. Aimoto organized a symposium entitlted "Perspective on the Peptide and Protein Preparation", Osaka, November, 2003. (in Japanese).
2. Dr. S. Aimoto, director of the Japanese Peptide Society(日本ペプチド学会理事)

A-d. Division of Nucleic Acid Chemistry Tsutomu Katayama Group

RESEARCH ACTIVITIES

(1) Transcriptional control for initiation of chromosomal replication in *Escherichia coli:* fluctuation of origin transcription ensures timely initiation[1]

Masayuki Su'etsugu, Akiko Emoto, Kazuyuki Fujimitsu, Kenji Keyamura and Tsutomu Katayama

During the cell cycle, the initiation of chromosomal replication is carefully controlled. In *E. coli,* the initiator DnaA and the replication origin *oriC* are major targets for this regulation. Here, we assessed the role of transcription of the *mioC* gene, which reads through the adjacent *oriC* region. This *mioC-oriC* transcription is regulated in a replication cycle-coordinated manner to be activated after initiation and to be repressed before initiation. We for the first time isolated a strain bearing a *mioC* promoter mutation that causes constitutive *mioC-oriC* transcription on the chromosome. In this mutant fluctuations of the transcription were abolished. Introduction of this mutation suppressed the growth defect of an overinitiation-type *dnaA*cos mutant, and severely inhibited growth of initiation-defective *dnaA* mutants at semi-permissive temperatures in a *dnaA* allele-specific manner. These results suggested that the *mioC-oriC* transcription inhibits initiation at *oriC*. Indeed, flow cytometry analysis and quantification of DNA replication in synchronized cultures revealed that the *mioC* promoter mutation reduces initiation activity of chromosomal replication, resulting in alterations in cell cycle-dependent initiation control such as delay in time. These results suggest that the transcriptional regulation of the *mioC* gene is required for cell cycle-coordinated initiation of chromosomal replication.

(2) Structural analysis of the DnaA DNA-binding domain complexed with DNA: Structural basis of replication origin recognition by the DnaA protein[2]

Norie Fujikawa, Hitoshi Kurumizaka, Osamu Nureki, Takaho Terada, Mikako Shirouzu, Tsutomu Katayama and Shigeyuki Yokoyama

Escherichia coli DnaA binds to 9 bp sequences (DnaA boxes) in the replication origin, *oriC*, to form a complex initiating chromosomal DNA replication. In the present study, we determined the crystal structure of its DNA-binding domain (domain IV) complexed with a DnaA box at 2.1 Å resolution. DnaA domain IV contains a helix-turn-helix motif for DNA binding. One helix and a loop of the helix-turn-helix motif are inserted into the major groove and 5 bp (3' two-thirds of the DnaA box sequence) are recognized through base-specific hydrogen bonds and van der Waals contacts with the C5-methyl groups of thymines. In the minor groove, Arg399, localized in the loop adjacent to the motif, recognizes three more base pairs (5' one-thirds of the DnaA box sequence) by base-specific hydrogen bonds. DNA bending by about 28° was also observed in the complex. These results well coincide with the data of solution structure of this complex obtained using NMR.[3] Base-specific interactions revealed here explain how DnaA exhibits higher affinity for the strong DnaA boxes (R1, R2 and R4) than the weak DnaA boxes (R3 and M) in the replication origin.

(3) Molecular mechanism of DNA replication-coupled inactivation of the initiator protein in *Escherichia coli*: Binding of DnaA to the sliding clamp-loaded DNA and binding of the sliding clamp to Hda

Masayuki Su'etsugu, Makoto Takata, Toshio Kubota and Tsutomu Katayama

In *Escherichia coli*, the ATP-DnaA protein initiates chromosomal replication. After the DNA polymerase III holoenzyme is loaded onto DNA, DnaA-bound ATP is hydrolyzed in a manner depending on Hda protein and the DNA-loaded form of the DNA polymerase III sliding clamp subunit, which results in yielding of ADP-DnaA, an inactivated form for initiation. This regulatory DnaA-inactivation is required to repress extra initiation events. In this study, *in vitro* replication intermediates and structured DNA mimicking replicational intermediates were first used to identify structural prerequisites in the process of DnaA-ATP hydrolysis. Unlike duplex DNA loaded with sliding clamps, primer RNA-DNA heteroduplexes loaded with clamps were not associated with DnaA-ATP hydrolysis, and duplex DNA provided *in trans* did not rescue this defect. Moreover, the DnaA-ATP hydrolysis was inhibited by excessive supply *in trans* of a DnaA box-bearing oligonucleotide that tightly binds DnaA. These results imply that the DnaA-ATP hydrolysis involves the interaction of ATP-DnaA with duplex DNA flanking the sliding clamp. Furthermore, Hda protein formed a stable complex with the sliding clamp, suggesting that this complex is an important structure for interaction with ATP-DnaA. These proposals provide a molecular basis in mechanisms of the DnaA-inactivation that depends on chromosomal DNA replication *in vivo*.

Publications

Papers

1. Su'etsugu, M., Emoto, A., Fujimitsu, K., Keyamura, K. and Katayama, T. (2003) Transcriptional control for initiation of chromosomal replication in *Escherichia coli*: fluctuation of the level of origin transcription ensures timely initiation. Genes Cells **8**, 731-745.
2. Fujikawa, N., Kurumizaka, H., Nureki, O., Terada, T., Shirouzu, M., Katayama, T. and Yokoyama, S. (2003) Structural basis of replication origin recognition by the DnaA protein. Nucleic Acid Res. **31**, 2077-2086.
3. Yoshida, Y., Obita, T., Kokusho, Y., Ohmura, T., Katayama, T., Ueda, T. and Imoto, T. (2003) Identification of the region of *Escherichia coli* DnaA protein required for specific recognition of the DnaA box. Cell. Mol. Life Sci. **60**, 1998-2008.
4. Higuchi, K., Katayama, T., Iwai, S., Hidaka, M., Horiuchi, T. and Maki, H. (2003) Fate of DNA replication fork encountering a single DNA lesion during *oriC* plasmid DNA replication *in vitro*. Genes Cells **8**, 437-449.

Reviews

5. 片山　勉(2003)「細菌染色体の複製制御機構」　薬学研究の進歩　19巻　19－28頁　薬学研究奨励財団.

EDUCATION

1. Dr. Katayama gave a lecture for extension courses at Kyushu University, Fukuoka, July, 2003 (in Japanese).
2. Dr. Katayama gave a lecture at Tochiku High School, Fukuoka, October, 2003 (in Japanese).
3. Dr. Katayama and others gave a poster presentation in "Genome Square 2003 in Fukuoka", Fukuoka, November, 2003 (in Japanese).

SOCIAL CONTRIBUTIONS AND OTHERS

A best paper award in the 75th annual meeting of the Genetic Society of Japan was conferred on the presentation by Kawakami, Su'etsugu and Katayama.

B. DEPARTMENT OF CELL GENETICS

B-a. Division of Cytogenetics Tamotsu Yoshimori Group

RESEARCH ACTIVITIES

Membrane traffic: the intracellular transport network organized by proteins and membranes

Tamotsu Yoshimori

Most of membrane-bound organelles in eukaryotic cells are linked each other by dynamic membrane trafficking regulated by proteins. Membrane traffic is involved not only in survival of each cell but also in various functions required for organizing the multi-cellular system, e.g., formation of cell polarity and intercellular communication. We aim to unravel molecular mechanisms of membrane traffic and their roles in higher physiological functions and diseases in animals, which must produce knowledge contributing clinical medicine. We are now focusing on two trafficking routes; autophagy and the endosomal system. Autophagy is membrane traffic delivering cytoplasmic components to lysosomes for bulk degradation. The process is mediated by the formation of the double membrane-bound autophagosomes. Endosomes received macromolecules taken up by endocytosis from outside. The cargo is then either sorted to lysosomes or recycled back to the plasma membrane.

(1) Identification of a new component of a large protein complex associated with the autophagic membrane

Noboru Mizushima[1], Akiko Kuma[1], Yoshinori Kobayashi[1], Akitsugu Yamamoto[2], Masami Matsubae[3], Toshifumi Takao[3], Tohru Natsume[4], Yoshinori Ohsumi[1] and Tamotsu Yoshimori ([1]National Institute for Basic Biology, [2]Nagahama Institute of Bio-Science and Technology, [3]Osaka University, [4]National Institute of Advanced Industrial Science and Technology)

We previously reported that association of the Atg12-Atg5 conjugate with the small precursor membranes is essential in autophagosome formation. In this work, we demonstrated that the mouse Atg12-Atg5 conjugate forms a ~800 kDa protein complex[2]. Moreover, we identified a novel WD-repeat protein as a component of the large protein complex[2]. We have designated it mouse Atg16-like protein (Atg16L), since its N-terminal region has homology with yeast Atg16. However, C-terminal WD domain of Atg16L is absent from the yeast homologue, suggesting possible higher-eukaryote-specific function of the complex. Atg16L binds to both Atg5 and additional Atg16L monomers. These interactions are, however, independent of the WD-repeat domain. Atg16L, together with Atg12-Atg5 conjugate, is associated with the membrane during formation of autophagosome. The membrane targeting of Atg16L requires Atg5 but not Atg12. Existence of the WD-repeat domain, which provides a platform for protein-protein interactions, suggests further interaction of Atg16L with unidentified protein(s) in mammalian cells.

(2) Involvement of autophagy in degradation of unfolded proteins causing diseases

Shisako Shouji, Takahiro Kamimoto, David H. Perlmutter[1], Akira Kakizuka[2] and Tamotsu Yoshimori ([1]University of Pittsburgh, [2]Kyoto University)

Intracelluar accumulation and aggregation of abnormal unfolded protein are linked to many diseases, including neurodegeneration. It has been believed that cells avoid accumulating potentially toxic aggregates by degradation of unfolded protein mainly via ubiquitin-proteasome system. We hypothesized that autophagy is also involved in degradation of such protein, since induction of autophagy has been often observed in the unfolded protein diseases. To examine the possibility, we have used disease models of cultured cells; we overexpressed the expanded polyQ fragment, which causes Huntington disease, or mutant α_1-antitrypsin Z (ATZ), which causes degeneration of liver cells. Atg5-deficient ES cells that we previously established shows defect of autophagosome formation.

In these cells, both polyQ and ATZ was accumulated and aggregated more than in the normal ES cells. The phenotype was suppressed by co-transfection of wild-type Atg5 cDNA but not by the KR-mutant Atg5 which cannot form autophagosomes. A pulse-chase experiment demonstrated that ATZ degradation was retarded in the Atg5-KO cells. The results suggest that autophagy is involved in degradation of at least the two distinct unfolded proteins.

(3) Analysis of autophagic response to invasion of pathogenic bacteria into cells

Kayoko Tsuda, Ichiro Nakagawa[1], Atsuo Amano[1] and Tamotsu Yoshimori ([1]Osaka University)

Some pathogenic bacteria invade into animal cells and survive or proliferate inside cells. Such bacteria often internalize to the membrane-bound compartments such as phagosomes and modulate them to avoid digestion in lysosomes. It was recently reported that some bacteria appears in autophagosomes after invasion. We confirm this about two pathogenic bacteria. They were colocalized with LC3, an autophagosome membrane marker that we reported previously. Autophagy was induced by infection of both bacteria in cultured cells. One of them significantly multiplied in the Atg5-KO cells, suggesting that autophagy functions in cellular protection against invaded bacteria.

(4) Reconstitution of early step of autophagy by using semi-intact cells

Shunsuke Kimura, Atsuki Nara and Tamotsu Yoshimori

In vitro reconstitution of membrane trafficking by using the semi-intact cell systems is a powerful tool to resolve its molecular machinery; application of the technique to secretory pathway has provided many crucial information about it. To elucidate mechanisms underlying autophagic membrane dynamics, we established semi-intact cells by treatment of cultured cells with a bacterial toxin, Streptolysin O, which forms micro pores on the plasma membranes and allow us to access the cytoplasm directly. We succeeded to reconstitute binding of GFP-Atg5 to the precursor membranes, the initial step of autophagosome formatrion, in cells by adding the cytosol fraction and ATP-regenerating system. The cytosol isolated from starved cells was more effective than that from cells cultured in nutrient-rich condition. Since autophagy is known to be induced by starvation, the result indicates that the starved cell cytosol contains factor(s) triggering autophagosome formation. We will identified such factor(s) by using the reconstitution system.

(5) Functional analysis of SKD1 by RNAi

Atsuki Nara, Masako Sakai and Tamotsu Yoshimori

We previously demonstrated that the E235Q mutant of mouse SKD1 AAA ATPase lacking ATP hydrolysis activity has dominant negative effect on transport from endosomes to both the plasma membrane and to lysosomes, suggesting that SKD1 is involved in sorting and transport out of cargos in endosomes. To address precise role of SKD1, we decreased the endogenous protein by RNA interference and showed significant inhibition of the endosome-to-lysosome transport. We are examining other transports including recycling and transport to the TGN. Moreover, we found that overexpression of SKD1 suppressed transport defect in some lipid storage disease.

(6) Interaction of Toml with Tollip, ubiquitin, and clathrin

Megumi Yamakami, Tamotsu Yoshimori and Hideyoshi Yokosawa[1] ([1]Hokkaido University)

We investigated protein-protein interaction of Toml, a member of the VHS domain-containing protein family. Although proteins belonging this family have been implicated in membrane trafficking, the role of Toml is unclear. We found that Toml binds to ubiquitin chains and Tollip, which is a mediator of interleukin-1 signaling[7]. Toml was also capable of binding to clathrin heavy chain[7]. Based on the results, we suggest that Toml plays a role in link of polyubiquitinated receptors to clathrin in endocytosis.

(7) Regulated transport of yeast amino acid permease

Kyohei Umebayashi and Akihiko Nakano[1] ([1]Molecular Membrane Biology Laboratory, RIKEN)

Although it can be speculated that nutrient uptake into cells is tightly regulated, there has been limited experimental evidence. We have studied intracellular transport of Tat2p, the yeast tryptophan permease, and shown that uptake of tryptophan is controlled by sorting of Tat2p to the plasma membrane[8]. When cells are grown in high tryptophan medium, Tat2p is not targeted to the plasma membrane. Instead, it is sorted to the vacuole for degradation. In contrast, Tat2p is targeted to the plasma membrane when cells are subjected to low tryptophan. Our detailed analysis indicates that the sorting occurs in early endosomes and that ubiquitination of Tat2p is the key determinant for the sorting[8]. Tat2p is polyubiquitinated on its way to the vacuole, and inhibiting the polyubiquitination results in efficient plasma membrane sorting. In addition to ubiquitination, the sorting of Tat2p is dependent on sterols in the membrane[8]. Association of Tat2p with a sterol-rich membrane domain, which is called "lipid raft", is required for its plasma membrane delivery. Failure in raft association results in missorting to the vacuole, and the sorting of Tat2p is drastically affected by changes in sterol composition of the membrane. These results lead to the hypothesis that polyubiquitinated cargo proteins are excluded from lipid rafts to be sorted to the vacuole.

(8) Ubiquitin-dependent protein sorting in the endocytic pathway

Kyohei Umebayashi, Min-Soo Kim[1], Yasue Hatanaka and Tamotsu Yoshimori ([1]Tokyo Metropolitan Institute of Medical Science)

Epidermal growth factor (EGF) receptor (EGFR) is ubiquitinated by the E3 ligase Cbl, and it has been considered that the ubiquitination is required for receptor internalization from the plasma membrane. We found, however, that Cbl is localized to early endosomes after cells are stimulated with EGF. Under this condition, coimmunoprecipitation of EGFR with Cbl has been confirmed. These results raise the possibility that ubiquitination and sorting of EGFR occurs also in endosomes. We are now examining the functions of SKD1 in the receptor ubiquitination. Recently, the concept has been emerging that receptor signaling can occur in endosomes. We will examine the possibility that EGFR can transmit the signal in endosomes.

Publications

Papers

1. Fujita, H., Yamanaka, M., Imamura, K., Tanaka, Y., Nara, A., Yoshimori, T., Yokota, S. and Himeno, M. (2003). A dominant negative form of the AAA ATPase SKD1/VPS4 impairs membrane trafficking out of endosomal/lysosomal compartments: Class E vps phenotype. J. Cell Sci. **116**, 401-414.

2. Mizushima, N., Kuma, A., Kobayashi, Y., Yamamoto, A., Matsubae, M., Takao, T., Natsume, T., Ohsumi, Y. and Yoshimori, T. (2003). Mouse Apg16L, a Novel WD Repeat Protein, Targets to the Autophagic Isolation Membrane with the Apg12-Apg5 Conjugate. J. Cell Sci. **116**, 1679-1688.

3. Saeki, K., Hong, Z., Nakatsu, M., Yoshimori, T., Kabeya, Y., Yamamoto, A., Kaburagi, Y. and You, A. (2003) on line. Insulin-dependent signaling regulates azurophil granule-selective macroautophagy in human myeloblastic cells. J. Leukoc. Biol. **74**, 1108-1116.

4. Ohashi, M., Mizushima, N., Kabeya, Y. and Yoshimori, T. (2003). Localization of Mammalian NAD(P)H Steroid Dehydrogenase-like Protein on Lipid Droplets. J. Biol. Chem. **278**, 36819-36829.

5. Katoh, K., Shibata, H., Suzuki, H., Nara, A., Ishidoh, K., Kominami, E., Yoshimori, T. and Maki, M. (2003). The ALG-2-interacting protein Alix associates with CHMP4b, a human homologue of yeast Snf7 that is involved in multivesicular body sorting. J. Biol. Chem. **278**, 39104-39113.

6. Kanazawa, C., Morita, E., Yamada, M., Ishii, N., Miura, S., Asao, H., Yoshimori, T. and Sugamura, K. (2003). Effects of deficiencies of STAMs and Hrs, mammalian class E Vps proteins, on receptor downregulation Biochem. Biophys. Res. Commun. **309**, 848-856.

7. Yamakami, M., Yoshimori, T. and Yokosawa H. (2003) on line. Tom 1, a VHS domain-containing protein, interacts with Tollip, ubiquitin, and clathrin. J. Biol. Chem. **278**, 52865-72.

8. Umebayashi, K. and Nakano, A. (2003). Ergosterol is required for targeting of tryptophan permease to the yeast plasma membrane. J. Cell Biol. **161**, 1117-1131.

Reviews

9. Mizushima, N., Yoshimori, T. and Ohsumi, Y. (2003). Role of the Apg12 conjugation system in mammalian autophagy. Int. J. Biochem. Cell Biol. **35**, 553-561.

10. 梅林恭平，吉森　保(2003)エンドサイトーシス経路における分子選別—輸送シグナルとしてのユビキチン　実験医学増刊　**21**, 1986-1990.
11. 吉森　保(2003)哺乳動物のオートファジー:分子機構と生理機能　生体の科学　**54**, 514-520.

Books

12. Yoshimori, T. and Mizushima, N. (2003) Mammalian homologues of Yeast Autophagy Proteins. In Autophagy. Klionsky D. J. ed. (Georgetown, Texas, USA: Eurekah.com/Landes Bioscience), pp.204-210.
13. 吉森　保(2003)リソソーム／ゴルジ体／ペルオキシソーム／KDEL配列／PDI／液胞型H^+－ATPアーゼ／カベオリン／共焦点顕微鏡／ラフト／レーザー共焦点顕微鏡　分子生物学・免疫学キーワード辞典第２版　医学書院.

EDUCATION

1. Dr. T. Yoshimori gave a lecture at Graduate School of Medicine, Osaka University, May, 2003 (in Japanese).
2. Dr. T. Yoshimori gave a lecture at Division of Pharmaceutical Science, Graduate School of Natural Science & Technology, Kanazwa University, July, 2003 (in Japanese).
3. Dr. T. Yoshimori gave a lecture at Graduate School of Bioagricultural Sciences, Nagoya University, July, 2003 (in Japanese).
4. Dr. T. Yoshimori gave a lecture at Graduate School of Medicine, The University of Tokushima, November, 2003 (in Japanese).

SOCIAL CONTRIBUTIONS AND OTHERS

1. Dr. T. Yoshimori is a councillor of the Japan Society for Cell Biology.
2. Dr. T. Yoshimori gave a lecture on Open Day of National Institute of Genetics, Mishima, April 2003.
3. Dr. T. Yoshimori organized a symposium entitled "Membrane trafficking to endosomes/lysosomes: The long and winding roads" at the 76th Annual Meeting of the Japanese Biochemical Society, Yokohama, August 2003.

B-b. Division of Microbial Genetics Hiroyuki Araki Group

RESEARCH ACTIVITIES

We have been studying on eukaryotic chromosomal DNA replication and its regulation by the cell cycle. For this purpose, we have employed budding yeast, *Saccharomyces cerevisiae*, as a model system of eukaryotic cells. Using strong genetics of budding yeast, we have identified novel factors involving in chromosomal DNA replication and revealed their functions in chromosomal DNA replication. We have been also interested in the S-phase checkpoint that monitors the status of DNA replication and arrest the cell cycle when DNA replication is inhibited since the Dpb11 protein we found seems to be related to the checkpoint. Furthermore, we have extended our study to duplication mechanism of chromatin structure accompanying DNA replication.

(1) The interactions between replication proteins for the initiation of DNA replication

Yoichiro Kamimura and Hiroyuki Araki

The initiation of eukaryotic chromosomal DNA replication requires two steps; pre-replicative complex (pre-RC) formation at replication origins from late M to G1 phase and replisome formation that is the assembly of factors involved in DNA synthesis in S phase. In budding yeast, Dpb11 forms a complex with essential DNA polymerase ε (Polε) and is required for assembly of DNA polymerases with origins. Thus, Dpb11 functions for replisome formation in chromosomal DNA replication. To know the molecular mechanism of replisome formation, we have identified Sld2, Sld3-Cdc45 and GINS (see below) as factors genetically interacting with Dpb11. All these factors function for chromosomal DNA replication. In summary, Sld2 forms a complex with Dpb11 and this complex is involved in assembly of DNA polymerases with origins.

On the other hand, Sld3-Cdc45 functions as a complex and the association of Sld3-Cdc45 with replication origins in the chromatin immunoprecipitation (ChIP) assay precedes Dpb11-Sld2 association with origins. We recently identified the new complex composed of Sld5, Psf1, Psf2 and Psf3 and named this

complex GINS (Go, Ichi, Ni, San; Five, One, Two, Three in Japanese).[1] The GINS complex was also identified in *Xenopus* egg extracts and found to function for DNA replication.[2] We analyzed the replication intermediates by N/N 2D agarose gel electrophoresis and found that the initiation frequencies of chromosomal DNA replication reduces in *sld5-12* and *psf1-1* cells. The ChIP assay showed that Psf1 associates with replication origins at the same timing with Pol ε in S phase and the association of Psf1, Sld3 and Dpb11 with origins are mutually dependent. These results suggest that Sld3-Cdc45, GINS, and Pol ε-Dpb11-Sld2 are required for replisome formation through some interactions. Indeed, Psf1 interacts with not only Dpb11 but also Sld3 in a two hybrid assay. We therefore investigate the interaction between Sld3 and GINS in detail. The C-terminal portion of Sld3 interacts with Psf1 (GINS). The *sld3-1* mutation, which was originally isolated by the synthetically lethal screening with *dpb11-1*, occurred in the C-terminal portion of Sld3 and Sld3-1 proteins reduce the interaction with Psf1. These data suggest that the interaction between Sld3 and GINS is important for chromosomal DNA replication. From these results we propose the following model as to replisome formation. First, Dpb11-Sld2 interacts with Pol ε in S phase. Next, Pol ε -Dpb11-Sld2 is competent to form a complex with GINS. Finally, this complex is loaded onto replication origins via the protein interaction between GINS and Sld3.

(2) Molecular mechanism of the initiation of DNA replication regulated by cyclin-dependent kinase (CDK)

Yon-Soo Tak, Yoichiro Kamimura, Seiji Tanaka and Hiroyuki Araki

The initiation of chromosomal DNA replication in eukaryotes requires the CDK activity. However, how CDK regulates the initiation of DNA replication is not well elucidated since the substrates of CDK in DNA replication have not been identified except the Sld2 protein. Sld2 has a cluster of eleven CDK-dependent phosphorylation sites (S/T-P), six of which are preferred CDK phosphorylation sites. Phosphorylation of Sld2 enhanced its interaction with Dpb11, which appears to be necessary for onset of DNA replication. We thus studied how the interaction between Dpb11 and Sld2 is regulated by CDK activity.

We showed that Sld2 forms a complex with Dpb11, independent of Cdc45 and Cdc7 kinase, both of which are required for the initiation of DNA replication. Thus, the complex formation seems to occur without any other replication proteins. In yeast two-hybrid assay, we mapped the interaction region of Sld2 and Dpb11 using various truncated forms of the proteins. Dpb11 has four copies of the BRCT domain, which is important for protein-protein interaction. Two BRCT domains at C terminal of Dpb11 bound to specific region of Sld2. We also delimitated a binding domain of Sld2 for Dpb11 into a 39-amino acids stretch, partially overlapping with a cluster of the phosphorylation sites. While a 39-aa stretch binds to Dpb11 in phosphorylation-independent manner, the binding of Dpb11 and Sld2 depended on phosphorylation when this 39-aa stretch was connected to a cluster of the phosphorylation sites of Sld2.

We then developed the *in vitro* binding assay of Sld2 and Dpb11. This *in vitro* assay demonstrated that the Sld2 protein directly interact with Dpb11 and CDK-dependent phosphorylation of Sld2 (39 aa plus a cluster of phosphorylation sites) enhances its binding to Dpb11. Therefore, we propose that a cluster of phosphorylation sites in Sld2 regulates binding of the 39-aa stretch to Dpb11.

The Sld2 protein is a good candidate for the substrate of CDK to regulate the initiation of DNA replication. We therefore constructed the phosphomimetic form of Sld2, in which all the SP and TP sites are substituted by DP. While this mutant protein is functional, it could not bypass the requirement of the CDK activity for the initiation of DNA replication. It suggests that there are other important CDK-substrates to initiate DNA replication.

(3) Functional analysis of the Dpb11 protein

Sachiko Muramatsu and Hiroyuki Araki

To elucidate the function of Dpb11 further, we explored defect in *dpb11-1* cells. Previous studies show that Dpb11 is required for the origin association of DNA polymerases at the initiation step of DNA replication and moves rapidly away from origins in ChIP assay, suggesting that Dpb11 functions only for the initiation of DNA replication. However, *dpb11-1* cells that were arrested by hydroxyurea (HU), an inhibitor of DNA replication, and released at the restrictive temperature lost their viability quickly. Moreover, the

cells that were released in medium with Nocodazole, an inhibitor of mitosis, from HU at the restrictive temperature did not restore viability. These results suggest two possibilities. First, Dpb11 plays roles not only before HU block but also after HU block in S phase. Second, the origin firing before HU block is not enough to complete DNA replication. In the presence of HU, cells released from G1 arrest activate only the origins firing in early S phase. Thus, replication forks travels longer distance than those in wild-type cells, which may lead to collapse of replication forks.

We therefore examined collapse of replication forks in *dpb11-1* cells. For this purpose, we observed focus formation of Ddc2-GFP. Ddc2 is a damage-checkpoint protein that forms a focus at the damaged DNA. We then found that the frequency of focus formation of Ddc2 increases in *dpb11-1*. This result suggests that Dpb11 is solely required for the initiation step of DNA replication and its defect causes collapse of replication forks indirectly.

(4) Counteractions of DNA polymerase ε and yCHRAC for epigenetic inheritance of telomere position effect variegation (TPE)[3]

Tetsushi Iida and Hiroyuki Araki

Relocation of euchromatic genes near heterochromatin region often results in mosaic gene silencing. In budding yeast, cells with the genes inserted at telomeric heterochromatin-like regions show phenotypic variegation known as telomere-position effect (TPE), and the epigenetic states are stably inherited to following generations. Here we show that the epigenetic states of the telomere gene are not stably inherited in cells either bearing a mutation in a catalytic subunit (Pol2) of replicative DNA polymeraseε(Polε) or lacking one of non-essential and histone-fold motif-containing subunits of Polε, Dpb3 and Dpb4. We also report a novel and putative chromatin-remodeling complex, ISW2/yCHRAC that contains Isw2, Itc1, Dpb3-like subunit (Dls1) and Dpb4. Using the single-cell method developed in this study, we demonstrated that without Pol ε and ISW2/yCHRAC, the epigenetic states of telomere are frequently switched. Furthermore, we revealed that Pol ε and ISW2/yCHRAC function independently: Polε operates for stable inheritance of a silent state, while ISW2/yCHRAC works for that of an expressed state. We therefore propose that inheritance of specific epigenetic states of telomere requires at least two counteracting regulators.

Publications

Papers

1. Takayama, Y., Kamimura, Y., Okawa, M., Muramatsu, S., Sugino, A. and Araki, H. (2003). GINS, a novel multi-protein complex required for chromosomal DNA replication in budding yeast. Genes & Dev. **17**, 1153-1165.

2. Kubota, Y., Takase, Y., Komori, Y., Hashimoto, Y., Arata, T., Kamimura, Y., Araki, H. and Takisawa, H. (2003). A novel ring-like complex of *Xenopus* proteins essential for the initiation of DNA replication. Genes & Dev. **17**, 1141-1152.

3. Iida, T. and Araki, H. (2004). Non-competitive counteractions of DNA polymerase ε and ISW2/yCHRAC for epigenetic inheritance of telomere-position effect in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **24**, 217-227.

Reviews

4. 荒木弘之(2003) S期サイクリン依存性キナーゼによる染色体複製制御，実験医学**21**，593-597.

5. 上村陽一郎，荒木弘之(2003)複製とクロマチン構造，「クロマチンと遺伝子機能制御」(堀越正美編)，シュプリンガー・フェアラーク東京，pp.121-129.

B-b. Division of Microbial Genetics Seiichi Yasuda Group

RESEARCH ACTIVITIES

(1) Mechanism of DnaA interaction with phospholipids

Seiichi Yasuda

DnaA is a protein essential for the initiation of replication that occurs at a unique locus, oriC, on the chromosome in *Escherichia coli*. DnaA binds to the oriC DNA and opens the double stranded DNA at an AT-rich region within oriC. The opened single-stranded region serves as the site of assembly of other proteins to form a replication complex. DnaA is made of four functional domains. Among them, domain 3 is responsible for ATP-binding, and domain 4 for

sequence-specific binding to oriC DNA. It has been known that acidic phospholipids such as cardiolipin release bound ATP and ADP from DnaA. This phenomenon was suggested to be involved in rejuvenation of used DnaA. It has been postulated that dnaA interacts with phospholipids at a site near the C-terminal portion of domain 3 because this site has an amino acid sequence that can form an alpha helix having a hydrophobic surface. To determine whether this site is really involved in the DnaA-phospholipid interaction, several mutant DnaA proteins were constructed and the release of bound ATP by cardiolipin treatment was measured. All mutant DnaA had ATP-binding activity that was comparable to the wild type DnaA, implying that the function of domain 3 is intact in these mutants. One mutant protein that has a complete deletion of the putative membrane-interaction site was found to release bound ATP as efficiently as wild type DnaA upon cardiolipin treatment. OriC DNA bound the protein normally and inhibited the nucleotide release almost completely as with wild type DnaA. Another mutant DnaA that has a deletion of almost all of domain 4 but that retains the membrane-binding site was also prepared. This mutant DnaA did not bind oriC DNA and showed no release of bound ATP at all upon cardiolipin treatment. These results suggest that the putative membrane-binding site may not be involved in the cardiolipin-induced release of nucleotides from DnaA. Recent analyses of DnaA structure revealed the presence of a large cavity between domain 3 and domain 4, where bound DNA is located. They also showed that many hydrogen bonds are formed between amino acid side chains of DnaA and phosphate moiety of DNA. Considering the partial structural resemblance between DNA and cardiolipin in which two phosphate groups are separated by three carbon atoms of glycerol, it might be possible to suppose that cardiolipin interacts with DnaA protein by binding to the DNA-binding site.

B-c. Division of Cytoplasmic Genetics
Takeo Yoshikawa Group

RESEARCH ACTIVITIES

(1) Linkage disequilibrium (LD) scanning of functional psychoses

Takeo Yoshikawa

We are continuing to collect human samples from families and unrelated individuals with schizophrenia and mood disorder (particularly bipolar disorder) and controls. For sample recruitment and genetic analyses, we are working closely with nation-wide collaborative networks for schizophrenia (JSSLG: Japanese Schizophrenia Sib-Pair Linkage Group)[2] and mood disorders (JGIMD: Japanese Genetics Initiative for Mood Disorders). In 2003, we completed an LD scan of schizophrenia pedigrees using 444 genome-wide microsatellite markers[15]. For chromosome 18p, we have completed LD mapping of schizophrenia families with a marker density of less than 1 cM, revealing C18orf1 as a tempting candidate gene, and published this result[9),10]. Currently, we are focusing on chromosome 6p where the dysbindin gene has been mapped as a schizophrenia susceptibility gene and chromosome 8p where the neuregulin 1 and calcineurin genes are located. Both genes are reported to confer a risk for schizophrenia. For the study of endophenotypes, we are preparing to use the measurement of prepulse inhibition (PPI) in schizophrenia.

(2) Genetic and functional analyses of candidate genes

Takeo Yoshikawa

Dysregulation of catecholaminergic neurotransmission has been proposed as a common pathophysiological basis for schizophrenia and mood disorders. In 2003, we analyzed PMX2B, a protein involved in the development and differentiation of midbrain dopaminergic and adrenergic neurons. PMX2B is also involved in the development of brain motor neurons that control the extraocular muscles for eye movement. This protein functions as a transcription factor and contains two alanine homopolymeric stretches. In general, the length of alanine tracts in transcription factors is

strictly conserved, and alterations in the number of alanine residues usually result in serious phenotypic defects. In the C-terminal alanine stretch canonically consisting of 20 alanine residues, we have found non-rare length polymorphisms that show association with schizophrenia, especially in those patients who carry ocular misalignments[14].

Another well established hypotheses for schizophrenia etiology, is that of hypoglutamatergic neurotransmission via NMDA receptors. We analyzed the NR2A subtype gene (*GRIN2A*) of the NMDA receptor complex, because its ontogenic expression corresponds to the peri-pubertal period of schizophrenia onset in humans and because it is abundantly expressed in the cortices, anatomical regions relevant to disease. We identified a polymorphic GT repeat in the promoter region of *GRIN2A*, and published the following results in 2003: (i) the longer the (GT)n stretch, the lower the promoter activity, (ii) the length of the (GT)n inversely correlates with MK801 binding sites (MK801 specifically binds to NMDA receptors) in cortical regions of postmortem brains, (iii) the mean (GT)n length is longer in schizophrenia than in control samples and this length correlated with the severity of chronic outcome in schizophrenia[7].

Continuing from 2003 and into 2004, we have reported on NR4A2[8] and we are studying the netrin G1 and G2 genes.

For mood disorders, in 2003 we examined the genes *PMX2B* and *GRIN2A*[6] mentioned above, as well as *AKT1*[13] and *NCAM1*[1]. We are also investigating additional genes in collaboration with other laboratories.

We have performed a genome-wide expression analysis of mRNA from the postmortem brains of bipolar disorder subjects, which were provided by our collaborator, Dr. Brian from Australia. We are now correlating gene mRNA changes with protein expression levels. Once this has been completed, the genes showing consistent changes will be treated as candidates for population genetic analysis.

Myo-inositol monophosphatase 2 (IMPA2) is thought to be a target for lithium, a therapeutic drug for mood swings, and it maps to 18p11.2 where linkage to both bipolar disorder and schizophrenia has been reported. We are currently determining the enzymatic function of IMPA2 and also making gene-manipulated animals.

(3) Studies on animal models

Takeo Yoshikawa

We studied gene expression profiles in the frontal cortex and hippocampus of brains from learned helplessness (LH) rats, a reliable depression model, and found that Limk1 is the gene of most dramatically reduced in the frontal cortex of LH rats[11]. Based on this result, we have obtained *Limk1* knockout mice and performed extensive behavioral analyses of these animals during the year 2003. We have obtained data suggesting that the gene-disrupted mice display anxiety-related behaviors that are associated with deficient serotonergic neurotransmission.

Previously, by performing QTL analysis, we reported the mouse chromosomal regions that control immobility times for the forced swim test and tail suspension test, behavioral paradigms for depression models. In 2003, we have attempted to identify responsible genes in these QTL, using two approaches: (i) continuing the preparation of consomic mice strains, (ii) narrowing down candidate genes by combining information on differential expressions between progenitor mouse strains, revealed by GeneChip experiments, and information on the positions of these genes (i.e. whether these gene are located in QTL regions or not). The latter strategy has led to the idea that genes involved in actin turnover may underlie the pathophysiology of depression.

Deficits in prepulse inhibition (PPI) have been reported in schizophrenics and first degree relatives suggesting that it may represent an endophenotype for schizophrenia. PPI is one of the few behavioral measures that can be examined by the same paradigm in both rodents and humans. Based on the assumption that genes controlling PPI should overlap with those for schizophrenia susceptibility, we have performed QTL analysis using mice. We first tested several inbred strains for their intrinsic PPI, and found that C57BL/6N (B6) mice showed the highest percentage of PPI and the C3H/HeN (C3) strain displayed the lowest. During 2003, we prepared 1000 F2 mice from B6 and C3 progenitor strains, scored their PPI and genotyped them using 80 genome-wide microsatellite markers. Interestingly, we found that one QTL for PPI mapped to the syntenic region for human chromosome 6p, a well documented linkage region for schizophrenia.

Publications

Papers

1. Arai, M., Itokawa, M., Yamada, K., Toyota, T., Arai, M., Haga, S., Ujike, H., Sora, I., Ikeda, K. and Yoshikawa, T. (2004). Association of neural cell adhesion molecule 1 gene polymorphisms with bipolar affective disorder in Japanese. Biol. Psychiatry. **55**: 804-810.

2. Arinami, T., Ishiguro, H., Minowa, Y., Ohtsuki, T., Tsujita, T., Imamura, A., Yoshikawa, T., Toyota, T., Yamada, K., Shimizu, H., Yoshitsugu, K., Shibata, H., Fujii, Y., Fukumaki1, Y., Tashiro, N., Inada, T., Iijima, Y., Kitao, Y., Furuno, T., Someya, T., Muratake, T., Kaneko, N., Tsuji, S., Mineta, M., Takeichi, M., Ujike, H., Takehisa, Y., Tanaka, Y., Nakata, K., Kitajima, T., Nishiyama, T., Yamanouchi, Y., Iwata, N., Ozaki, N., Ohara, K., Ohara, K., Suzuki, Y., Shibuya, H., Ohmori, O., Shinkai, T., Hori, H., Nakamura, J., Kojima, T., Takahashi, S., Tanabe, E., Yara, K., Nanko, S., Yoneda, H., Kusumi, I., Kameda, K., Koyama, T., Fukuzako, H., Hashiguchi, T., Tanabe, K. and Okazaki, Y. (2003). Initial genome-wide scan for linkage with schizophrenia in the Japanese Schizophrenia Sib-pair Linkage Group (JSSLG) families. Am. J. Med. Genet. **120B**: 22-28.

3. Ebihara, M., Ohba, H., Hattori, E., Yamada, K. and Yoshikawa, T. (2003). Transcriptional activities of cholecystokinin promoter haplotypes and their relevance to panic disorder susceptibility. Am. J. Med. Genet. Neuropsychiatric Genet. **118B**: 32-35.

4. Ebihara, M., Ohba, H., Kikuchi, M. and Yoshikawa, T. (2004). Structural characterization and promoter analysis of human potassium channel Kv8.1 (*KCNV1*) gene. Gene **325**: 89-96.

5. Itokawa, M., Kasuga, T., Yoshikawa, T. and Matsushita, M. (in press). Identification of a male schizophrenic patient carrying a de novo balanced translocation, t(4;13)(p16.1;q21.31). Psychiatry Clin. Neurosci.

6. Itokawa, M., Yamada, K., Iwayama-Shigeno, Y., Ishitsuka, Y., Detera-Wadleigh, S.D. and Yoshikawa, T. (2003). Genetic analysis of a functional GRIN2A promoter (GT)n repeat in bipolar disorder pedigrees in humans. Neurosci. Lett. **345**: 53-56.

7. Itokawa, M., Yamada, K., Yoshitsugu, K., Toyota, T., Suga, T., Ohba, H., Watanabe, A., Hattori, E., Shimizu, H., Kumakura, T., Ebihara, M., Meerabux, J.M.A., Toru, M. and Yoshikawa, T. (2003). A microsatellite repeat in the promoter of the NMDA receptor 2A subunit (*GRIN2A*) gene suppresses transcriptional activity and correlates with chronic outcome in schizophrenia. Pharmacogenetics, **13**: 271-278.

8. Iwayama-Shigeno, Y., Yamada, K., Toyota, T., Shimizu, H., Hattori, E., Yoshitsugu, K., Fujisawa, T., Yoshida, Y., Kobayashi, T., Toru, M., Kurumaji, A., Detera-Wadleigh, S. and Yoshikawa, T. (2003). Distribution of haplotypes derived from three common variants of the NR4A2 gene in Japanese schizophrenic patients. Am. J. Med. Genet. Neuropsychiatric Genet. **118B**: 20-24.

9. Kikuchi, M., Yamada, K., Toyota, T., Itokawa, M., Hattori, E., Yoshitsugu, K., Shimizu, H. and Yoshikawa, T. (2003). Two-step association analyses of the chromosome 18p11.2 region in schizophrenia detect a locus encompassing *C18orf1*. Mol. Psychiatry 8: 467-469.

10. Kikuchi, M., Yamada, K., Toyota, T. and Yoshikawa, T. (2003). C18orf located on chromosome 18p11.2 may confer susceptibility to schizophrenia. J. Med. Dent. Sci. **50**: 225-229.

11. Nakatani, N., Aburatani, H., Nishimura, K., Semba, J. and Yoshikawa, T. (2004). Comprehensive expression analysis of a rat depression model. Pharmacogenomics J. **4**: 114-126..

12. Segurado, R., Detera-Wadleigh, S.D., Levinson, D.F., Lewis, C.M., Gill, M., Nurnberger, J.I.Jr., Craddock, N., Raymond, DePaulo, J., Baron, M., Gershon, E.S., Ekholm, J., Cichon, S., Tureck, G., Claes, S., Kelsoe, J.R., Schofield, P.R., Badenhop, R.F., Morissette, J., Coon, H., Blackwood, D., Curtis, D., McInnes, LA., Foroud, T., Edenberg, H.J., Reich, T., Rice, J.P., Goate, A., McInnis, M.G., McMahon, F.J., Badner, J.A., Goldin, L.R., Bennett, P., Willour, V.L., Zand, P.P., Jianjun, Liu, J., Gilliam, C., Juo, S-H., Wade, H., Berrettini, W.H., Yoshikawa, T., Peltonen, L., Lonndnqvist, J., Nothen, M.M., Schumacher, J., Windemuth, C., Rietschel, M., Propping, P., Maier, W., Alda, M., Grof, P., Rouleau, G.A., Del-Favero, J., Van, Broeckhoven, C., Mendlewicz, J., Adolfsson, R., Spence, M.A., Luebbert, H., Adams, L.J., Donald, J.A., Mitchell, P.B., Barden, N., Shink, E., Byerley, W., Muir, W., Visscher, P.M., Macgregor, S., Gurling, H., Kalsi, G., McQuillan, A., Escamilla, M.A., Reus, V.I., Leon, P., Freimer, N.B., Ewald, H., Kruse, T.A., Mors, O., Radhakrishna, U., Blouin, J-L., Antonarakis, S.E. and Akarsu, N. (2003). Genome scan meta-analysis of schizophrenia and bipolar disorder part Ⅲ: bipolar disorder. Am. J. Hum. Genet. **73**: 49-62.

13. Toyota, T., Yamada, K., Detera-Wadleigh, SD.

and Yoshikawa, T. (2003). Analysis of a cluster of polymorphisms in *AKT1* gene in bipolar pedigrees: a family-based association study. Neurosci. Lett. **339**: 5-8.
14. Toyota, T., Yoshitsugu, K., Ebihara, M., Yamada, K., Ohba, H., Fukasawa, M., Minabe, Y., Nakamura, K., Sekine, Y., Takei, N., Suzuki, K., Itokawa, M., Meerabux, J.M.A., Iwayama-Shigeno, Y., Tomaru, Y., Shimizu, H., Hattori, E., Mori, N. and Yoshikawa, T. (2004). Association between schizophrenia with ocular misalignment and polyalanine length variation in *PMX2B*. Hum. Mol. Genet. **13**: 551-561.
15. Yamada, K., Iwayama-Shigeno, Y., Yoshida, Y., Toyota, T., Itokawa, M., Hattori, E., Shimizu, H. and Yoshikawa, T. (in press). Family-based association study of schizophrenia with 444 markers and analysis of a new susceptibility locus mapped to 11q13.3. Am. J. Med. Genet. (Neuropsychiatr. Genet.)
16. Yamada, K., Watanabe, A., Iwayama-Shigeno, Y. and Yoshikawa, T. (2003). Evidence of association between gamma-aminobutyric acid type A receptor genes located on 5q34 and female patients with mood disorders. Neurosci. Lett. **349**: 9-12.
17. Yoshitsugu, K., Meerabux, J.M.A., Asai, K. and Yoshikawa, T. (2003). Fine mapping of an isodicentric Y chromosomal breakpoint from a schizophrenic patient. Am. J. Med. Genet. Neuropsychiatric Genet. **116B**, 27-31.

EDUCATION

1. Dr. T. Yoshikawa gave a lecture at the 37th Training Seminar for Psychiatric Medical Staff of Nagano Prefecture, under the title of "Psychiatric Illnesses and Genes", March, 2003 (in Japanese).
2. 東京医科歯科大学医学部非常勤講師

SOCIAL CONTRIBUTIONS AND OTHERS

Psychiatry and Clinical Neuroscience-editorial board
日本精神・行動遺伝医学会理事

C. DEPARTMENT OF DEVELOPMENTAL GENETICS

C-a. Division of Developmental Genetics Yasushi Hiromi Group

RESEARCH ACTIVITIES

(1) Longitudinal axon guidance in the *Drosophila* CNS

Masaki Hiramoto and Yasushi Hiromi

The anterior-posterior organization of the animal body plan requires axons to interconnect successive segments. How the longitudinal axons that initiate their trajectory using intra-segmental guidance signals interpret segmentally-repeated cues along the entire length of the body is poorly understood. *Roundabout (robo)* is a gene required for the correct axonal guidance, named after its mutant phenotype in *Drosophila*; longitudinal axons fail to enter the next segment and instead project towards the midline, as if running along a roundabout (rotary). This phenotype implies that there is a pathway choice point at the segment boundary: whether or not to observe an attractive signal towards the midline. While the role of ROBO as a receptor for the midline repellant Slit is well established, neither the nature of the attractive field that generates the "roundabout" phenotype nor the function of ROBO in the pathway choice is known. We showed that in *robo* mutant longitudinal axons are steered toward midline by responding to Netrin, an evolutionary conserved midline attractant. Whilst a gradient of Netrin can attract axons *in vitro*, Netrin that misguides longitudinal axons is presented on the commissural axons by the Netrin receptor Frazzled, thereby exerting a long-range effect. ROBO appears to counteract Netrin signaling, allowing longitudinal axons to enter the next segment.

(2) Cell-autonomous axonal patterning in *Drosophila* neurons

Takeo Katsuki, Masaki Hiramoto and Yasushi Hiromi

Expression of axon guidance receptors in an axon is temporally and spatially regulated, presumably because it has a significant influence on growth cone navigation. In the *Drosophila* central nervous system, members of the Roundabout (ROBO) receptor family (ROBO, ROBO2 and ROBO3) play crucial roles in axon tract formation. Notably, ROBO proteins are accumulated specifically on the longitudinal axon segment but remain at undetectable levels on commissures. This raises a question of how neurons create such spatially regulated patterns of expression within axons. Can neurons autonomously generate an intra-axonal pattern, or are extrinsic factors required, such as signals provided by midline cells? To address this issue, we used a low-density cell culture system where neurons grow axons *in vitro*, devoid of cell-cell contacts. In cultured neurons, ROBO2 and ROBO3 were localized to the distal segment of axons, consistent with the patterns observed *in vivo*. In contrast, ROBO was uniformly distributed along the axon. These results indicate that neurons possess an ability to regulate the localization of axon guidance receptors cell-autonomously. Furthermore, the differences observed among ROBO family members suggest that the localization is regulated in a highly molecule-specific manner.

(3) How is the positional information reorganized?

Tsuyoshi Nagasaka, Masaki Hiramoto and Yasushi Hiromi

Positional information that guides organogenesis is temporally multi-layered, changing its pattern and character as development proceeds. Even a single protein that provides positional information can be used many times during organogenesis, in different developmental processes. For example, Netrin, a secreted axon guidance molecule, has its first role in attracting commissural axons to the midline. Subsequently, Netrin is captured by its receptor Frazzled, and is presented on the surface of the commissural axons as a positional information for the pathfinding of longitudinal axons (Nature 406: 886,

2000). Since axons that are initially guided by Netrin are also those that later present Netrin, there might be a causal link between these two processes. We are trying to understand how Netrin secreted at one place travels to the final location for presentation as a local guidance cue. To identify components that are necessary for the correct positioning of Netrin within the *Drosophila* CNS, we are screening a set of deletion strains that lack a part of the genome, focusing on the relationship between the pathfinding of axons and the distribution patterns of Netrin and other guidance molecules. We find three typical abnormal distribution patterns of Netrin: (1) Netrin completely absent, (2) Netrin absent on commissural axons, while present in cells that secrete Netrin, (3) Netrin localized ectopically in the CNS. These phenotypes likely represent distinct steps in Netrin expression, secretion and localization.

(4) Nuclear receptor Seven-up is essential for the temporal switching of Neuroblast fate in the *Drosophila* CNS.

Makoto Kanai, Masataka Okabe and Yasushi Hiromi

A critical step in generating neuronal diversity in the *Drosophila* CNS is the production of various neuronal and glial cell types by the sequential division of the precursor cell, neuroblast. Neuroblast undergoes a number of asymmetric divisions generating a chain of secondary precursor cells. Rather than self-renewing itself like a typical stem cell, neuroblasts change their character upon division, so that they can produce different type of progeny at the next division. Molecular determinants of the temporal character of the neuroblast are transcription factors HB, KR, PDM, etc., which are expressed sequentially in the developmental history of each neuroblast. How neuroblasts switch its expression profile from one transcription factor to the next one is poorly understood. We focused on the gene *seven-up*, which encode a nuclear receptor class of transcription factors, and demonstrated that it has an essential function for a switch from the HB+, KR+ state to the KR+ state. While seven-up is expressed in almost all neuroblasts, it expression is restricted to the subsection within the neuroblast lineage. Loss of function mutant of *seven-up* increases the number of cells of the early cell type (HB+, KR+) within several neuroblast lineages. Lineage analysis provide the evidence that in mutant embryo neuroblasts fail to switch off HB+ at proper timing. These findings indicate that *seven-up* is a fundamental regulator of neuroblast switching.

(5) A search for Seven-up-interacting proteins

Takayuki Hondoh and Yasushi Hiromi

Seven-up is a transcription factor containing a DNA binding domain and an evolutionarily-conserved domain that has homology to the ligand binding domains of nuclear receptors. The spatio-temporal expression pattern of *seven-up* is tightly linked to the cell fate; in the developing *Drosophila* eye, *seven-up* is expressed in only four of the eight photoreceptor neurons, and in *seven-up* mutant these neurons are transformed into another neuronal fate (Cell 60: 211, 1990). In the embryonic CNS *seven-up* expression is temporally restricted to a subsection of the neuroblast lineage, and its absence appears to cause a "temporal transformation" of the fate of the neuroblast and its progeny (see above). This suggests that *seven-up* performs an instructive role in choosing particular cell fates. Misexpression of *seven-up* interferes with many cell fate decisions, causing transformation towards a different fate, which depends on the cell that *seven-up* is misexpressed (Development 118: 1123, 1993, Development 121: 1361, 1995). Interestingly, many of such phenotypes induced by misexpression do not require the DNA binding domain of Seven-up, suggesting that they are caused by titration of proteins that interact with the ligand binding domain of Seven-up. By studying the mechanism of such unusual cell fate control, we expect to better understand the signalling cascade that occur downstream of Seven-up. Toward this goal, we are searching Seven-up interacting proteins by biochemical strategies. Such efforts should also lead to identification of components of other, yet unknown, cell fate switches.

(6) Diversification and differentiation of lateral glia in the *Drosophila* CNS

Yoshihiro Yuasa and Yasushi Hiromi

Glial cells are important elements of the nervous system, comprising more than 90% of the cells in the vertebrate brain. In the *Drosophila* embryo, there are approximately 60 glial cells per abdominal neuromere.

Majority of these are "lateral glia", whose differentiation is regulated by the homeodomain protein REPO[2]. Lateral glia is a heterogeneous population of cells, consisting of various glial subtypes such as longitudinal glia, subperineural glia and channel glia, classified based on the location, cell shape and lineage. Very little is known about the processes of the diversification and differentiation of lateral glia cell types. In order to identify genes involved in the development of lateral glia, we are screening enhancer-trap lines for those that are expressed in the lateral glial cells. In addition to genes previously known to be expressed in lateral glia, such as *loco* and *spinster*, we identified eleven lines that are expressed in subsets of lateral glia.

(7) Development of a novel fluorescent probe to visualize caspase activity in a living organism

Shu Kondo and Yasushi Hiromi

Visualization of intracellular events in a living organism has discovered various amazing phenomena that were unanticipated from in vitro experiments. During animal development, many unnecessary cells are removed by apoptosis. This is especially important for constructing fine structures such as the central nervous system. However, compared to the current knowledge of apoptosis *in vitro*, little is known about naturally occurring cell death during development. This is largely due to the lack of tools to detect apoptosis in its early phase. To overcome this problem, we created a genetically encoded fluorescent probe that allows *in vivo* detection of the activity of caspase, a sequence-specific protease that executes the final step of apoptosis. The efficacy of this probe was validated by time-lapse analysis of apoptosis in cultured cells. Transgenic *Drosophila* harboring this probe were generated and several cases of apoptosis were successfully visualized in live samples.

(8) The establishment of the stem cell fate in the *Drosophila* female germline: A cell lineage study

Miho Asaoka and Haifan Lin[1] ([1]Dept. Cell Biology, Duke University)

Stem cells are characterized by their ability to self-renew and to produce numerous differentiated daughter cells. These properties enable stem cells to play a central role in generating and maintaining most adult tissues in higher organisms. A fundamental yet unexplored question in stem cell biology is how the stem cell fate is initially determined during development. In *Drosophila* germline, stem cells descend from a subset of primordial germ cells (PGCs) at the onset of oogenesis. However, it remains unknown when and how this subpopulation is selected. It is possible that the stem cell fate is determined at the onset of oogenesis. Alternatively, stem cell fate determination may start earlier, as early as the beginning of the expansion of PGC population. As a first step to understand the mechanisms underlying germline stem cell (GSC) fate determination, we did a lineage tracing analysis for individual PGCs from the embryonic stage to the adult stage. We found that at the embryonic stage, PGCs located in the anterior part of the gonad produced PGC population that became GSCs in the adult ovary. In contrast, PGCs in the posterior part of the gonad produced PGC population that differentiate directly to produce eggs. These results suggest that GSC fate determination starts before PGC proliferation. The anterior half of the somatic embryonic gonads may provide a niche for the establishment of GSCs.

Publications

Papers

1. Liu, Q-X., Jindra, M., Ueda, H., Hiromi, Y. and Hirose, S. (2003). *Drosophila* MBF1 is a coactivator for Tracheae Defective and contributes to the formation of tracheal and nervous systems. Development **130**, 719-728.

2. Yuasa, Y., Okabe, M., Yoshikawa, S., Tabuchi, K., Xiong, W.-C., Hiromi, Y. and Okano, H. (2003). *Drosophila* homeodomain protein REPO controls glial differentiation by cooperating with ETS and BTB transcription factors. Development **130**, 2419-2428.

3. Yamada, T., Okabe, M. and Hiromi, Y. (2003). EDL/MAE regulates EGF-mediated induction by antagonizing Ets transcription factor Pointed. Development **130**, 4085-4096.

EDUCATION

1. Dr. Y. Hiromi gave a lecture course at Tokyo University of Agriculture and Technology. January,

2003 (in Japanese).

2. Dr. Y. Hiromi gave a two-day lab course on "Genetic transformation" at Gyoshu High School. December, 2003.

SOCIAL CONTRIBUTIONS AND OTHERS

1. Dr. Y. Hiromi served as an associate editor for Genes to Cells.

2. Dr. Y. Hiromi served as an editor for Development, Growth & Differentiation.

3. Dr. Y. Hiromi served as a member of the council of The Genetics Society of Japan.

4. Dr. M. Okabe developed "Barrier-free presentation that is friendly to colorblind people", and enhanced its public awareness through web pages:
http://www.nig.ac.jp/color/
http://jfly.iam.u-tokyo.ac.jp/color/

C-a. Division of Developmental Genetics Toshitaka Fujisawa Group

RESEARCH ACTIVITIES

(1) Compartments of Neuron Subtypes Revealed by in situ Hybridization of Neuropeptide Genes in *Hydra*.

Toshitaka Fujisawa, Chiemi Fujisawa, Yasuharu Takaku, Yukihiko Noro, Shiho Hyayakawa[1] and Takashi Gojobori[1] ([1]DNA Data Analysis laboratory, Center for Information Biology)

We have identified new members of several neuropeptide gene families in the *Hydra* ESTs. The in situ hybridization analysis revealed that some of these genes were expressed in particular regions along the body column. Double in situ hybridization showed that the subpopulations of neurons expressing different neuropeptide genes did not overlap thereby forming a sharp boundary between two regions. Meinhardt (2000) incorporating expression patterns of various axis forming genes suggested that the *Hydra* body column can be divided into several distinct regions. He further suggested that the *Hydra* body could be an ancestral form of brain. The present results are consistent with his view and suggest that the *Hydra* nervous system (or brain) is composed of several compartments. Furthermore, the neuropeptides present in each compartment seem to have a distinct function specific to the compartment.

(2) Evolutionary Origin of Autonomic Nervous System in *Hydra*.

Hiroshi Shimizu, Masataka Okabe and Toshitaka Fujisawa

Autonomic Nervous System (ANS) is a subpopulation of peripheral nervous system in mammals, which regulates physiological activities of organs. The mechanism of action of the ANS has been well studied, however, it remains unraveled when and how this nervous system appeared in evolution and developed. We propose that the most primitive form of ANS was invented in lower metazoan phyla such as Cnidaria and Plathyhelminthes. On capturing prey, mammals change their behavioral features from so-called "Fight or Flight stage" to "Rest and Digest stage". The heart rate is reduced whereas digestion is activated after feeding. Although defined solely in mammals, similar changes of behavior and physiological activities can be found also in other vertebrates or even in invertebrates. We examined the physiological activities of *Hydra* and found that *Hydra* and mammals show similar changes after feeding, the motility and circulation being reduced whereas digestion elevated. We interpret these similarities to demonstrate that the most primitive form of ANS was invented in lower metazoa and developed later with evolution.

(3) Motility of Endodermal Epithelial Cells Plays a Major Role in Reorganizing Endodermal and Ectodermal Tissue Layers.

Yasuharu Takaku, Takahiko Hariyama[1] and Toshitaka Fujisawa ([1]Hamamatsu Univ. School of Medicine, Hamamatsu)

Hydra has a simple body plan with only two layers of epithelial cells. It exhibits a strong regenerative capacity from dissociated single cells. When dissociated cells are allowed to form aggregates, the first event to occur is sorting of cells to establish two cell layers. In this study, we examined double-layer formation under various situations. (1) When the aggregate of separated ectodermal cells and that of endodermal cells were placed in direct contact, the

endodermal cells migrated into the ectodermal aggregate until entirely covered up by ectodermal cells (epiboly). This process was completely inhibited by cytochalasin B although initial firm attachment between the two aggregates was not blocked. In addition, cytochalasin pretreatment of only an endodermal aggregate delayed the onset of epiboly. (2) A single endodermal epithelial (endoepi-) cell placed in contact with an ectodermal aggregate, actively extended pseudopod-like structures and migrated toward the center of the ectodermal aggregate. In contrast, an ectoepi-cell remained in contact with an endodermal aggregate and never exhibited the ingressive behavior. The cytochalasin B treatment of only endodepi-cells abolished the ingress movements. (3) Single endoepi- and/or ectoepi-cells were adhered one by one up to 4-cell aggregates. Endoepi-cells showed high motility that can be attributed to the ingress movement described above. Finally, visualization of actin bundles with rhodamine-phalloidin always correlated with pseudopod formation except for the later stage of aggregate regeneration where actin bundles were incorporated into smooth muscle fibers at the base of epithelial cells and no longer sensitive to cytochalasin. These results indicate that the high motility of endoepi-cells plays an important role in reorganizing endodermal and ectodermal tissue layers.

Publications

Papers

1. Shimizu, H. and Fujisawa, T. (2003). Genesis **36**, 182-186.

2. Takahashi, T., Kobayakawa, Y., Muneoka, Y., Fujisawa, Y., Mohri, S., Shimizu, H., Fujisawa, T., Sugiyama, T., Takahara, M. and Koizumi, O. (2003). *Comparative Biochemistry and Physiology* **135**, 309-324.

3. Morishita, F., Nitagai, Y., Furukawa, Y., Matsushima, O., Takahashi, T., Hatta, M., Fujisawa, T., Tsunamoto, S. and Koizumi, O. (2003). *Peptides* **6452**, 1-10.

Reviews

4. Fujisawa, T. (2003). *Developmental Dynamics* **226**, 182-189.

C-b. Division of Gene Expression Susumu Hirose Group

RESEARCH ACTIVITIES

(1) Role of GAGA factor-dependent chromatin remodeling in epigenenetic gene expression

Tsukasa Shimojima, Masahiro Okada[1], Takahiro Nakayama, Hitoshi Ueda, Kenichi Nishioka, Hiroshi Handa[2], Tadashi Wada[2] and Susumu Hirose ([1]Present address, Division of Molecular Genetics; [2]Faculty of Bioscience and Biotechnology, Tokyo Institute of Technology, Nagatsuda, Midori-ku, Yokohama, 226-8501, Japan)

There are two types of epigenetic gene expression in *Drosophila*. One is maintenance of *Hox* gene expression governed by *Polycomb (Pc)* and *trithorax (trx)* group genes. The other is position effect variegation (PEV). *Trithorax-like (Trl)* encoding GAGA factor plays a role in the both types of regulation: *Trl* is a member of *trx* group and *Trl* mutation is an enhancer of PEV.

We found that *Drosophila* FACT, a heterodimer of dSPT16 and dSSRP1, is associated with GAGA factor through its dSSRP1 subunit, binds to a nucleosome and faciltitates GAGA factor-directed chromatin remodeling. Immunostaining of polytene chromosomes revealed colocalization of GAGA factor and FACT in many specific loci. Mutation in *Trl* abolished the colocalization, indicating that GAGA factor recruits FACT to the specific loci in the genome. Genetic interactions between *Trl* and *spt16* implicate the GAGA factor-FACT complex in the expression of *Hox* genes. Chromatin immunoprecipitation experiments indicated presence of the GAGA factor-FACT complex in the *bxd* region of *Ultrabithorax* and the *iab-6* element of *Abdominal-B*. These data illustrate a crucial role of the GAGA factor-FACT complex in the modulation of chromatin structure for the maintenance of *Hox* gene expression against Pc silencing[6][10].

When an actively transcribed *white (w)* gene is juxtaposed with heterochromatin by chromosome rearrangement such as w^{m4}, its expression is subject to variable but heritable silencing (PEV). We found that the silencing of *w* was enhanced by *spt16* mutation and further enhanced by *Trl*, *spt16* double mutation.

Chromatin immunoprecipitation assays revealed presence of the GAGA factor-FACT complex on a site just downstream of the coding region of w^+ in euchromatin. However, we observed significant reduction in the occupancy of the complex on the same site in w^{m4} and further decrease in w^{m4}, *Trl*/+. These results indicate that the GAGA factor-FACT complex contributes to maintenance of *w* expression against heterochromatin silencing. We also continued studies on chromatin transcription in collaboration with Drs. T. Wada and H. Handa.

(2) Tracking of FACT and RNA polymerase II elongation complex through chromatin in vivo

Abbie Saunders[1], Janis Werner[1], Erik D. Andrulis[1], Takahiro Nakayama, Susumu Hirose, Danny Reinberg[2] and John T. Lis[1] ([1]Molecular Biology and Genetics, Biotechnology Building, Cornell University, Ithaca NY 14850, USA; [2]Howard Hughes Medical Institute, Department of Biochemistry, Robert Wood Johnson Medical School, University of Medicine and Dentistry of New Jersey, Piscataway, NJ 08854, USA).

RNA polymerase II (Pol II) transcription through necleosomes is facilitated in vitro by the protein complex FACT (Facilitates Chromatin Transcription). We showed that FACT is associated with actively transcribed Pol II genes on *Drosophila* polytene chromosomes. Immunostaining of polytene chromosomes and chromatin immunoprecipitation experiments revealed that FACT displays kinetics of recruitment and of chromosome tracking in vivo similar to Pol II and elongation factors Spt5 and Spt6. Interestingly, FACT does not colocalize with Pol III-transcribed genes, which are known to undergo nucleosome transfer rather than disassembly in vitro. Our observations are consistent with FACT being restricted to transcription that involves nucleosome disassembly mechanisms[5].

(3) Role of DNA topology in the formation of active chromatin

Kuniharu Matsumoto, Hirofumi Furuhashi, Youhei Ogasawara, Hitoshi Ueda, Kazuma Hanai[1], Koji Akasaka[1] and Susumu Hirose ([1]Graduate University of Gene Science, Hiroshima University, Kagamiyama, Higashi-Hiroshima 739-8526, Japan)

Recent studies have established that chromatin structure is important for transcriptional regulation. However, our knowledge on conformation of chromatin DNA was elusive due to lack of proper probes for analyses of DNA topology in vivo. To circumvent the situation, we have developed a method for visualization of negatively supercoiled DNA within the interphase genome. Using the methods, we observed transcription-coupled, unconstrained negative supercoils of DNA in approximately 150 loci on polytene chromosomes of *Drosophila melanogaster*.

Supercoiling factor (SCF) is a protein capable of introducing negative supercoils into DNA in conjuction with DNA topoisomease II. We have identified a fly line carrying non-sense mutation within the coding region of SCF. RNAi and genetic studies revealed that SCF is involved in dosage compensation of X chromosome in *Drosophila*.

We also made studies on a *Drosophila* counterpart of sea urchin insulator binding protein in collaboration with Dr. K. Akasaka.

(4) Functional analysis of transcriptional coactivator MBF1

Qin-Xin Liu, Marek Jindra, Hitoshi Ueda, Masataka Okabe[1], Yasushi Hiromi[1] and Susumu Hirose ([1]Division of Developmental Genetics)

During gene activation, the action of sequence-specific regulators is transmitted to RNA polymerase by means of coactivators. Although many coactivators have been reported, their developmental roles are poorly understood. We conducted molecular and genetic dissection of an evolutionarily conserved but unique coactivator, Multiprotein Bridging Factor 1(MBF1) in *Drosophila*. The results of these studies demonstrate that MBF1 serves as a coactivator of a bZIP protein Tracheae defective/Apontic during development of the tracheal and central nervous systems[3].

Despite the evolutionary conservation of MBF1 suggests an essential role for the protein, null mutants lacking MBF1 proved to be viable under laboratory conditions. However, MBF1 is essential during stress situation encountered in the real world; *mbf1* mutants are sensitive to oxidative stress induced by H_2O_2. A comparative advantage provided by MBF1 under the stress condition is thus likely cause of its evolutionary

conservation.

(5) Mechanism of transcriptional regulation of the FTZ-F1 gene

Yasuo Agawa, Masa-aki Yamada, Susumu Hirose and Hitoshi Ueda

Insect FTZ-F1 is a unique transcription factor, because it is induced after a pulse exposure of ecdysteroids and is expressed just before hatching, larval ecdysis and pupation in a stage specific manner. The stage specific expression of FTZ-F1 is necessary for embryogenesis, larval ecdysis and metamorphosis, indicating that temporary precise regulation of the gene is important for the development of *Drosophila*. Transcription factor p170 has been identified as a sequence specific binding factor that is present during high ecdysteroid periods and that binds to a *cis*-regulatory region of the FTZ-F1 gene. To examine function of p170 during development, we first observed loss of function phenotype. RNAi of p170 induced premature expression of FTZ-F1, suggesting that p170 works as a repressor for the FTZ-F1 gene. On the other hand, over expression of p170 during high ecdysone period from heat shock promoter-p170 cDNA fusion gene resulted in super induction of FTZ-F1 after decline of ecdysone level, indicating that the factor potentiates expression of the FTZ-F1 gene during high ecdysone periods. Transgenic fly reporter assays supported the observations. From these results, we propose p170 to be a novel type transcriptional regulator.

(6) Establishment of a new mutant screening system in the silkworm *Bombyx mori*

Hitoshi Ueda, Yuko Nagashima and Toshiki Tamura[1] ([1]National Institute of Agrobiological Science)

Silkmoth, *Bombyx mori* is a lepidopteran insect and has been used for genetical, biochemical, endocrinological, neurobiological and pathological researches as a useful research organism due to its character that have not present in other insects. However, molecular genetic analyses were delayed compared with other organisms because of lacking a convenient system to analyze established mutants. To overcome the problem, we started a project to collect mutants induced by a transposon in the silkworm. To make a mutant screen easily in the silkworm, we developed new *piggyBac* transposone vectors that have a system to distinguish homozygous and heterozygous mutants easily. Usefullness of the system was confirmed in *Drosophila melanogaster* and now is testing in the silkworm in collaboration with Toshiki Tamura.

(7) Histone lysine methylation: a signature for chromatin function

Robert J. Sims, III[1], Kenichi Nishioka and Danny Reinberg[1] ([1]Howard Hughes Medical Institute, Division of Nucleic Acids Enzymology, Department of Biochemistry, University of Medicine and Dentistry of New Jersey, Robert Wood Johnson Medical School, Piscataway, NJ 08854, USA)

The rapid progress in deciphering the processes of covalent histone modifications has broadened our understanding of transcriptional regulation. Histone lysine methylation, along with DNA methylation, establishes the framework for long-term epigenetic maintenance. Recent studies of the mechanisms of specific histone lysine methylation have revealed a complex process that controls aspects of short- and long-term transcriptional regulation, in addition to the propagation of bulk chromosome structure and stability. In this article, we review the functional properties of histone lysine methylation and the enzymes that catalyze this covalent modification[7].

(8) Facile synthesis of site-specifically acetylated and methylated histone proteins: Reagents for evaluation of the histone code hypothesis

Shu He[1], David Bauman[1], Jamaine S. Davis[1], Alejandra Loyola[2], Kenichi Nishioka, Jennifer L. Gronlund[1], Danny Reinberg[2], Fanyu Meng[3], Neil Kelleher[3] and Dewey G. Mccafferty[1] ([1]Johnson Research Foundation and Department of Biochemistry and Biophysics, University of Pennsylvania School of Medicine, Philadelphia, PA 19104-6059; [2]Howard Hughes Medical Institute and Department of Biochemistry, University of Medicine and Dentistry of New Jersey/Robert Wood Johnson Medical School, Piscataway, NJ 08854; and [3]Department of Chemistry, University of Illinois at Urbana-Champaign, Urbana, IL 61801)

The functional capacity of genetically encoded histone proteins can be powerfully expanded by posttranslational modification. A growing body of biochemical and genetic evidence clearly links the unique combinatorial patterning of side chain acetylation, methylation, and phosphorylation mainly within the highly conserved N termini of histones H2A, H2B, H3, and H4 with the regulation of gene expression and chromatin assembly and remodeling, in effect constituting a "histone code" for epigenetic signaling. Deconvoluting this code has proved challenging given the inherent posttranslational heterogeneity of histone proteins isolated from biological sources. Here we describe the application of native chemical ligation to the preparation of full-length histone proteins containing site-specific acetylation and methylation modifications. Peptide thioesters corresponding to histone N termini were prepared by solid phase peptide synthesis using an acid labile Boc/HF assembly strategy, then subsequently ligated to recombinantly produced histone C-terminal globular domains containing an engineered N-terminal cysteine residue. The ligation site is then rendered traceless by hydrogenolytic desulfurization, generating a native histone protein sequence. Synthetic histones generated by this method are fully functional, as evidenced by their self-assembly into a higher order H3/H4 heterotetramer, their deposition into nucleosomes by human ISWI-containing (Imitation of Switch) factor RSF (Remodeling and Spacing Factor), and by enzymatic modification by human Sirt1 deacetylase and G9a methyltransferase. Site-specifically modified histone proteins generated by this method will prove invaluable as novel reagents for the evaluation of the histone code hypothesis and analysis of epigenetic signaling mechanisms[2].

(9) Methods and tips for the purification of human histone methyltransferases

Kenichi Nishioka and Danny Reinberg[1] ([1]Howard Hughes Medical Institute, Division of Nucleic Acids Enzymology, Department of Biochemistry, University of Medicine and Dentistry of New Jersey, Robert Wood Johnson Medical School, Piscataway, NJ 08854, USA)

Recently developed biochemical techniques have enabled researchers to study histone modifications more easily and accurately. One of these modifications, histone lysine methylation, has been shown to be highly stable and to represent an epigenetic alteration. Extensive biochemical analyses have led to discoveries about the nature and functions of this modification, thus accelerating our understanding of this crucial epigenetic event. Here we describe basic methods for purification and biochemical analysis of lysine-directed, histone methyltransferases from HeLa cell-derived extracts. In the section on substrate preparation, we describe a simple method for the preparation of recombinant substrates, although we recommend using native substrates for initial detection of the activities. The purification protocols for several histone methyltransferases have been streamlined so that those researchers with a basic understanding of biochemistry can perform them. We also describe many tips and provide suggestions to avoid common pitfalls in the biochemical analysis of histone methyltransferases[4].

(10) Construction of a replication-selective adenovirus for ovarian cancer therapy

Katsuyuki Hamada[1], Shohei Kohno[2], Mari Iwamoto[1], Hiroko Yokota[1], Masato Okada[4], Masatoshi Tagawa[5], Susumu Hirose, Kenshi Yamasaki[3], Yuji Shirakata[3], Koji Hashimoto[3] and Masaharu Ito[1] ([1]Departments of Obstetrics and Gynecology [2]Neurosurgery and [3]Dermatology, School of Medicine, Ehime University, Ehime 791-0295, Japan [4]Division of Oncogene Research, Research Institute for Microbial Diseases, Osaka University, Osaka 565-0871, Japan. [5]Division of Pathology, Chiba Cancer Center Research Institute, Chiba 260-8717, Japan)

Little is known concerning promoters or gene therapy specific for ovarian cancer. To explore the potential use of *IAI.3B* isolated from ovarian cancer cells in gene therapy for ovarian cancer, we identified the promoter region of the *IAI.3B* gene and created a replication-selective adenovirus, *AdE3-IAI.3B*, driven by the promoter. Transient transfection experiments showed that the DNA segment located between -1816 and -1 bp resulted in preferential expression in ovarian cancer cells with negligible expression in squamous cell carcinoma and normal cells. The promoter activity of *IAI.3B* was almost the same as that of *cytomegalovirus* and an order of magnitude higher than those of *midkine* and *cyclooxygenase-2* in ovarian cancer cells. *AdE3-IAI.3B* replicated as efficiently as the wild-type

adenovirus and caused extensive cell killing in a panel of ovarian cancer cells *in vitro*. In contrast, squamous cell carcinoma and normal cells were not able to support *AdE3-IAI.3B* replication. In animal studies, *AdE3-IAI.3B* administered to flank and i.p. xenografts of ovarian cancer cells led to a significant therapeutic effect. These results demonstrate the usefulness of the *IAI.3B* promoter for generation of ovarian cancer-specific adenoviral vectors and provide a potential for the development of ovarian cancer-specific oncolytic viral therapies.[1)]

Publications

Papers

1. Hamada, K., Kohno, S., Iwamoto, M., Yokota, H., Okada, M., Tagawa, M., Hirose, S., Yamasaki, K., Shirakata, Y., Hashimoto, K. and Ito, M. (2003). Identification of the human IAI.3B promoter element and its use in the construction of a replication-selective adenovirus for ovarian cancer therapy. *Cancer Res.*, **63**, 2506-2512.
2. He, S., Bauman, D., Davis, J.S., Loyola, A., Nishioka, K., Gronlund, J.L., Reinberg, D., Meng, F., Kelleher, N., McCafferty, D.G. (2003) Facile synthesis of site-specifically acetylated and methylated histone proteins: reagents for evaluation of the histone code hypothesis. *Proc Natl Acad Sci U S A.* **100**, 12033-12038.
3. Liu, Q.-X., Jindra, M., Ueda, H., Hiromi, Y. and Hirose, S. (2003). *Drosophila* MBF1 is a co-activator for Tracheae Defective and contributes to the formation of tracheal and nervous systems. *Development*, **130**, 719-728.
4. Nishioka, K. and Reinberg, D. (2003) Methods and tips for the purification of human histone methyltransferases. *Methods.* **31**, 49-58.
5. Saunders, A., Werner, J., Andrulis, E. D., Nakayama, T., Hirose, S., Reinberg, D. and Lis, J. T. (2003). Tracking FACT and RNA polymerase II elongation complex through chromatin in vivo. *Science*, **301**, 1094-1096.
6. Simojima, T., Okada, M., Nakayama, T., Ueda, H., Okawa, K., Iwamatsu, A., Handa, H. and Hirose, S. (2003). Drosophila FACT contributes to *Hox* gene expression through physical and functional interactions with GAGA factor. *Genes Dev.* **17**, 1605-1616.
7. Sims, R.J. 3rd, Nishioka, K. and Reinberg, D. (2003) Histone lysine methylation: a signature for chromatin function. *Trends Genet.* **19**, 629-639.
8. Markova, E. P., Ueda, H., Sakamoto, K., Oishi, K., Shimada, T. and Takeda, M. (2003) Cloning of Cyc (Bmal-1) homolog in *Bombyx mori*, Structural analyses and the tissue specific distributions. *Comp.Biochem. Physiol.* B. **134**, 535-542.

Reviews

9. 西岡憲一(2003)「ヒストンH４リジン20のメチル化によるクロマチン高次構造の制御」細胞工学**22**, 669-674.
10. 広瀬　進(2003)「クロマチンリモデリングとGAGA因子」生体の科学　**55**, 193-196.
11. 広瀬　進(2003)「ショウジョウバエのクロマチンと遺伝子制御」"クロマチンと遺伝子機能制御". 掘越正美編(シュプリンガー・フェアラーク東京), pp233-240.
12. 上田　均(2003)「昆虫発生過程におけるホルモン制御による遺伝子カスケード」蛋白質核酸酵素　48, 2254-2260.

EDUCATION

1. Dr. S. Hirose was invited to give a seminar on "Chromatin remodeling as a boundary against silencing" at the Institute of Molecular and Cellular Biology, The University of Tokyo, Tokyo, June, 2003 (in Japanese).
2. Dr. S. Hirose gave a lecture at University of Shizuoka, Shizuoka, December, 2003 (in Japanese).
3. Dr. S. Hirose was invited to give a seminar on "Chromatin remodeling serves as boundary against silencing" at Department of Medicine, Okayama University, Okayama, November, 2003 (in Japanese).

SOCIAL CONTRIBUTIONS AND OTHERS

特許

1. 出願番号：2003-146059, 発明の名称：負の超らせんDNA検出方法, 発明者：広瀬　進・松本国治, 出願人：国立遺伝学研究所長

C-c. Division of Molecular and Developmental Biology Koichi Kawakami Group

RESEARCH ACTIVITIES

(1) Gene trap approaches in zebrafish

Koichi Kawakami, Yasuyuki Kishimoto and Ghislaine Morvan

In order to understand the genetic basis for developmental processes in vertebrate, we have been using a small tropical fish, the zebrafish, as a model animal. Because it is practically possible to breed and maintain very large numbers of fish in the lab, and because zebrafish embryos develop in water and are transparent, forward genetic approaches (i.e., collecting a large number of mutations affecting developmental processes and analyzing genes responsible for the mutant phenotypes) are feasible in the fish. Taking advantage of these merits, large-scale chemical mutagenesis screens in zebrafish had been successfully carried out and hundreds of mutants defective in every aspect of early developmental processes had been collected. It has, however, not been easy to identify the mutated genes since most of the chemically induced mutations were point mutations and time-consuming positional cloning approaches are required to clone the genes. Therefore it is important to develop insertional mutagenesis methods in zebrafish, such as the P transposon system in Drosophila.

The *Tol2* element is a transposable element identified from the genome of the medaka fish. Previously we have found that the *Tol2* element encodes a fully functional transposase and have shown that the *Tol2* element can transpose into the zebrafish genome in the germ lineage. To date, the *Tol2* element is the only natural transposon in vertebrate, that has ever been shown to encode a functional transposase, and thereby from which an autonomous element has been identified. We have been interested in developing novel insertional mutagenesis methods in zebrafish using the *Tol2* element. Using this *Tol2* transposon system, we successfully established a protocol to generate transgenic zebrafish very efficiently. We then made an exon trap construct with a promoter-less GFP gene using the *Tol2* transposon, and generated hundreds of insertions of the exon trap construct in the zebrafish genome. 10% of the insertions yielded "specific" GFP expression, indicating that the gene (exon) trap method using the *Tol2* transposon system is successfully working. In those fish, GFP was expressed the heart, forebrain, midbrain, midbrain-hindbrain boundary, hindbrain, notochord, floorplate, etc. Our current goal is to establish methods to identify genes expressed in a spatially and temporally regulated fashion and play an important role in development of the structure or the organ where the GFP expression is detected. Efforts are in progress toward this goal.

(2) Genetic analysis of zebrafish maternal-effect mutations affecting early embryogenesis

Yasuyuki Kishimoto, Koichi Kawakami, Sumito Koshida[2], Makoto Furutani-Seiki[1] and Hisato Kondoh[1,3] ([1]Kondoh differentiation project (ERATO) JST; [2]Center for Integrative Bioscience, Okazaki National Research Institute; [3]Osaka University)

Maternal-effect genes play essential roles in early embryogenesis particularly before activation of the zygotic genes. We previously carried out a genetic screen for mutations affecting such maternal-effect genes employing an F3 screen strategy, identifying 6 recessive mutations out of 60 mutagenized genomes[1]. 4 mutations exhibiting characteristic phenotype were analyzed. Three of the mutations *(acytokinesis* mutations: ack^{kt5}, ack^{kt62} and ack^{kt119}*)*caused absence of cell cleavage, without affecting nuclear divisions. These embryos are defective in generating contractile rings. ack^{kt62} mutation abolishing reactions to organize cortical F-actin, while other mutations causing abortive contractile ring-like structures at ectopic sites. Defect of contractile ring formation in the affected embryos leads to the absence of microtubule arrays at the prospective cleavage plane. Thus, these mutations reveal the sequence of events associated with cytokinesis, in particular, the cortical actin dynamics. It is remarkable that in all acytokinetic embryos, daughter nuclei after mitosis are arranged in spatially normal positions, and maternal *vasa* mRNAs accumulate in the prospective planes of the first and second cell cleavages in the total absence of cytokinesis. This indicates that the basic cell architectures of early embryos are largely established by the autonomous activities of the mitotic apparatus, without much dependence on the cell cleavage machinery.

The *bobtail (btl)* mutant exhibited a recessive maternal effect causing a strong reduction of the tailbud outgrowth and abnormal posterior mesodermal development. Whole mount in situ hybridization analysis revealed that, in *btl* mutant embryos, *myoD* expression in adaxial and paraaxial mesoderm is strongly reduced without affecting the level of *no tail* expression in axial mesoderm. *btl* is mapped on chromosome 17, and positional cloning of the gene mutated in *btl* is under way.

(3) Transposition of the *Tol2* element in mouse embryonic stem cells.

Koichi Kawakami and Tetsuo Noda[1] ([1]Tohoku University)

The *Tol2* transposable element of the Japanese medaka fish belongs to the hAT family of transposons including hobo of Drosophila, Ac of maize, and Tam3 of snapdragon. To date, *Tol2* is the only natural transposon in vertebrate that has ever been shown to encode a fully functional transposase. It has not been known, however, whether *Tol2* can transpose in vertebrates other than fish. We demonstrated transposition of *Tol2* in mouse embryonic stem (ES) cells. We constructed a transposon donor plasmid containing a nonautonomous *Tol2* element with the neomycin resistance gene and a helper plasmid capable of expressing the transposase, and introduced the donor plasmid with various amounts of the helper plasmid by electroporation into mouse ES cells. The number of G418-resistant ES colonies increased as the amount of helper plasmid was increased, in a dose-dependent manner, indicating that the transposase activity elevated the integration efficiency. These G418-resistant ES colonies were cloned and the structure of the junction of the integrated *Tol2* element and the genomic DNA was analyzed by inverse PCR. In those clones, *Tol2* was surrounded by mouse genomic sequences and an 8-bp direct repeat was created adjacent to the both ends of *Tol2*, indicating that *Tol2* was integrated in the genome through transposition. The *Tol2* transposon system is thus active in mouse as well as in fish. We propose that it should be used as a genetic tool to develop novel gene transfer, transgenesis and mutagenesis methods in mammals.

(4) The Fugu EST projects

Melody S. Clark[1], Yvonne J.K. Edwards[1], Dan Peterson[2], Sandra W. Clifton[2], Amanda J. Thompson[1], Masahide Sasaki[3], Yutaka Suzuki[3], Kiyoshi Kikuchi[4], Shugo Watabe[4], Koichi Kawakami, Sumio Sugano[3], Greg Elgar[1] and Stephen L. Johnson[2] ([1]MRC Rosalind Franklin Centre for Genomics Research, UK; [2]Washington University Medical School, USA; [3]The Institute of Medical Science, University of Tokyo; [4]Graduate School of Agricultural and Life Sciences, University of Tokyo)

The draft Fugu rubripes genome was released in 2002, at which time relatively few cDNAs were available to aid in the annotation of genes. We sequenced and analyzed 24,398 expressed sequence tags (ESTs) generated from 15 different adult and juvenile Fugu tissues, 74% of which matched protein database entries. Analysis of the EST data compared with the Fugu genome data predicts that approximately 10,116 gene tags have been generated, covering almost one-third of Fugu predicted genes. This represents a remarkable economy of effort. Comparison with the Washington University zebrafish EST assemblies indicates strong conservation within fish species, but significant differences remain. This potentially represents divergence of sequence in the 5' terminal exons and UTRs between these two fish species, although clearly, complete EST data sets are not available for either species. This project provides new Fugu resources, and the analysis adds significant weight to the argument that EST programs remain an essential resource for genome exploitation and annotation. This is particularly timely with the increasing availability of draft genome sequence from different organisms and the mounting emphasis on gene function and regulation.

(5) Characterization of the *hagoromo* gene in East African cichlids

Yohei Terai[1], Naoko Morikawa[1], Koichi Kawakami and Norihiro Okada[1] ([1]Graduate School of Bioscience and Biotechnology, Tokyo Institute of Technology)

The adaptive radiation of cichlid fishes in the lakes of East Africa is a prime example of speciation. The choice of cichlid mates on the basis of a variety of coloration represents a potential basis for speciation that led to adaptive radiation. We characterized the cichlid homolog of the zebrafish hagoromo *(hag)* gene that was recently cloned and characterized from a pigmentation mutant. Although only one *hag* mRNA was reported in zebrafish, cichlids express nine different hag mRNAs resulting from alternative splicing. The *hag* mRNAs are expressed between the myotome and the epidermis where pigment cells are located, suggesting the cichlid hag gene is involved in pigmentation. The *hag* mRNA splicing pattern does not fluctuate among individuals from each of two species, suggesting that alternative splice site choice

is fixed within species. Furthermore, cichlids in lineages that underwent explosive speciation expressed a greater variety of *hag* mRNAs than those in lineages that did not undergo such a degree of speciation, suggesting that species in the explosively speciated lineage acquired a complex regulatory mechanism of alternative splicing over a very short evolutionary period. Thus we provide an example in which alternative splicing may play a role in mate choice, leading to cichlid speciation through diversification of gene function by production of multiple mRNAs from a single gene.

Publications

Papers

1. Kishimoto, Y., Koshida, S., Furutani-Seiki, M. and Kondoh H. (2004). Zebrafish maternal-effect mutations causing cytokinesis defect without affecting mitosis or equatorial vasa deposition. Mech Dev. **121**, 79-89.
2. Kawakami, K. and Noda, T. (2004). Transposition of the *Tol2* element, an *Ac*-like element from the Japanese medaka fish Oryzias latipes, in mouse embryonic stem cells. Genetics. **166**, 895-899.
3. Clark, M.S., Edwards, Y.J.K., Peterson, D., Clifton, S.W., Thompson, A.J., Sasaki, M., Suzuki, Y., Kikuchi, K., Watabe, S., Kawakami, K., Sugano. S., Elgar, G. and Johnson, S.L. (2003). Fugu ESTs: New resources for transcription analysis and genome annotation. Genome Research **13**, 2747-2753.
4. Terai, Y., Morikawa, N., Kawakami, K. and Okada, N. (2003). The complexity of alternative splicing of hagoromo mRNAs is increased in an explosively speciated lineage in East African cichlids. Proc. Natl. Acad. Sci. USA **100**, 12798-12803.

EDUCATION

K. Kawakami gave a lecture at Sars Center for Marine Molecular Biology, Norway, March, 2003.

SOCIAL CONTRIBUTIONS AND OTHERS

K. Kawakami organized a symposium at 26th the molecular biology society of Japan annual meeting, Kobe, Dec. 2003.

C-d. Division of Physiological Genetics Haruhiko Koseki Group

RESEARCH ACTIVITIES

(1) Functional and biochemical analyses of mammalian Polycomb-group proteins

Haruhiko Koseki

1) Association of mammalian PcG proteins at Hoxb8 gene in mice

Mammalian Ring1B is involved in at least two disticnt multimeric protein complexes both are closely related to Drosophila Polycomb reppressive complex-1 (PRC1). We have previously shown that Ring1B mediates transcriptional repression of Hox cluster genes (Suzuki et al., 2002). To understand how Ring1B functions on Hox cluster, we have compared the binding pattern of Ring1B on Hoxb7/8/9 region by using chromatin immunoprecipitation with 500 to 1000 bp resolution between cranial and caudal regions of 11.5 dpc embryos where Hoxb7/8/9 are repressed or transcribed, respectively. Ring1B turned out to associate predominantly to promotor and enhancer regions in cranial region where Hoxb7/8/9 are transcriptionally inactive. Closely related protein Ring1A distribution was very similar to Ring1B, however, other PcG components, Rae28/Mph1 and M33, were associated with Hoxb7/8/9 region in both cranial and caudal regions. This implies Polycomb complexes are associated with chromatin in not only transcriptionally inactive but also active region. We further examined degree of HistonH3 acetylation and methylation on Hoxb7/8/9 regions. Acetylated HistonH3 predominantly associated to Hoxb7/8/9 regions in the caudal region. In addition, we found colinearity between gene order and acetylation of HistonH3. We did not find out significant methylation of HostonH3. These observations suggest that inversely graded associations of mammalian polycomb Ring1B and acetylated HistoneH3 associations upon Hoxb7/8/9 region is involved in the maintenance of anterior boundaries of their expression.

2) Role of PcG proteins during organ development

We have isolated Mph2 (Mouse polyhomeotic-2) as an interacter of Mel18, a homologue of Drosophila

posterior sex combs (Psc) and demonstrated that Mph2 is an component of mammalian PcG complexes. We have previously reported Mel18 mutants show defects in lymphoid development that include subtle hypoplasia of Peryer's patches (PPs). Mph2 single deficient mice showed posterior transformations in the axial skeleton but did not exhibit obvious defects in lymphoid development. Mph2 mutation, however, was shown to enhance defects in PP development observed in Mel18 mutants. In Mph2/Mel18 doubly deficient mice, accumulation of haematopoietic cells into prospective PP anlagen may be significantly affected and subsequently condensation of PP anlagen were clearly impaired. Either number and proliferation of PP inducer cells in the developing intestine or their migratory activity upon BLC signaling was not significantly affected in double mutants. Immunohistochemical analysis revealed BrdU-labeled CD45-positive cells were reduced exclusively in PP anlagen in gene-dosage dependent manner. It is thus suggested PcG multimeric complexes may be essential for proliferation of lymphoid-lineage cells immigrated into PP anlagen. Mechanisms underlying this defect is currently addressed.

Publications

Papers

1. Shirasawa, T., Izumizaki, M., Suzuki, YI., Ishihara, A., Shimizu, T., Tamaki, M., Huang, F., Koizumi, KI., Iwase, M., Sakai, H., Tsuchida, E., Ueshima, K., Inoue, H., Koseki, H., Senda, T., Kuriyama, T. and Homma, I. (2003) Oxygen affinity of hemoglobin regulates O2 consumption, metabolism, and physical activity. J Biol Chem **278**, 5035-5043.

2. Fukuda, K., Yoshida, H., Sato, T., Furumoto, T., Mizutani-Koseki, Y., Suzuki, Y., Saito, Y., Takemori, T., Kimura, M., Sato, H., Nakayama, T., Taniguchi, M., Nishikawa, S. and Koseki, H. (2003) Mesenchymal expression of Foxl1, a winged helix transcriptional factor, regulates generation and maintenance of gut associated lymphoid organs. Dev. Biol. **255**, 278-289.

3. Yamada, R., Mizutani-Koseki, Y., Hasegawa, T., Osumi, N., Koseki, H. and Takahashi. N. (2003). Requirement for Mab21l1 during development of murine eye and preputial gland. Development **130**, 1759-1770.

4. Horie, A., Isono, K. and Koseki, H. (2003) Generation of a monoclonal antibody against the mouse Sf3b1 (SAP155) gene product for U2 snRNP component of spliceosome. Hybridoma and Hybridomics **22**, 117-119.

5. Kaneko, T., Miyagishima, H., Hasegawa, T., Mizutani-Koseki, Y., Isono, K. and Koseki, H. (2003) Mouse YAF2 gene generates two distinct transcripts and is expressed in pre- and postimplantation embryos. Gene **315**, 183-192.

6. Miyagishima, H., Isono, K., Fujimura, Y., Iyo, M., Takihara, Y., Masumoto, H., Vidal, M. and Koseki, H. (2003) Dissociation of mammalian Polycomb-group proteins, Ring1B and Rae28/Ph1, from the chromatin correlates with configuration changes of the chromatin in mitotic and meiotic prophase. Histochem Cell. Biol **120**, 111-119.

7. Nakamura, Y., Ozaki, T., Koseki, H., Nakagawara, A. and Sakiyama, S. (2003) Accumulation of p27(KIP1) is associated with BMP2-induced growth arrest and neuronal differentiation of human neuroblastoma-derived cell lines. Biochem Biophys Res Commun. **307**, 206-213.

8. Shin, T., Kennedy, G., Gorski, K., Tsuchiya, H., Koseki, H., Azuma, M., Yagita, H., Chen, L., Powell, J., Pardoll, D. and Housseau, F. (2003) Cooperative B7-1/2 (CD80/CD86) and B7-DC Costimulation of CD4+ T Cells Independent of the PD-1 Receptor. J Exp Med. **198**, 31-38.

9. Nakajima, M., Yuasa, S., Ueno, M., Takakura, N., Koseki, H. and Shirasawa, T. (2003) Abnormal blood vessel development in mice lacking presenilin-1. Mech Dev. **120**, 657-667.

10. Koike, J., Wakao, H., Ishizuka, Y., Sato, TA., Hamaoki, M., Seino, K., Koseki, H., Nakayama, T. and Taniguchi, M. (2004) Bone Marrow Allograft Rejection Mediated by a Novel Murine NK Receptor, NKG2I. J Exp Med.**199**,137-144.

11. Okazaki, N., Kikuno, R., Ohara, R., Inamoto, S., Koseki, H., Hiraoka, S., Saga, Y., Nagase, T., Ohara, O. and Koga, H. (2003) Prediction of the coding sequences of mouse homologues of KIAA gene: Ⅲ. the complete nucleotide sequences of 500 mouse KIAA-homologous cDNAs identified by screening of terminal sequences of cDNA clones randomly sampled from size-fractionated libraries. DNA Res.**10**,167-80.

12. D, Graaff, W., Tomotsune, D., Oosterveen, T., Takihara, Y., Koseki, H. and Deschamps, J. (2003) Randomly inserted and targeted Hox/reporter fusions transcriptionally silenced in Polycomb mutants. Proc

Natl Acad Sci U S A. **100**, 13362-13367.
13. Hoki, Y., Araki, R., Fujimori, A., Ohhata, T., Koseki, H., Fukumura, R., Nakamura, M., Takahashi, H., Noda, Y., Kito, S. and Abe, M. Growth retardation and skin abnormalities of the Recql4-deficient mouse. Hum Mol Genet.**12**, 2293-2299.
14. Murata, K., Inami, M., Hasegawa, A., Kubo, S., Kimura, M., Yamashita, M., Hosokawa, H., Nagao, T., Suzuki, K., Hashimoto, K., Shinkai, H., Koseki, H., Taniguchi, M., Ziegler, SF. and Nakayama, T. (2003) CD69-null mice protected from arthritis induced with anti-type II collagen antibodies. Int Immunol.**15**, 987-992.
15. Yamamoto, K., Hamada, H., Shinkai, H., Kohno, Y., Koseki, H. and Aoe, T. (2003) The KDEL receptor modulates the endoplasmic reticulum stress response through mitogen-activated protein kinase signaling cascades. J Biol Chem. **278**, 34525-34532.
16. Wang, T., Tamakoshi, T., Uezato, T., Shu, F., Kanzaki-Kato, N., Fu, Y., Koseki, H., Yoshida, N., Sugiyama, T. and Miura, N. Forkhead transcription-factor Foxf2 (LUN)-deficient mice exhibit abnormal development of secondary palate. Dev Biol.**259**, 83-94.

Books

17. 古関　明彦「発生・細胞分化過程におけるエピジェネティックな転写制御の役割」, クロマチンと遺伝子機能制御(シュプリンガー・フェアラーク・堀越正美編), 187-195, (2003).

EDUCATION

1. 古関明彦　“理研筑波セミナー”理化学研究所バイオリソースセンター　2003年5月
2. 古関明彦　“第2回千葉循環器フロンティア研究会”かずさアカデミアパーク　2003年5月
3. 古関明彦　千葉市民文化大学「ヒトのからだのでき方」千葉市文化センター　2003年6月

C-d. Division of Physiological Genetics
Yukiko Gotoh Group

RESEARCH ACTIVITIES

Regulation of neural development and cell death

Koji Oishi, Maiko Higuchi, Fuminori Tsuruta, Jun Sunayama, Yoko Ogawara, Norihisa Masuyama and Yukiko Gotoh

During neural development, a large number of cells undergo programmed cell death to eliminate damaged cells and developmentally unwanted cells, mostly through a process called apoptosis. Caspases, a family of cysteine proteases, play a central role in induction of apoptosis. Gene disruption of caspase-9 results in exencephaly due to a marked increase of neural precursor cells (NPCs), suggesting the cell death/survival regulation of NPCs is critical for neural development. We found that NPCs prepared from mouse forebrain neuroepithelial cultures survived better under high-density conditions. The density-dependent survival-promoting effect was likely to require the Notch signaling, since a gamma-secretase inhibitor, which inhibits the Notch processing, suppressed it. Expression of an active form of Notch was sufficient for promotion of NPC survival. Importantly, Hes1 and Hes5, two effectors of the Notch signaling in inhibition of neuronal differentiation, could not promote NPC survival. Therefore, Notch appears to have two independent functions in NPCs, namely, Hes-dependent inhibition of neuronal differentiation and promotion of survival through unknown mechanisms.

This year we have also found that phosphorylation of 14-3-3 proteins by the stress-activated kinase JNK is a key event in regulation of cell death and that Akt plays a crucial role in regulation of neurite morphogenesis during neuronal differentiation.

Publications

Papers

1. Kamakura, S., Oishi, K., Yoshimatsu, T., Nakafuku, M., Masuyama, N. and Gotoh, Y. (2004). Hes binding to STAT3 mediates crosstalk between Notch and JAK-STAT signaling. Nat. Cell Biol. In press.

2. Hirabayashi, Y., Itoh, Y., Tabata, H., Nakajima, K., Akiyama, T., Masuyama, N. and Gotoh, Y. (2004). The Wnt-beta-catenin pathway directs neuronal differentiation of cortical neural precursor cells. Development **131**, 2791-2801.

3. Tsuruta, F., Sunayama, J., Mori, Y., Shimizu, S., Tsujimoto, Y., Yoshioka, K., Masuyama, N. and Gotoh, Y. (2004). JNK promotes Bax translocation to mitochondria through phosphorylation of 14-3-3 proteins. EMBO J. **23**, 1889-1899.

4. Miyagi, S., Saito, T., Mizutani, K., Masuyama, N., Gotoh, Y., Iwama, A., Nakauchi, H., Masui, S., Niwa, H., Nishimoto, M., Muramatsu, M. and Okuda, A. (2004). The sox-2 regulatory regions display their activities in two distinct multipotent stem cells. Mol. Cell. Biol. In press.

5. Higuchi, M., Onishi, K., Masuyama, N. and Gotoh, Y. (2003). The phosphatidylinositol-3 kinase (PI3K)-Akt pathway suppresses neurite branch formation in NGF-treated PC12 cells. Genes Cells 8, 657.

6. Suzawa, M., Takada, I., Yanagisawa, J., Ohtake, F., Ogawa, S., Yamaguchi, T., Kadowaki, T., Takeuchi, Y., Shibuya, H., Gotoh, Y., Matsumoto, K. and Kato, S. (2003). Cytokines suppress adipogenesis and PPAR-gamma function through the TAK1/TAB1/NIK cascade. Nat. Cell Biol. **5**, 224-230.

7. Fujishiro, M., Gotoh, Y., Katagiri, H., Sakoda, H., Ogihara, T., Anai, M., Onishi, Y., Ono, H., Abe, M., Shojima, N., Fukushima, Y., Kikuchi, M., Oka, Y. and Asano, T. (2003). Three Mitogen-Activated Protein kinases inhibit Insulin signaling by different mechanisms in 3T3-L1 adipocytes. Mol. Endocrinology **17**, 487-497.

8. Sakoda, H., Gotoh, Y., Katagiri, H., Kurokawa, M., Ono, H., Onishi, Y., Anai, M., Ogihara, T., Fujishiro, M., Fukushima, Y., Abe, M., Shojima, N., Kikuchi, M., Oka, Y., Hirai, H. and Asano, T. (2003). Differing roles of Akt and SGK in glucose metabolism, DNA synthesis and oncogenic activity. J. Biol. Chem. **278**, 25802-25807.

9. Katome, T., Obata, T., Matsushima, R., Masuyama, N., Cantley, L.C., Gotoh, Y., Kishi, K., Shiota, H. and Ebina, Y. (2003). Use of RNA-interference-mediated gene silencing and adenoviral overexpression to elucidate the roles of AKT/PKB-isoforms in insulin actions. J. Biol. Chem. **278**, 28312-28323.

Reviews

10. 増山典久・後藤由季子, 神経系細胞の生存シグナル, 実験医学　増刊, 21(2), pp258-264, 2003.

11. 樋口麻衣子・後藤由季子, PI3K-Akt経路による癌の悪性化, 実験医学, 21(18), pp2499-2504, 2003.

EDUCATION

東京大学工学系研究科化学生命工学専攻・兼担
東京大学新領域創成科学研究科先端生命科学専攻・兼担
岡崎国立共同研究機構生理学研究所生体情報研究系 客員助教授

SOCIAL CONTRIBUTIONS AND OTHERS

Editor:
Oncogene
Journal of Biochemistry (Associate Editor)
Cell Structure and Function (2001)
日本細胞生物学会　庶務幹事
日本学術会議・細胞生物学研究連絡委員会(第18期)委員
第1回日本分子生物学会奨励賞受賞

D. DEPARTMENT OF POPULATION GENETICS

D-a. Division of Population Genetics Naruya Saitou Group

RESEARCH ACTIVITIES

(1) Construction of a gorilla fosmid library and its PCR screening system

Choong-Gon Kim, Asao Fujiyama[1] and Naruya Saitou ([1]National Institute of Informatics)

A gorilla fosmid library of 261,120 independent clones was constructed and characterized. The fosmid vector is similar to the cosmid in average insert size of ca. 40 kb but contains the F factor for replication, and it is more resistant to recombination. This clone library represents about 3.7 times coverage of the gorilla genome. A simple screening system by PCR was established, and we successfully found 9 clones that cover the entire Hox A gene cluster of the gorilla genome. This gorilla fosmid DNA library is a useful resource for comparative genomics of human and apes. For details, see ref. (1).

(2) Netview: Aplication software for constructing and visually exploring phylogenetic networks

Kirill Kryukov and Naruya Saitou

Reconstructing evolutionary history of a group of species is a major task in biological study. Many methods exist for reconstructing such history or phylogeny, but most of them are based on an assumption that evolutoin of given gene family can be represented as a tree. However some families of genes may have alternative historical structure that cannot be represented as a tree. Such history can result from events such as recombination, gene conversion and horizontal gene transfer, and require not tree but network for accurate representation. Parallel substitutions are also sources of non-tree networks. For the purpose of understanding such history we developed a program Netview, which enables constructing phylogenetic network based on the sequence data. For details, see ref. (2).

(3) Understanding the dynamics of Spinocerebellar Ataxia 8 (SCA8) locus through a comparative genetic approach in humans and apes

Aida M. Andres[1], Marta Soldevila[1], Naruya Saitou, Victor Volpini[1], Francesc Calafell[1] and Jaume Bertranpetit[1] ([1] Unitat de Biologia Evolutiva, Facultat de Ciencies de la Salut i de la Vida, Universitat Pompeu Fabra)

Spinocerebellar Ataxia 8 (SCA8) is a neurodegenerative disorder caused by expansion of a trinucleotide repeat. We undertake a comparative genetic analysis among human populations and primate species in the normal variation range, where forces that shaped present diversity can be recognised. We determinate number of repeats of the short tandem repeat through allele length sizing and sequencing methods. Human allele distributions are very similar among populations, ruling out ethnicity as a genetic risk for allele expansion. Primate comparison shows human-specific features, with longer human alleles due to a novel variable trinucleotide repeat, not present in non-human primates, which increased the disease-causing expansion likelihood. SCA8 seems to be a human specific disease. For details, see ref. (3).

(4) Evolution of the cystatin B gene: implications for the origin of its variable dodecamer tandem repeat in humans

Motoki Osawa.[1], Mika Kaneko[1], Hidekazu Horiuchi[1], Takashi Kitano, Yoshi Kawamoto[2], Naruya Saitou and Kazuo Umetsu[1] ([1]Yamagata University School of Medicine, [2]Kyoto University)

The human cystatin B gene contains a variable number of 12-bp tandem repeats in its promoter region, of which the common alleles contain two or three copies and unusual expansion causes progressive myoclonus epilepsy of the Unverricht-Lundborg type. We undertook a comprehensive analysis of the

genomic sequence to address the evolutionary events of this variable repeat. By examination of a contiguous genome sequence spanning 5.0 kb and linkage analysis of detected polymorphic changes, we identified six major intragenic haplotypes in unrelated Japanese subjects. The number of normal repeats was closely correlated with these alleles, indicating that changes in the array should be comparatively rare events during human evolution. To examine the origin of the repeat array further, we also analyzed five primate genomes. Repetitive polymorphism was unlikely in hominoids, and the array originated with the dodecamer itself in the course of primate evolution. The variability conceivably developed after the separation to humans. For details, see ref.(4).

(5) Synonymous mutations in the human dopamine receptor D2 (DRD2) affect mRNA stability and synthesis of the receptor

Jubao Duan[1], Mark S. Wainwright[1], Josep M. Comeron[1], Naruya Saitou, Alan R. Sanders[1], Joel Gelernter[1] and Pablo V. Gejman[1] ([1]Department of Psychiatry, The University of Chicago, Jules F. Knapp Medical Research Center)

Although changes in nucleotide sequence affecting the composition and the structure of proteins are well known, functional changes resulting from nucleotide substitutions cannot always be inferred from simple analysis of DNA sequence. Because a strong synonymous codon usage bias in the human DRD2 gene, suggesting selection on synonymous positions, was revealed by the relative independence of the G+C content of the third codon positions from the isochoric G+C frequencies, we chose to investigate functional effects of the six known naturally occurring synonymous changes (C132T, G423A, T765C, C939T, C957T, and G1101A) in the human DRD2. We report here that some synonymous mutations in the human DRD2 have functional effects and suggest a novel genetic mechanism. 957T, rather than being 'silent', altered the predicted mRNA folding, led to a decrease in mRNA stability and translation, and dramatically changed dopamine-induced up-regulation of DRD2 expression. 1101A did not show an effect by itself but annulled the above effects of 957T in the compound clone 957T/1101A, demonstrating that combinations of synonymous mutations can have functional consequences drastically different from those of each isolated mutation. C957T was found to be in linkage disequilibrium in a European-American population with the -141C Ins/Del and TaqI 'A' variants, which have been reported to be associated with schizophrenia and alcoholism, respectively. These results call into question some assumptions made about synonymous variation in molecular population genetics and gene-mapping studies of diseases with complex inheritance, and indicate that synonymous variation can have effects of potential pathophysiological and pharmacogenetic importance. For details, see ref. (5).

(6) Mitochondrial DNA polymorphisms in nine aboriginal groups of Taiwan: implications for the population history of aboriginal Taiwanese

Atsushi Tajima[1], Takafumi Ishida[2], Naruya Saitou and Satoshi Horai[1] ([1]Department of Biosystems Science, Graduate University for Advanced Studies [2] Department of Biological Sciences, Graduate School of Science, University of Tokyo)

Mitochondrial DNA (mtDNA) polymorphisms in the D-loop region and the intergenic COII/tRNA(Lys) 9-bp deletion were examined in 180 individuals from all nine aboriginal Taiwanese groups: Atayal, Saisiat, Bunun, Tsou, Rukai, Paiwan, Ami, Puyuma, and Yami. A comparison of 563-bp sequences showed that there were 61 different sequence types, of which 42 types were specific to respective aboriginal groups. D-loop sequence variation and phylogenetic analysis enabled the 180 aboriginal lineages to be classified into eight monophyletic clusters (designated C1-C8). Phylogeographic analysis revealed that two (C2 and C4) of the eight clusters were new characteristic clusters of aboriginal Taiwanese and accounted for 8.3% and 13.9% of the aboriginal lineages, respectively. From the estimated coalescent times for the two unique clusters, the mtDNA lineages leading to such clusters were inferred to have been introduced into Taiwan approximately 11,000-26,000 years ago, suggesting ancient immigrations of the two mtDNA lineages. Genetic distances, based on net nucleotide diversities between populations, revealed three distinct clusters that were comprised of northern mountain (Atayal and Saisiat), southern mountain (Rukai and Paiwan), and middle mountain/east coast (Bunun, Tsou, Ami, Puyuma, and Yami) groups, respectively. Furthermore, phylogenetic

analysis of 16 human populations (including six other Asian populations and one African population) confirmed that the three clusters for aboriginal Taiwanese had remained largely intact. Each of the clusters (north, south, and middle-east coast) was characterized by a high frequency of a particular lineage (C4, C2, and 9-bp deletion, respectively). This may result from random genetic drift among the aboriginal groups after a single introduction of all the mtDNA lineages into Taiwan, but another plausible explanation is that at least three genetically distinct ancestral populations have contributed to the maternal gene pool of aboriginal Taiwanese. For details, see ref. (6).

(7) Sequence polymorphisms of the mitochondrial DNA control region and phylogenetic analysis of mtDNA lineages in the Japanese population

Sayaka Maruyama[1], Kiyoshi Minaguchi[1] and Naruya Saitou ([1]Department of Forensic Odontology, Tokyo Dental College)

Sequence polymorphisms of the hypervariable mitochondrial DNA (mtDNA) regions HVI and HVII, and coding region polymorphisms were investigated in 211 unrelated individuals from the Japanese population. Sequence comparison of the HVI and HVII regions led to the identification of 169 mitochondrial haplotypes defined by 147 variable positions. Among them 145 types were observed in only 1 individual; the other 24 types were shared by 2 or more individuals. The gene diversity was estimated at 0.9961, and the probability of two randomly selected individuals from the population having identical mtDNA types was 0.86%. We also established phylogenetic haplogroups in the Japanese population based on the coding and control region polymorphisms and compared the haplotypes with those in other Japanese, Korean and Chinese populations. As a result, three new sub-haplogroups, G4a, G4b, and N9b, and several haplotypes specific for the Japanese and Korean populations were identified. The present database can be used not only for personal identification but also as an aid for geographic or phenotype (race) estimation in forensic casework in Japan. For details, see ref. (7).

(8) Microsatellite variation in Japanese and Asian horses and their phylogenetic relationship using a European horse outgroup

Teruaki Tozaki[1], Naoko Takezaki[2], Telhisa Hasegawa[1], Nobushige Ishida[1], Masahiko Kurosawa[1], Motowo Tomita[1], Naruya Saitou and Harutaka Mukoyama[1] ([1]Department of Molecular Genetics, Laboratory of Racing Chemistry, [2]Max Planck Institute, Tubingen)

The genetic relationships of seven Japanese and four mainland-Asian horse populations, as well as two European horse populations, were estimated using data for 20 microsatellite loci. Mongolian horses showed the highest average heterozygosities (0.75-0.77) in all populations. Phylogenetic analysis showed the existence of three distinct clusters supported by high bootstrap values: the European cluster (Anglo-Arab and thoroughbreds), the Hokkaido-Kiso cluster, and the Mongolian cluster. The relationships of these clusters were consistent with their geographical distributions. Basing our assumptions on the phylogenetic tree and the genetic variation of horse populations, we suggest that Japanese horses originated from Mongolian horses migrating through the Korean Peninsula. The genetic relationship of Japanese horses corresponded to their geographical distribution. Microsatellite polymorphism data were shown to be useful for estimating the genetic relationships between Japanese horses and Asian horses. For details, see ref. (8).

(9) The diversity of bovine MHC class II DRB3 genes in Japanese Black, Japanese Shorthorn, Jersey and Holstein cattle in Japan.

Shin-noske Takeshima[1], Naruya Saitou, Mitsuo Morita[1], Hisetoshi Inoko[2] and Yoko Aida[1] ([1]Retrovirus Research Unit, RIKEN, [2]Tokai University)

We sequenced exon 2 of the major histocompatibility complex (MHC) class II DRB3 gene from 471 individuals in four different Japanese populations of cattle (201 Japanese Black, 101 Holstein, 100 Japanese Shorthorn, and 69 Jersey cattle) using a new method for sequence-based typing (SBT). We identified the 34 previously reported alleles and four novel alleles. These alleles were 80.0-100.0% identical at the nucleotide level

and 77.9-100.0% identical at the amino acid level to the bovine MHC (BoLA)-DRB3 cDNA clone NR1. Among the 38 alleles, eight alleles were found in only one breed in this study. However, these alleles did not form specific clusters on a phylogenetic tree of 236-base pairs (bp) nucleotide sequences. Furthermore, these breeds exhibited similar variations with respect to average frequencies of nucleotides and amino acids, as well as synonymous and non-synonymous substitutions, in all pairwise comparisons of the alleles found in this study. By contrast, analysis of the frequencies of the various BoLA-DRB3 alleles in each breed indicated that DRB3 *1101 was the most frequent allele in Holstein cattle (16.8%), DRB3*4501 was the most frequent allele in Jersey cattle (18.1%), DRB3*1201 was the most frequent allele in Japanese Shorthorn cattle (16.0%) and DRB3 *1001 was the most frequent allele in Japanese Black cattle (17.4%), indicating that the frequencies of alleles were differed in each breed. In addition, a population tree based on the frequency of BoLA-DRB3 alleles in each breed suggested that Holstein and Japanese Black cattle were the most closely related, and that Jersey cattle were more different from both these breeds than Japanese Shorthorns. For details, see ref. (9).

Kenta Sumiyama, Assistant Professor

Evolutionary study of cis-element in mammalian development

Kenta Sumiyama studied evolution of genetic regulatory mechanism of developmental controlling genes, including Dlx and Hox genes. Dlx genes are indispensable factors for forming proper jaw structure. We have identified branchial arch specific cis-element by using both large scale genomic clone and transgenic mouse technologies (ref. 10). Gene duplication and subsequent functional diversification was analyzed on three Dlx clusters in mammal (ref. 11). Hoxc8 gene is regulated by an element named "hoxc8 early enhancer" located intergenic region of Hox cluster. We have analyzed evolutionary change in the Hoxc8 early enhancer throughout many mammalian species (ref. 12).

Publications

Papers

1. Kim, C.-G., Fujiyama, A. and Saitou, N. (2003) Construction of a Gorilla Fosmid Library and its PCR Screening System. Genomics, **82**, 571-574.
2. Kryukov, K. and Saitou, N. (2003) Netview: pplication Software for Constructing and Visually Exploring Phylogenetic Networks. Genome Informatics. **14**, 280-281.
3. Andres, A.M., Soldevila, M., Saitou, N., Volpini, V., Calafell, F. and Bertranpetit, J. (2003) Understanding the dynamics of Spinocerebellar Ataxia 8 (SCA8) locus through a comparative genetic approach in humans and apes. Neuroscience Letters, **336**, 143-146.
4. Osawa, M., Kaneko, M., Horiuchi, H., Kitano, T., Kawamoto, Y., Saitou, N. and Umetsu, K. (2003) Evolution of the cystatin B gene: implications for the origin of its variable dodecamer tandem repeat in humans. Genomics, **81**, 78-84.
5. Duan, J., Wainwright, M.S., JComeron, J.M., Saitou, N., Sanders, A.R., Gelernter, J. and Gejman, P.V. (2003) Synonymous mutations in the human dopamine receptor D2 (DRD2) affect mRNA stability and synthesis of the receptor. Human Molecular Genetics, **12**, 205-216.
6. Tajima, A., Cheih-Shan, Sun, C.-S., Pan, I-H., Ishida, T., Saitou, N. and Horai, S. (2003) Mitochondrial DNA polymorphisms in nine aboriginal groups of Taiwan: implications for the population history of aboriginal Taiwanese. Human Genetics, **113**, 24-33.
7. Maruyama, S., Minaguchi, K. and Saitou, N. (2003) Sequence polymorphisms of the mitochondrial DNA control region and phylogenetic analysis of mtDNA lineages in the Japanese population. International Journal of Legal Medicine, **117**, 218-225.
8. Tozaki, T., Takezaki, N., Hasegawa, T., Ishida, N., Kurosawa, M., Tomita, M., Saitou, N. and Mukoyama, H. (2003) Microsatellite variation in Japanese and Asian horses and their phylogenetic relationship using a European horse outgroup. Journal of Heredity, vol. 94, no. **5**, pp.374-380.
9. Takeshima, S., Saitou, N., Morita, M., Inoko, H. and Aida, Y. (2003) The diversity of bovine MHC class II DRB3 genes in Japanese Black, Japanese Shorthorn, Jersey and Holstein cattle in Japan. Gene **316**, 111-118.
10. Sumiyama, K. and Ruddle, F.H. (2003). Regulation of Dlx3 gene expression in visceral arches by evolutionarily conserved enhancer elements. Proceedings of the National Academy of Sciences of the United States of America **100**, 4030-4034.
11. Sumiyama, K., Irvine, S.Q. and Ruddle, F.H. (2003). The role of gene duplication in the evolution and

function of the vertebrate distal-less/Dlx bigene cluster. Journal of Structural and Functional Genomics 3, 151-159.
12. Kim, C.B., Shashikant, C.S., Sumiyama, K., Amemiya, C.T., Wang, W.C.H. and Ruddle, F.H. (2003). Phylogenetic Analysis of the Mammalian Hoxc8 Non-coding Region. Journal of Structural and Functional Genomics 3, 195-199.
13. 斎藤成也監訳〔ジョンジョー・マクファデン著，十河和代・十河誠治訳〕(2003)『量子進化』. 共立出版.
14. 斎藤成也(2003)第4章：遺伝子の多様性. 西田利貞・佐藤矩行編著『新しい教養のすすめ・生物学』, 73-88頁. 昭和堂.
15. 斎藤成也(2003)自然科学におけるデータ生成とその解釈に伴う問題.『第1回二十一世紀の人間科学』, フォーラム人文第5号, 61-69頁. 札幌学院大学人文学部.
16. 斎藤成也(2003)霊長類ゲノムの進化. 五條堀孝編著『ゲノムからみた生物の多様性と進化』, 171-176頁. シュプリンガー・フェアラーク東京.
17. 斎藤成也(2003)ゲノム進化. 田村・山本編『分子生物学イラストレイテッド改訂第2版』, 348-350頁. 羊土社.
18. 瀬名秀明・斎藤成也(2003)時空の旅：類人猿のゲノムで探る人間らしさの起源(対談). 日経サイエンス, 6月号, 94-99頁.

EDUCATION

1. 斎藤成也：お茶の水女子大学集中講義　3月
2. 斎藤成也：福井医科大学法医学講義　3月
3. 斎藤成也：総合研究大学院大学先導科学研究科生命体科学専攻集中講義　3月
4. 斎藤成也：東京大学理学部講義　5月，6月
5. 斎藤成也：北海道大学大学院農学研究科集中講義　7月
6. 斎藤成也：伊達市立有珠中学校講義　10月
7. 斎藤成也：静岡県立大学講義　10月
8. 斎藤成也：東京大学農学部集中講義　12月

SOCIAL CONTRIBUTIONS AND OTHERS

1. Molecular Biology and Evolution, Associate Editor
2. 日本遺伝学会　会計幹事
3. 日本進化学会　庶務幹事
4. 財団法人遺伝学普及会　監事

D-a. Division of Population Genetics Toshiyuki Takano Group

RESEARCH ACTIVITIES

(1) Non-random association between variants at the Drosophila olfactory and gustatory receptor genes

Akira Kawabe, Yuriko Ishii, Masanobu Itoh[1], Nobuyuki Inomata[2], Rumi Kondo[3], Noriko Nanba[1], Yutaka Inoue[4] and Toshiyuki Takano-Shimizu ([1]Kyoto Institute of Technology, [2]Kyushu University, [3]Ochanomizu University, [4]Osaka University of Foreign Studies)

Multilocus selection, besides genetic drift and gene flow, can generate linkage disequilibrium. Many previous allozyme analyses of Drosophila have failed to detect long-distance linkage disequilibrium, but this does not prove a general lack of linkage disequilibrium. Indeed, previous Drosophila studies have demonstrated the existence of recombination load, meaning that some natural variants are not randomly combined in an individual, but in linkage disequilibria because of advantageous and disadvantageous combinations of natural variants. With the availability of the complete DNA sequence, we are now in a position to be able to measure the degree of linkage disequilibria at the whole-genome level. As a first step, we took a genome-wide analysis of sequence variation in the olfactory and gustatory receptor families, identifying about 1700 nucleotide segregating sites including more than 300 replacement variations. What is more, we found twelve different putative null alleles at ten of 104 loci analyzed. By extrapolation, a single fly of *D. melanogaster* may have eight or more null alleles for these genes.

Using the variations identified, we genotyped about 400 wild-caught flies, 800 chromosomes from two natural populations of *D. melanogaster*. In contrast to the previous studies, many pairs of marker sites at the olfactory and gustatory receptor genes were in significant linkage disequilibrium. Indeed, about 50 pairs of polymorphic sites showed significant linkage disequilibrium at the 0.5% level and 15 pairs of sites did with Bonferroni correction for the whole tests (at the 0.0017%) in both samples. Significant linkage disequilibria were not necessarily between polymor-

phisms at the different olfactory receptor genes or between those at the gustatory receptor genes, but they were also observed between an olfactory receptor gene polymorphism and a gustatory receptor gene polymorphism, suggesting functional relationship between these two classes of genes. Furthermore the observed linkage disequilibrium between replacement-site polymorphisms showed a significant excess of haplotypes composed of one frequent and one rarer allele. This was not observed for marker pairs consisting of two silent-site polymorphisms or one replacement polymorphism and one silent polymorphism. This finding suggested synergistic deleterious effects of replacement changes.

(2) DNA variation at Odorant binding protein genes in Drosophila

Aya Takahashi and Toshiyuki Takano-Shimizu

Odorant binding proteins (OBPs) are low-molecular-weight proteins that are known to bind and solubilize hydrophobic odorants. The specific function of OBPs in olfaction is still not known. However, they are found in olfactory and gustatory organs and seem to play an important role in odor detection by restricting the odorants accessible to specific receptors. Among around 40 OBP genes annotated in the *Drosophila* genome, we have focused on *Obp57d* and *Obp57e* genes because of their specific expression at tarsi. DNA sequences of these two genes were obtained from 18 inbred lines of *D. melanogaster* collected in Kyoto, 4 *D. melanogaster* lines collected around the world, and 1 *D. simulans* line. The sequence variations showed a pattern that deviates from the neutral expectation, thus indicating a signature of natural selection.

(3) Searching for genes involved in sexual isolation between *Drosophila simulans* and *Drosophila melanogaster*

Aya Takahashi, Kuniaki Takahashi[1], Ryu Ueda[1] and Toshiyuki Takano-Shimizu ([1]Invertebrate Genetics Laboratory)

The interspecific mating frequencies between *D. melanogaster* and *D. simulans* were surveyed in seven lines of *D. melanogaster* and seven lines of *D. simulans*. Ten two-day-old virgin females of one species and ten two-day-old males were placed in a food vial for one day. The mating frequency was assessed by the number of females with sperm in their spermathecae and/or ventral receptacles. The data showed large variation among lines in mating frequencies between the two sibling species, suggesting multiple gene involvement. Two *D. melanogaster* lines of high and low heterospecific mating frequencies were chosen for QTL mapping of the genes responsible for the differences in interspecific mating behavior.

As a candidate gene for sexual isolation, *yellow* gene has long been one of them. Its mutant females have repeatedly been observed to show higher mating frequency against males of other species. Strains for inducible RNAi of this gene were constructed. Several aspects of mating behavior are being tested in the *yellow* knock-down mutants by RNAi.

(4) Genetic and molecular basis of the within- and between-species variation in the sex comb teeth number in Drosophila.

Haruki Tatsuta, Yuriko Ishii and Toshiyuki Takano-Shimizu

Sex comb of *Drosophila melanogaster* is a specific row of enlarged bristles on male foreleg and a good representative of sexually dimorphic characters. In general, such characters are likely driven by sexual selection. Using two inbred *Drosophila simulans* lines of the large (Tananarive) and the small (Sim3) number of sex-comb teeth, we made QTL analyses for the sex-comb-tooth-number variation in five mapping populations of whole genome F_2, two second-chromosome substitution strains (STSS and STTT), and two third-chromosome congenic strains. While no QTL was detected on the X chromosome, we found three QTL on the third chromosome and two of them were detected in both the whole genome F_2 and Sim3 backgrounds. Notably, we found strong background dependency of second-chromosome QTL identification: two QTL in the whole genome F_2 background, a single QTL in the Sim3 background (STSS), and three QTL in the Tananarive background (STTT). Although the two QTL in STTT were possibly identical with the QTL detected in the other backgrounds, the remaining two QTL identified were background-specific, implying extensive inter-chromosomal epistatic interactions.

Indeed, we found a significant interaction between the second-chromosome QTL and the third-chromosome one. Background dependency of second-chromosome QTL effects but independence of third-chromosome QTL effects may indicate the gene-action order of the QTL involved: the third-chromosome QTL act upstream of the second-chromosome QTL. For fine mapping, we are now studying the level and pattern of linkage disequlibrium in the candidate gene region.

SOCIAL CONTRIBUTION

国家公務員試験　試験専門委員

D-b. Division of Evolutionary Genetics Toshimichi Ikemura Group

RESEARCH ACTIVITIES

(1) A novel bioinformatics strategy for unveiling hidden genome signatures

Takashi Abe, Yuta Ichiba and Toshimichi Ikemura

In addition to protein-coding information, genome sequences contain a wealth of information of interest in many fields of biology from molecular evolution to genome engineering and biomedicine. G+C% is used as a fundamental characteristic of individual genomes but too simple a parameter to differentiate a wide variety of genomes. Because oligonucleotide frequencies vary significantly among genomes, they can be used to distinguish genomes and genomic regions. We used an unsupervised neural network algorithm, a self-organizing map (SOM), to analyze di-, tri-, and tetranucleotide frequencies in a wide variety of prokaryotic and eukaryotic genomes. The SOM, which can cluster complex data efficiently, was shown to be an excellent tool for analyzing global characteristics of genome sequences and for revealing key combinations of oligonucleotides representing individual genomes. From analysis of 1-kb and 10-kb genomic sequences derived from 65 bacteria (a total of 170 Mb) and from 6 eukaryotes (460 Mb), clear species-specific separations of major portions of the sequences were obtained with the di-, tri-, and tetranucleotide SOMs. The unsupervised algorithm could recognize, in most 10-kb sequences, the species-specific characteristics (key combinations of oligonucleotide frequencies) that are signature features of each genome. We were able to classify DNA sequences within one and between many species into subgroups that corresponded to biological categories. Because the classification power is very high, the SOM is an efficient and fundamental bioinformatic strategy for extracting a wide range of genomic information from a vast amount of sequences. (Abe et al. *Genome Research*, **13**, 693-702, 2003).

(2) Self-organizing map reveals sequence characteristics of 90 prokaryotic and eukaryotic genomes on a single map

Takashi Abe, Atushi Fukushima,Yoko Kosaka and Toshimichi Ikemura

With the increasing amount of available genome sequences, novel tools are needed for comprehensive analysis of species-specific sequence characteristics for a wide variety of genomes. An unsupervised neural network algorithm, Kohonen's self-organizing map (SOM), is an effective tool for clustering and visualizing high-dimensional complex data on a two-dimensional array of weight vectors. We adapted SOM to genome informatics, making the learning process and resulting map independent of the order of data input. We used the SOM to characterize oligonucleotide frequencies in a total of 1.4 Gb derived from 90 prokaryotic and eukaryotic genomes for which complete genomic sequences are available. SOMs classified 140,000 10-kb sequences from the 90 genomes mainly according to species and could unveil hidden sequence characteristics of each genome. Because the classification power is very high, SOM is an efficient and fundamental bioinformatic strategy for extracting a wide range of genomic information from a vast amount of sequence data. (Abe et al., *Workshop* 2003 *on Self-Organizing Maps*, pp. 95-100, 2003)

(3) Novel bioinformatics developed for phylogenetic classifications of genomic sequences from uncultured microorganisms in environmental samples

Takashi Abe, Yoko Kosaka, Jian-Ping Song and Toshimichi Ikemura

Self-organizing map (SOM) is a powerful tool for clustering and visualizing high-dimensional complex data on a two-dimensional plane. Oligonucleotide

frequency is an example of high-dimensional data. We constructed SOMs of tri- and tetranucleotide frequencies in 1- and 5-kb sequence fragments from bacterial genomes for which complete sequences are available. The sequence fragments could be classified primarily according to species and thus to 11 major bacterial groups without information regarding the species. SOM also classified 16S rRNA gene (16S rDNA) sequences accurately into the phylogenetic groups. For example, approximately 97% of rDNA sequences and 80% of non-rDNA sequences longer than 1 kb were classified into the correct phylogenetic groups. Because the classification power is very high, SOM is a powerful tool for phylogenetic classification of sequence fragments obtained from mixed genomes of uncultured environmental microorganisms. With this method, all rDNA and non-rDNA sequences including sargasso sea sequences in GenBank that were from unidentified bacteria and longer than 1 kb were classified into 11 major bacterial groups. The result indicated that the method is also useful for survey of pathogenic microorganisms causing novel and unclear infectious diseases.

(4) Inter- and intraspecies characterizations of eukaryotic genome sequences and *in silico* prediction of genetic signal sequences

Takashi Abe, Yoko Kosaka, Yuta Ichiba, Kiyomi Kita and Toshimichi Ikemura

Novel tools are needed for comprehensive comparisons of inter- and intraspecies characteristics of massive amounts of increasingly available genome sequences. An unsupervised neural network algorithm, self-organizing map (SOM), is an effective tool for clustering and visualizing high-dimensional complex data on a single map. We generated SOMs for tri-, tetra-, and pentanucleotide frequencies in 300,000 10-kb sequences from 13 eukaryotes for which almost complete genomic sequences are available (a total of 3 Gb). SOM recognized in most 10-kb sequences species-specific characteristics (key combinations of oligonucleotide frequencies), permitting species-specific classification of sequences without information regarding the species. Because the classification power is very high, SOM is an efficient and powerful tool for extracting a wide range of genomic information. SOM constructed with oligonucleotide frequencies in 10-kb sequences from 2.8 Gb of human sequences identified oligonucleotides with frequencies characteristically biased from random occurrence predicted from the mononucleotide composition, and 10-kb sequences rich in these oligonucleotides were self-organized on a map. Because these oligonucleotides often corresponded to genetic signals or the constituent elements, we propose an *in silico* method that should be useful for identification of genetic signal sequences in genomes for which large amounts of sequence data are available but additional experimental data are lacking.

Publications

Papers

1. Abe, T., Kanaya, S., Kinouchi, M., Ichiba, Y., Kozuki, T. and Ikemura, T. (2003). Informatics for unveiling hidden genome signatures. Genome Research **13**, 693-702.

2. Abe, T., Kozuki,T., Kosaka, Y., Fukushima, A., Nakagawa, S. and Ikemura T. (2003). Self-organizing map reveals sequence characteristics of 90 prokaryotic and eukaryotic genomes on a single map. Workshop 2003 on Self-Organizing Maps **3**, 95-100.

3. Kato, M., Onishi, Y., Wada-Kiyama, Y., Abe, T., Ikemura, T., Kogan, S., Bolshoy, A., Trifonov, E.N. and Kiyama, R. (2003). Dinucleosome DNA of human K562 cells: experimental and computational characterizations. J. Mol. Biol. **332**, 111−125.

SOCIAL CONTRIBUTIONS AND OTHERS

特許

4. 出願番号：2003-343862, 発明の名称：マイクロアレイ実験から得られたデータ解析の新手法, 発明者：池村淑道・金谷重彦・西　達也・和田健之介・小笠原直毅・小林和夫・増田　泰, 出願人：国立遺伝学研究所長

5. 出願番号：2003-328845, 発明の名称：塩基配列の分類システムおよびオリゴヌクレオチド出現頻度の解析システム, 発明者：池村　淑道, 出願人：国立遺伝学研究所長

E. DEPARTMENT OF INTEGRATED GENETICS

E-a. Division of Human Genetics Hiroyuki Sasaki Group

RESEARCH ACTIVITIES

(1) Analyses of the distal imprinted domain on mouse chromosome 7 and its orthologue on chicken chromosome 5

Hiroyuki Sasaki, Takaaki Yokomine, Hisao Shirohzu, Chikako Suda, Wahyu Purbowasito[1], Masaoki Tsuzuki[2], Atsushi Toyoda[3], Masahira Hattori[3] and Yoshiyuki Sakaki[3] ([1]Kyushu Univ.; [2]Hiroshima Univ.; [3]GSC, RIKEN)

Genomic imprinting, an epigenetic gene-marking phenomenon in mammals, causes parent-of-origin-specific monoallelic expression of a subset of genes. Imprinted genes tend to form clusters in the genome (imprinted domains), which may be related to the mechanism of imprinting or to the evolution of imprinting. As a step to understand the structural and functional characteristics of the imprinted domains, we have characterized a 1-Mb imprinted domain in mouse chromosome 7F4/F5 and its orthologous domain in chicken chromosome 5 (~0.5 Mb). We found that the genes of the chicken domain are not imprinted and, furthermore, that the chicken domain lacks the unique tandem repeat cluster of ~0.2 Mb, the *H19* gene, and the imprinting control elements, all of which are present in the mouse. The results indicate that the mammalian imprinted genes were already clustered in the common ancestors of mammals and birds and that the imprinting mechanism, which can affect multiple genes in the cluster, came in later during mammalian evolution (in preparation). We also mapped a total of 52 nuclear matrix attachment regions (MARs) in the imprinted mouse domain. We found that the MARs are unevenly distributed within the domain and that there is a large MAR cluster in the boundary region of two imprinted subdomains (in preparation).

(2) Imprinting mechanisms of the mouse *Igf2/H19* subdomain

Hiroyuki Sasaki, Yuzuru Kato, Ko Ishihara[1], Melanie Ehrlich[2] and Mitsuyoshi Nakao[1] ([1]Kumamoto Univ.; [2]Tulane Univ.)

The imprinted mouse 7F4/F5 domain contains two linked imprinted genes *Igf2* and *H19* near its centromeric boundary: *Igf2* is paternally expressed and *H19* maternally expressed. It is known that the paternal-specific methylation of the differentially methylated region (DMR) located upstream of *H19* is the primary cause for the *Igf2/H19* imprinting. We found that a winged-helix type DNA binding protein called RFX1 or MDBP binds to the conserved sequences within the DMR. Interestingly, this protein binds to the target sequence preferentially when they are methylated at CpG sites. We are currently knocking out the target sequences within the DMR in mice to see whether these proteins play a role in *Igf2/H19* imprinting.

(3) Establishment and maintenance of genomic imprinting in the germline and in early embryos

Hiroyuki Sasaki, Masahiro Kaneda, Masaki Okano[1], Kenichiro Hata, Takashi Sado, Naomi Tsujimoto[2], En Li[2], Kenji Kumaki, Hiroyasu Furuumi, Tomohiro Suzuki[3], Shigeharu Wakana[3] and Toshihiko Shiroishi[3] ([1]CDB, RIKEN; [2]Harvard Med. Sch.; [3]GSC, RIKEN)

DNA methylation serves as an important gene-marking mechanism for discrimination of the parental alleles of imprinted genes. Although *de novo* DNA methyltransferases of the Dnmt3 family are implicated in maternal imprinting, the lethality of conventional *Dnmt3a* and *Dnmt3b* knockout mice has precluded further studies. We have disrupted *Dnmt3a* and *Dnmt3b* in male and female germ cells, leaving them intact in somatic cells, by conditional knockout technology. Offspring from the *Dnmt3a* conditional mutant females died *in utero* and lacked methylation and allele-specific expression at maternally imprinted loci. The *Dnmt3a* conditional mutant males showed impaired spermatogenesis and a lack of methylation

at paternally imprinted loci in spermatogonia. Although these defects closely resembled those of *Dnmt3L* knockout mice, exact contribution of Dnmt3a and Dnmt3L to paternal imprinting varied from locus to locus. By contrast, the *Dnmt3b* conditional mutants and their offspring showed no phenotype. These results indicate that Dnmt3a is the critical enzyme responsible for both paternal and maternal imprinting (submitted). We also study how the primary imprints are maintained in preimplantation mouse embryos, and set out to screen ENU-treated mutant mouse stocks for mutations that affect the establishment of germline imprints in collaboration with a group in GSC, RIKEN.

(4) Computer-assisted search for sequence features common to imprinted DMRs

Hiroyuki Sasaki, Hisato Kobayashi, Takashi Abe[1] and Toshimichi Ikemura[1] ([1]Div. Evol. Genet., NIG)

Although the imprinted DMRs, which show differential methylation depending on parental origin, often play crucial roles in imprinting, features common to the DMRs have not been identified. We therefore set out to look for sequence features common to the DMRs by computer-assisted programs. We first located the DMR sequences on self-organizing maps (SOMs) produced from the mouse genome sequences for di-, tri- and tetra-nucleotides. We found that most DMRs are located in the periphery of the SOMs: they are more CpG-rich than most of the genome but less CpG-rich than the CpG islands. More detailed studies on the DMR sequences are ongoing.

(5) Molecular pathology of ICF syndrome

Hiroyuki Sasaki, Hiroyasu Furuumi and Takeo Kubota[1] ([1]Yamanashi Univ.)

We previously studied two Japanese families with ICF (immunodeficiency, centromeric instability, facial anomalies) syndrome, an autosomal recessive disorder with hypomethylation of satellite DNA, and found that they have mutations in the *de novo* DNA methyltransferase gene *DNMT3B*. We have now studied a new Japanese ICF case and found that this patient does not have any mutation in *DNMT3B* (submitted). This suggests that the molecular pathology of ICF syndrome is heterogeneous and that a gene other than *DNMT3B* can affect the methylation of pericentromeric satellite DNA.

(6) Investigation on the possibility of Z chromosome dosage compensation in chicken

Hiroyuki Sasaki, Takaaki Yokomine, Asato Kuroiwa[1], Yoichi Matsuda[1] and Masaoki Tsuduki[2] ([1]Hokkaido Univ.; [2]Hiroshima Univ.)

We studied whether chicken Z chromosome is subject to inactivation for gene dosage compensation in ZZ males. It was found that Z-linked genes are biallelically expressed in ZZ males (ref. 2). The results suggest that sex-chromosome inactivation of a mammalian X-chromosome type does not occur in the chicken.

(7) Development of a universal DNA chip system applicable to any organism

Hiroyuki Sasaki, Shin-ichi Mizuno[1], Tadafumi Iino[1], Hidetoshi Ozawa[1], Teruhisa Otsuka[1], Kosuke Tashiro[1] and Takashi Gojohbori ([1]Kyushu Univ.)

We carried out a collaborative research project with groups in Kyushu University to develop a universal DNA chip system that can be used to study expression of any gene in any organism. We established the basic chip design and protocols for this innovative chip system. Development of the universal DNA chip system for practical use is now underway.

(8) Role of *de novo* DNA methyltransferases in X chromosome inactivation

Takashi Sado, Masaki Okano[1], En Li[2] and Hiroyuki Sasaki ([1]CDB, RIKEN; [2]Harvard Med. Sch.)

Xist (X-inactive specific transcript) plays a critical role in X-inactivation. This non-coding RNA becomes upregulated on the X chromosome that is to be inactivated upon differentiation. Previous studies revealed that although maintenance-type DNA methylation is not essential for X-inactivation to occur, it is required for the stable repression of *Xist* in differentiated cells. However, it is unknown whether differential *de novo* methylation at the *Xist* promoter, which is mediated by *Dnmt3a* and/or *Dnmt3b*, is a

cause or a consequence of monoallelic expression of *Xist*. In this study, we showed that *Xist* expression was appropriately regulated in the absence of *Dnmt3a* and *Dnmt3b* and that a single X chromosome underwent proper inactivation in mutant females. Our results indicate that a mechanism(s) other than DNA methylation plays a principal role in initiating X-inactivation. We also demonstrated that delayed upregulation of *Xist* did not induce X-inactivation, consistent with a critical developmental window for the chromosomal silencing (ref. 5).

(9) Effect of *Tsix* disruption on *Xist* expression

Takashi Sado and Hiroyuki Sasaki

Xist and its antisense partner, *Tsix*, are the key players in X inactivation, both of which encode a non-coding RNA. Targeted disruption of *Tsix* causes ectopic expression of *Xist* in the extraembryonic tissues upon maternal transmission, which subsequently causes embryonic lethality due to inactivation of both X chromosomes in females and a single X chromosome in males. *Tsix* , therefore, plays a crucial role in maintaining *Xist* silencing in *cis* and in the regulation of imprinted X-inactivation in the extraembryonic tissues. We examined the effect of *Tsix*-disruption on *Xist* expression in the embryonic lineage using embryonic stem (ES) cells as a model system. Upon differentiation, *Xist* is ectopically activated in a subset of nuclei of male ES cells harboring the Tsix-deficient allele on the single X chromosome. Such ectopic expression, however, ceased during prolonged culture. We are tempted to speculate that surveillance by the counting mechanism somehow shuts off ectopic *Xist* expression before that X chromosome undergoes inactivation. Counting does not seem to be impaired in *Tsix*-deficient ES cells (ref. 1).

(10) X-inactivation in mouse embryos deficient for histone methyltransferase G9a

Tatsuya Ohhata, Makoto Tachibana[1], Hiroyuki Sasaki, Yoichi Shinkai[1] and Takashi Sado ([1]Kyoto Univ.)

Accumulating evidence suggests that methylation of histone H3 at lysine 9 (K9) and 27 (K27) is implicated in X-inactivation. Histone methyltranferase G9a is the enzyme that catalyzes methylation of K9 and perhaps K27 in euchromatic region. We studied X-inactivation in mouse embryos deficient for G9a, which die around the early somite stage. RNA-FISH revealed that *Xist* was appropriately regulated in both males and females. Taking advantage of X-linked GFP transgenes, effects of functional loss of G9a on the maintenance of X-inactivation was analyzed. We did not observe reactivation of the hitherto inactivated GFP transgenes in both the embryonic and extraembryonic tissues, suggesting that X-inactivation is stably maintained in G9a-null embryos. The results suggest that the X-inactivation process is properly regulated in the absence of G9a. It is likely that methylation of histone H3 at K9 and K27 on the inactive X chromosome is mediated by an enzyme(s) other than G9a.

(11) Role of Dnmt3L in spermatogenesis and in genomic imprinting during oogenesis

Kenichiro Hata, En Li[1] and Hiroyuki Sasaki ([1]Harvard Med. Sch.)

Dnmt3L (DNA cytosine-5-methyltransferase 3-Like) encodes a protein of 421 amino acid residues and harbors a putative zing finger domain that shares a high degree of homology with the PHD-like domain of DNA methyltransferases Dnmt3a and Dnmt3b. The C-terminal part of Dnmt3L is related to DNA cytosine-5-methyltransferase, but it does not possess critical motifs for methyltransferase activity. We have generated *Dnmt3L*-deficient mice by gene targeting. While Dnmt3L−/− female mice grew normally, all embryos from pregnant *Dnmt3L*−/− mothers died around E10.5. The maternally methylated imprinted genes, e.g. *Igf2r* and *Peg1*, were hypomethylated in embryos derived from *Dnmt3L* −/− females x *Dnmt3L* +/+ males, but paternally methylated imprinted genes were unaffected. Also, *Dnmt3L*−/− male mice showed severe defects in spermatogenesis, which is similar to, but severer than, the phenotype displayed by *Dnmt3a*−/− mice. We speculate that Dnmt3L functions via interactions with Dnmt3a and/or Dnmt3b to control DNA methylation in developing germ cells. Dnmt3L may be involved in not only the establishment of genomic imprinting but also DNA methylation of other regions.

Publications

Papers

1. Sado, T., Li, E. and Sasaki, H. (2002). Effect of *Tsix*-disruption on *Xist* expression in male ES cells. Cytogenet. Genome Res. **99**, 115-118.
2. Kuroiwa, A., Yokomine, T., Sasaki, H., Tsudzuki, M., Tanaka, K., Namikawa, T. and Matsuda, Y. (2002). Biallelic expression of Z-linked genes in male chickens. Cytogenet. Genome Res. **99**, 310-314.
3. Ishida, C., Ura, K., Hirao, A., Sasaki, H., Toyoda, A., Sakaki, Y., Niwa, H., Li, E. and Kaneda, Y. (2003). Genomic organization and promoter analysis of the Dnmt3b gene. Gene **310**, 151-159.
4. Gribnau, J., Hochedlinger, K., Hata, K., Li, E. and Jaenisch, R. (2003). Asynchronous replication timing of imprinted loci is independent of DNA methylation, but consistent with differential subnuclear localization. Genes Dev. **17**, 759-773.
5. Sado, T., Okano, M., Li, E. and Sasaki, H. (2004). De novo methylation is dispensable for the initiation and propagation of X chromosome inactivation. Development **131**, 975-982.

Reviews

6. Hata, K. and Sasaki, H. (2003). Genomic imprinting: Mechanisms, significance and evolution. J. Mamm. Ova Res. **20**, 64-68.
7. 金田正弘, 佐々木裕之：DNAメチル化とクロマチンによるゲノムインプリンティング制御機構. 生体の科学 **54**, 206-210(2003).
8. 秦健一郎, 佐々木裕之：ゲノム刷り込みのファンダメンタルズ. 現代医療 **35**, 965-970(2003).
9. 金田正弘, 熊木健治, 佐々木裕之：ゲノムインプリンティング. Molecular Medicine **40**(増刊号)再生医学, 18-24(2003).
10. 秦健一郎, 佐々木裕之：ゲノムインプリンティング. 小児科 **44**, 254-255(2003).
11. 佐渡敬：X染色体不活性化を制御するnon-coding RNA. 実験医学 **21**(増刊号)エピジェネティクスと遺伝子発現機構, 129-135(2003).
12. 秦健一郎：ゲノム刷り込みにおけるDNAメチル化酵素の役割. 分子細胞治療 **2**, 60-65(2003).
13. 金田正弘, 佐渡敬, 佐々木裕之：遺伝子に刷り込まれた記憶－細胞分化とDNAのメチル化・ゲノムインプリンティング. 生物の科学 遺伝 **58**, 69-74(2004).
14. 佐々木裕之：エピジェネティクスと疾患. Molecular Medicine **41**(増刊号)ヒトゲノム－ヒトの理解と疾病の克服, 332-338(2004).

Books

15. 佐渡敬, 佐々木裕之：性染色体の構造と機能. Springer Reviewsシリーズ－クロマチンと遺伝子機能制御(堀越正美編), 15-24, シュプリンガー・フェアラーク東京(2003).
16. 横峯孝昭, 佐々木裕之：ゲノムインプリンティングの進化. Springer Reviewsシリーズ－ゲノムからみた生物の多様性と進化(五條堀孝編), 164-170, シュプリンガー・フェアラーク東京(2003).
17. 佐々木裕之編：Springer Reviewsシリーズ－エピジェネティクス, シュプリンガー・フェアラーク東京(2004).
18. 佐渡敬：性染色体の遺伝子量補償. Springer Reviewsシリーズ－エピジェネティクス(佐々木裕之編), 117-127, シュプリンガー・フェアラーク東京(2004).
19. 古海弘康, 佐々木裕之：哺乳類のメンデル遺伝するエピジェネティクス. Springer Reviewsシリーズ－エピジェネティクス(佐々木裕之編), 129-134, シュプリンガー・フェアラーク東京(2004).
20. 秦健一郎：生殖, 発生の異常とエピジェネティクス. Springer Reviewsシリーズ－エピジェネティクス(佐々木裕之編), 183-190, シュプリンガー・フェアラーク東京(2004).

EDUCATION

1. 秦健一郎：配偶子形成のエピジェネティクス. 九州大学生体防御研究所セミナー, 福岡, 4月.
2. 佐々木裕之：クローン技術の功罪. 御殿場市市民大学講座, 御殿場, 6月.
3. 佐々木裕之：山梨大学医学部講義, 玉穂町, 7月.
4. 佐々木裕之：生殖細胞におけるde novoメチル化の役割. 千里神経懇話会, 吹田, 9月.
5. 佐々木裕之：ゲノムインプリンティングと個体の発生・クローン技術. 染色体研究会, 東京, 10月.
6. 佐々木裕之：北里大学理学部講義, 相模原, 10月.
7. Sasaki, H.: Role of *de novo* DNA methyltransferases in genomic imprinting revealed by conditional gene knockout in mice. Seminar in the Department of Genetics, Universität of Saarland, Saarbrücken, Germany, November.
8. 秦健一郎：神経管発生に関与するエピジェネティクス機構. 平成15年度研究報告会「二分脊椎症の診断・治療及び予防システムに関する研究」, 東京, 12月.

SOCIAL CONTRIBUTIONS AND OTHERS

佐々木裕之：Associate Editor, Journal of Biochemistry (Tokyo).

E-b. Division of Agricultural Genetics Tetsuji Kakutani Group

RESEARCH ACTIVITIES

(1) Developmental abnormalities induced by DNA methylation mutation of Arabidopsis

Tetsuji Kakutani, Tetsu Kinoshita, Asuka Miura, Yuki Kinoshita and Masaomi Kato Hodetoshi Saze

Arabidopsis provides a genetically tractable system to learn role of DNA methylation, since viable mutants with reduced genomic DNA methylation are available. Arabidopsis MET1 (METHYLTRANSFERASE 1, ortholog of mammalian DNA methyltransferase Dnmt1) is necessary for maintaining genomic cytosine methylation at 5'-CG-3' sites. Arabidopsis additionally methylates non-CG sites using CHROMOMETHYLASE3 (CMT3). A third Arabidopsis gene necessary for DNA methylation is *DDM1 (DECREASE IN DNA METHYLATION)*, which encodes a chromatin remodeling factor (Jeddeloh et al., 1999, Nat. Genet 22, 94-). The *ddm1* mutation affects both CG and non-CG methylation. A striking feature of *ddm1* mutation is that it induces a variety of developmental abnormalities by causing heritable change in other loci (Kakutani et al., 1996, PNAS 93, 12406-). The molecular basis has been clarified in two of the loci directly causing the developmental abnormalities (see below).

(2) Epigenetic control of transposons and their behavior in natural population

Asuka Miura, Masaomi Kato, Kazuya Takashima, Yuki Kinoshita and Tetsuji Kakutani

Through genetic characterization of one of the *ddm1*-induced developmental abnormalities, we identified a novel endogenous Arabidopsis transposon, named *CACTA1*. This transposon transposes and increases in the copy number in DNA hypomethylation background in *ddm1* mutant (Miura et al., 2001, Nature 411, 212-). Loss of DNA methylation seems to be sufficient for mobilization of *CACTA1*, because it was mobilized in mutants of DNA methyltransferase genes, *MET1* and *CMT3*. High frequency transposition of *CACTA* elements was detected in *cmt3-met1* double mutants. Single mutants in either *met1* or *cmt3* were much less effective in mobilization. Thus CG and non-CG methylation systems redundantly function for immobilization of transposons (Kato et al., 2003). *CMT3* gene and non-CG methylation in plants may have evolved as an additional epigenetic tag dedicated to transposon control.

Differentiation of gene-rich and transposon-rich (gene-poor) regions is a common feature in plant genomes. That may be due to preferential integration of transposons to gene-poor regions or may be due to purifying selection against transposon insertion to gene-rich regions in natural populations. To evaluate the possible contribution of natural selection to the formation of transposon distribution pattern, we examined the distribution of the *CACTA* transposons in genomes of 19 natural variants (ecotypes) and compared that to integration induced in laboratory in the *ddm1* mutants. Sequences similar to mobile *CACTA1* copy distribute among the ecotypes and show high polymorphism in genomic localization. Despite the high polymorphism, the copy number was low in all the examined ecotypes and they localized preferentially in pericentromeric and transposon-rich regions. This contrasts to transposition induced in laboratory, in which the integration sites are less biased and the copy number frequently increases. The differences in the integration sites may be due to natural selection against deleterious insertion into chromosomal arm regions (Miura et al, 2004).

(3) Inheritance of epigenetic developmental abnormality.

Yuki Kinoshita, Asuka Miura, Tetsu Kinoshita and Tetsuji Kakutani

Another developmental abnormality, late flowering trait, was induced by ectopic expression of *FWA* gene associated with hypomethylation of tandem repeat upstream of the coding region (Soppe et al., 2000, Mol Cell 6, 791-). Interesting thing is that change in nucleotide sequence was also not observed in *fwa-1* and *fwa-2* alleles isolated by conventional mutagenesis. In both cases, over-expression associated with the hypomethylation resulted in the phenotypes. Combining *ddm1* mutation and linkage analysis is useful for identifying epigenetically regulated genes affecting plant development (Kakutani, 1997, Plant J., 12, 1447-). The *ddm1*-induced late flowering trait as well as the

hypomethylation and ectopic expression of the *FWA* gene were stably inherited over generations even in the presence of the wild type *DDM1* copy (manuscript in preparation).

(4) Epigenetic control of *FWA* gene expression in endosperm

Tetsu Kinoshita, Asuka Miura, Yuki Kinoshita and Tetsuji Kakutani

Although *FWA* is ectopically expressed in the epigenetic alleles stated above, the role of *FWA* gene product in normal development remained unknown. To understand why the *FWA* gene is epigenetically controlled, we further examined expression of this gene during normal development in wild type. Results of GFP reporter system and direct detection of the transcript both suggest that *FWA* is expressed specifically in the endosperm. Endosperm is a plant tissue analogous to mammalian placenta; it serves as nutritional support to the embryo. Furthermore, the *FWA* gene was expressed in parent-of-origin-specific manner; only maternal gene is expressed. The *FWA* imprint depends on the maintenance DNA methyltransferase *MET1*, as is the case in mammals. Unlike mammals, however, the *FWA* imprint is not established by allele-specific *de novo* methylation. It is established by maternal gametophyte-specific gene activation, which depends on a DNA glycosylase gene, *DEMETER*. Since endosperm does not contribute to the next generation, the activated *FWA* gene need not be silenced again. Double fertilization enables plants to use such 'one-way' control of imprinting and DNA methylation in endosperm (Kinoshita et al, 2004).

Publications

Papers

1. Kato, M., Miura, A., Bender, J., Jacobsen, S.E. and Kakutani, T. (2003) Role of CG and non-CG methylation in immobilization of transposons in Arabidopsis. Current Biology **13**, 421-426.
2. Kinoshita, T., Miura, A., Choi, Y., Kinoshita, Y., Cao, X., Jacobsen, SE., Fischer, RL. and Kakutani, T. (2004) One-way control of *FWA* imprinting in *Arabidopsis* endosperm by DNA methylation. Science, **303**, 521-523. (online publication, Nov 20, 2003).
3. Miura, A., Kato, M., Watanabe, K., Kawabe, A., Kotani, H. and Kakutani, T. (2004) Genomic localization of endogenous mobile CACTA family transposons in natural variants of *Arabidopsis thaliana*. MGG, **270**, 524-532. (online publication, Nov 8, 2003).

EDUCATION

1. Kakutani T, a seminar at Cold Spring Harbor Laboratory, August, 2003
2. Kakutani T, a seminar at UCLA, August, 2003
3. Kakutani T, a seminar at California Institute of Technology, August, 2003
4. Kinoshita T, a seminar at UC Berkeley, August, 2003
5. 角谷徹仁　北海道大学理学部で集中講義，2003年2月

E-b. Division of Agricultural Genetics Keiichi Shibahara Group

RESEARCH ACTIVITIES

(1) Mechanism of nucleosome assembly during DNA replication

Tomohiro Kubo[1], Akira Shimizu[1] and Keiichi Shibahara[2] ([1]Graduate School of Biostudies, Kyoto University, [2]Japan Science and Technology Agency)

A newly replicated DNA is assembled into nucleosome soon after the passage of replication fork. This rapid formation of nucleosome is functionally linked to DNA replication machineries and plays a critical role for the maintenance of genome integrity and epigenetic states of chromatin in proliferating cells. Two histone binding proteins, CAF-1 (Chromatin Assembly Factor-1) and ASF1 (Anti-Silencing Function1), are found to be involved in some process of this nucleosome assembly reaction.

We recently developed an *in vitro* reconstitution system with human cell extract to analyze a mechanism of the DNA replication-dependent nucleosome assembly. The nucleosome assembly reaction was formerly analyzed in an ongoing replication reaction of SV40 *in vitro* system. This replication-coupled system has proven particularly useful in identifying CAF-1. However, its use to identify other activities involved in the replication-dependent nucleosome

assembly has been limited, because biochemical fractionations of the human cell cytosolic extract (the S100 extract) used in the SV40 DNA replication (which contains all the necessary DNA replication factors, histones, and other chromatin assembly factors) often resulted in a severe loss of DNA replication activity. In order to overcome these limitations, we successfully used replicated DNA purified by a gel filtration spin column as a substrate for the nucleosome assembly reaction. The newly replicated and separated DNA was competent for the replication-dependent nucleosome assembly even after separation from free protein fractions in the S100 extract because the DNA remains marked, at least by PCNA (*Cell 96*, 575-585, 1999).

With this "two step system", we showed that unidentified activities in the S100 extract were required for the nucleosome assembly in addition to CAF-1 and histones. We are now on the way to identify additional activities required for the DNA replication-dependent nucleosome assembly.

(2) Physiological implications of CAF-1 and ASF1 in *Arabidopsis*

Hidetaka Kaya[1,2], Takashi Araki[1] and Keiichi Shibahara[3] ([1]Department of Botany, Graduate School of Science, Kyoto University, [2]Japan Society for the Promotion of Science, [3]Japan Science and Technology Agency)

The genetic approach of *Arabidopsis* was a powerful tool to see physiological implications of CAF-1 and ASF1. We analyzed loss-of-function mutants of *caf-1* (*fasciata: fas*) in *Arabidopsis* and showed that those *fas* mutants displayed severely disturbed cellular and functional organization of both shoot apical meristem and root apical meristem (*Cell* **104**, 131-142. 2001). Those mutants also showed a varied pattern of distorted expression of both *WUS* and *SCR*, which play key roles in the organization of SAM and RAM, respectively. Recently, we found that gene silencing of *CACTA* and *TSI*, which expressions are repressed in wild type plants, was de-repressed in a small number of cells, and the percentage of the cells express those genes was increased with growth of the plants. These observations suggested that silent status of gene expression cannot be maintained stably in *fas* mutants and thus those cells express CACTA and TSI accidentally appear in a stochastic manner.

We proposed several models to explain those observations, and the most plausible is that CAF-1 ensures stable propagation of epigenetic states of chromatin through facilitating nucleosome assembly during DNA replication. Rapid re-formation of nucleosomes onto newly replicated DNA by CAF-1 would prevent DNA binding factors like transcriptional regulators from being targeted to the DNA in a non-regulated manner, thereby preventing random changes in gene expression patterns in daughter cells.

(3) Identification of a novel regulatory element in the X- inactivation Center *(Xic)*

Yuya Ogawa and Jeannie T. Lee[1] ([1]Massachusetts General Hospital, Harvard Medical School and Howard Hughes Medical Institute)

During X-chromosome inactivation in mammalian female, one X-chromosome is randomly silenced by *Xist*. X-chromosome choice is determined by asymmetric expression of *Tsix* whose antisense action represses *Xist*. In mice, choice is also regulated by the modifier, *Xce*. We identified a novel element in the X inactivation center *(Xic)* region near *Xist* and *Tsix* that regulates allelic choice. Termed *Xite*, the element harbors two clusters of intergenic transcription start sites and series of DNase I hypersensitive sites with allelic differences. Deleting *Xite* skews choice to favor the mutated X for inactivation, but truncating the transcripts associated with *Xite* does not abolish this effect. Evidence suggests that, at the onset of XCI, *Xite* enables the persistence of *Tsix* expression on the future active X. We propose that *Xite* is a candidate for the *Xce* and promotes allelic inequality by biasing *Tsix* expression.

Publications

Papers

1. Ogawa, Y. and Lee, J. T. (2003). *Xite*, X-inactivation intergenic transcription elements that regulate the probability of choice, Mol Cell **11**, 731-743.

2. Takeda, S., Tadele, Z., Hofmann, I., Angelis, K.J., Kaya, H., Araki, T., Mengiste, T., Scheid, OM, Probst, AV., Shibahara, K-i., Scheel, D. and Paszkowski, J.: *BRU1*, a novel link between responses to DNA damage and epigenetic gene silencing in *Arabidopsis*. (2004) Genes & Dev. **18**. 782-793

Reviews
3. 小川裕也(2003), 不活性化X染色体の選択：*Xite*領域による*Tsix*の発現制御, 実験医学21, 1653-1656.
4. 久保知大, 柴原慶一：DNA複製期のヌクレオソームアセンブリー, 実験医学増刊「エピジェネティックスと遺伝子発現制御」, VOL.21 No.11, 24-28, 2003.
5. 久保知大, 柴原慶一：DNA複製及びヌクレオチド除去修復に伴うヌクレオソーム形成, わかる実験医学「DNA複製とDNA修復」, 2004. 88-92.

E-c. Division of Brain Function Tatsumi Hirata Group

RESEARCH ACTIVITIES

(1) Theoretical Consideration of Olfactory Axon Targeting with an Activity-Dependent Neural Network Model

Hirokazu Tozaki and Tatsumi Hirata

The olfactory sensory neurons arranged on the olfactory epithelium express odorant receptors (ORs), which capture odorous chemicals in the air. The ORs are encoded by about 1000 genes in mice. Among the large repertoires, however, only one allele of the single OR gene is expressed in individual sensory neurons. The neurons expressing the same OR are randomly scattered over the olfactory epithelium, but converge their axons on a few glomeruli, specialized synaptic structures, connecting with the second-order neurons. For the glomerular convergence, ORs play an indispensable role. Deletion of an OR coding sequence by genetic disruption destroys the glomerular convergence by sensory neurons that are committed to express the OR. OR swap experiments in which one OR coding sequence is replaced by another induce a positional shift of the glomerulus targeted by the OR-expressing neurons from the endogenous glomeruli defined by either of the donor or acceptor ORs. These results suggest that the ORs instruct the axons where to converge.

How do the ORs instruct the convergence of olfactory axons? Several studies have hypothesized a model in which ORs presented on axon terminals recognize positional cues in the olfactory bulb as a guidance receptor. Another possibility is activity-dependent guidance of olfactory axons through the ORs. Activity-dependency is one of the fundamental mechanisms involved in the construction of neural networks, and basically represented by the Hebbian rule that simultaneous electrical activities between pre- and post-synaptic cells reinforce their synaptic connections, whereas incoherent activities do not. With regard to the olfactory projection, the activity-dependent versus -independent issue is still much controversial.

We computationally simulated how olfactory axons target if activity-dependent mechanisms were considered. We modified Tanaka's activity-dependent self-organization model, which is based on the Hebbian rule. In many nervous systems, spatially neighboring relationships of the presynaptic neurons are the basis for the correlated activities. By contrast, olfactory sensory neurons with adjacent cell bodies on the olfactory epithelium do not necessarily mean their correlated activities, but it is more likely that recognition of the same OD as a common ligand triggers correlated activities in the neurons. Therefore, we formulated the OD-evoked activities of olfactory sensory neurons and evaluated them as the force that organizes glomeruli. This model successfully generated a glomerular map, which represents various aspects of the glomerular layer *in vivo*. Furthermore, our model explained wide range of perplexing phenomena and results reported in the olfactory system. For example, local fluctuation of glomerular positioning in individuals, failure of olfactory targeting in OR-gene knockout mice, segregated projection of olfactory axons in OR-gene transgenic mice, and seemingly inconsistent targetings of olfactory axons in the knockout mice for activity-evoking ion channel genes, are simply reproducible if only activity-dependent mechanisms are taken into consideration. Thus, we propose the activity-dependent targeting of olfactory axons as a simple probable mechanism of glomerular formation.

(2) Chronotopic Organization of Olfactory Bulb Axons in the Lateral Olfactory Tract

Hitoshi Yamatani, Yasufumi Sato, Hajime Fujisawa* and Tatsumi Hirata (*Nagoya University Graduate School)

The olfactory system senses volatile chemicals in the air with high sensitivity, compromising spatial resolution of the stimuli. Odorous chemicals are first

perceived by odorant receptors expressed on olfactory sensory neurons of the olfactory epithelium. Because the sensory neurons expressing the same odorant receptor converge their axons onto a few topographically fixed glomeruli, the axons creates a stereotyped spatial map on the OB that represents odor codes extracted from molecular features of odorants. This odor map on the OB is subsequently transmitted to the higher-order centers through mitral and tufted cells, the output neurons of the OB. The primary projections of these neurons collectively construct the lateral olfactory tract (LOT). The LOT axons give off collateral branches outside, which eventually make synaptic connections with third-order neurons in the olfactory target areas such as the anterior olfactory nucleus, the piriform cortex and the olfactory tubercle. The principle underlying this projection remains obscure. Axonal tracing studies have failed to find a point-to-point topographic relationship between the spatial map on the OB and any of the olfactory target areas. Thus, the stereotyped odor map on the OB is believed to be completely disorganized in the higher-order centers. Furthermore, it is evident that the spatial representation of the OB is already lost at the level of the LOT, in which mitral/tufted cell axons are intermingled randomly regardless of positions of their cell bodies.

We isolated monoclonal antibody (mAb) H2C7 that recognizes a segregated subset of LOT axons at each developmental stage. The antigen recognized by mAb H2C7, which was identified as c-kit receptor tyrosine kinase, was only transiently expressed by newborn mitral/tufted cells that were radially migrating in the intermediate zone of the OB. These findings reveal the chronotopic nature of the organization of LOT axons; the mitral/tufted cells that differentiate at the same developmental stage assemble their axons together within the LOT, regardless of positions of their cell bodies in the OB. Because newly arriving axons were sequentially added to the ventral superficial part of the tract, displacing the older axons, there seems to be a developmental gradient in organization of LOT axons from the dorsal depth to ventral surface. This special assembly of LOT axons explains the seemingly disordered arrangement of LOT axons that has been reported previously, and could influence the subsequent target selection by collateral branches projected from the LOT.

E-d. Division of Applied Genetics
Hirohiko Hirochika Group

RESEARCH ACTIVITIES

(1) Target site specificity of the *Tos17* retrotransposon shows a preference for insertion within genes and against insertion in retrotransposon-rich regions of the genome

Akio Miyao[1], Katsuyuki Tanaka[2], Kazumasa Murata[2], Hiromitsu Sawaki[2], Shin Takeda[1], Kiyomi Abe[1], Yoriko Shinozuka[2], Katsura Onosato[2] and Hirohiko Hirochika[1] ([1]National Institute of Agrobiological Sciences and .[2]Institute of the Society for Techno-innovation of Agriculture, Forestry and Fisheries)

Because retrotransposons are the major component of plant genomes, analysis of the target site selection of retrotransposons is important for understanding the structure and evolution of plant genomes. Here, we examined the target site specificity of the rice retrotransposon *Tos17*, which can be activated by tissue culture. We have produced 47,196 *Tos17*-induced insertion mutants of rice. This mutant population carries approximately 500,000 insertions. We analyzed >42,000 flanking sequences of newly transposed *Tos17* copies from 4316 mutant lines. More than 20,000 unique loci were assigned on the rice genomic sequence. Analysis of these sequences showed that insertion events are three times more frequent in genic regions than in intergenic regions. Consistent with this result, *Tos17* was shown to prefer gene-dense regions over centromeric heterochromatin regions. Analysis of insertion target sequences revealed a palindromic consensus sequence, ANGTT-TSD-AACNT, flanking the 5-bp target site duplication. Although insertion targets are distributed throughout the chromosomes, they tend to cluster, and 76% of the clusters are located in genic regions. The mechanisms of target site selection by *Tos17*, the utility of the mutant lines, and the knockout gene database are discussed.

(2) Three distinct rice cellulose synthase catalytic subunit genes required for cellulose synthesis in the secondary wall

Katsuyuki Tanaka K[1], Kazumasa Murata[1], Muneo Yamazaki[2], Katsura Onosato[1], Akio Miyao[2] and Hirohiko Hirochika[2] ([1]Institute of the Society for Techno-innovation of Agriculture, Forestry and Fisheries and [2]National Institute of Agrobiological Sciences)

Several brittle culm mutations of rice *(Oryza sativa)* causing fragility of plant tissues have been identified genetically but not characterized at a molecular level. We show here that the genes responsible for three distinct brittle mutations of rice, induced by the insertion of the endogenous retrotransposon *Tos17*, correspond to *CesA* (cellulose synthase catalytic subunit) genes, *OsCesA4*, *OsCesA7* and *OsCesA9*. Three *CesA* genes were expressed in seedlings, culms, premature panicles, and roots but not in mature leaves, and the expression profiles were almost identical among the three genes. Cellulose contents were dramatically decreased (8.9%-25.5% of the wild-type level) in the culms of null mutants of the three genes, indicating that these genes are not functionally redundant. Consistent with these results, cell walls in the cortical fiber cells were shown to be thinner in all the mutants than in wild-type plants. Based on these observations, the structure of a cellulose-synthesizing complex involved in the synthesis of the secondary cell wall is discussed.

(3) Transcriptional activation mediated by binding of a plant GATA-type zinc finger protein AGP1 to the AG-motif (AGATCCAA) of the wound-inducible Myb gene NtMyb2.

Kazuhiko Sugimoto, Shin Takeda and Hirohiko Hirochika (National Institute of Agrobiological Sciences)

NtMyb2 is a regulator of the tobacco retrotransposon *Ttol* and the defense-related gene phenylalanine ammonia lyase (PAL), which are induced by various stress stimuli such as wounding or elicitor treatment. *NtMyb2* is also induced by wounding or elicitor treatment and is regulated at the transcriptional level. In this study, mutational analysis of the promoter of *NtMyb2* and gain-of-function analysis in vivo showed that the sequence AGATCCAA, named the AG-motif, is a cis-element sufficient to confer responsiveness to wounding and elicitor treatment. Furthermore, by using the south-western method, we cloned cDNAs encoding a GATA-type zinc finger protein, which can specifically bind to the AG-motif, named AG-motif binding Protein (AGP1). Domain analysis revealed that not only the GATA-type zinc finger region but also the downstream His2 motif of AGP1 is required for binding activity, showing that the AGP has a novel GATA-type zinc finger domain. AGP1 can activate expression from promoters containing the AG-motif in tobacco protoplasts, indicating that AGP1 is a positive regulator of NtMyb2. We also found that the AGP1 binding activity is highly enhanced by adenine methylation of the AG-motif by bacterial dam methylase.

(4) Characterization of a rice chlorophyll-deficient mutant using the T-DNA gene-trap system

Ki-Hong Jung[1], Junghe Hur[1], Choong-Hwan Ryu[1], Youngju Choi[1], Yong-Yoon Chung[1], Akio Miyao[2], Hirohiko Hirochika[2] and Gynheung An[1] ([1]Pohang University of Science and Technology and [2]National Institute of Agrobiological Sciences)

We have previously generated a large pool of T-DNA insertional lines in rice. In this study, we screened those T-DNA pools for rice mutants that had defective chlorophylls. Among the 1,995 lines examined in the T2 generation, 189 showed a chlorophyll-deficient phenotype that segregated as a single recessive locus. Among the mutants, 10 lines were beta-glucuronidase (GUS)-positive in the leaves. Line 9-07117 has a T-DNA insertion into the gene that is highly homologous to *XANTHA-F* in barley and *CHLH* in AR*ABIDOPSIS*. This *OsCHLH* gene encodes the largest subunit of the rice Mg-chelatase, a key enzyme in the chlorophyll branch of the tetrapyrrole biosynthetic pathway. In the T2 and T3 generations, the chlorina mutant phenotypes are co-segregated with the T-DNA. We have identified two additional chlorina mutants that have a Tos17 insertion in the *OsCHLH* gene. Those phenotypes were co-segregated with *Tos17* in the progeny. GUS assays and RNA blot analysis showed that expression of the *OsCHLH* gene is light inducible, while TEM analysis revealed that the thylakoid membrane of the mutant

chloroplasts is underdeveloped. The chlorophyll content was very low in the *OschlH* mutants. This is the first report that T-DNA insertional mutagenesis can be used for functional analysis of rice genes.

(5) The *MSP1* gene is necessary to restrict the number of cells entering into male and female sporogenesis and to initiate anther wall formation in rice

Ken-ichi Nonomura[1], Kazumaru Miyoshi[1], Mitzugu Eiguchi[1], Tazunu Suzuki[1], Akio Miyao[2], Hirohiko Hirochika[2] and Nori Kurata[1] ([1]National Institute of Genetics and [2]National Institute of Agrobiological Sciences)

The function of the novel gene *MSP1 (MULTIPLE SPOROCYTE)*, which controls early sporogenic development, was elucidated by characterizing a retrotransposon-tagged mutation of rice. The *MSP1* gene encoded a Leu-rich repeat receptor-like protein kinase. The *msp1* mutation gave rise to an excessive number of both male and female sporocytes. In addition, the formation of anther wall layers was disordered and the tapetum layer was lost completely. Although the mutation never affected homologous chromosome pairing and chiasma maintenance, the development of pollen mother cells was arrested at various stages of meiotic prophase I, which resulted in complete male sterility. Meanwhile, plural megaspore mother cells in a mutant ovule generated several megaspores, underwent gametogenesis, and produced germinable seeds when fertilized with wild-type pollen despite disorganized female gametophytes. In situ expression of *MSP1* was detected in surrounding cells of male and female sporocytes and some flower tissues, but never in the sporocytes themselves. These results suggest that the MSP1 product plays crucial roles in restricting the number of cells entering into male and female sporogenesis and in initiating anther wall formation in rice.

(6) Loss-of-Function Mutations of the Rice GAMYB Gene Impair α-Amylase Expression in Aleurone and Flower Development.

Miyuki Kaneko[1], Yoshiaki Inukai[1], Miyako Ueguchi-Tanaka[1], Hironori Itoh[1], Takeshi Izawa[2], Yuhko Kobayashi[1], Tsukaho Hattori[1], Akio Miyao[2], Hirohiko Hirochika[2], Motoyuki Ashikari[1] and Makoto Matsuoka[1] ([1]Nagoya University and [2]National Institute of Agrobiological Sciences)

GAMYB was first isolated as a positive transcriptional regulator of gibberellin (GA)-dependent alpha-amylase expression in barley aleurone cells, and its molecular and biochemical properties have been well characterized. However, the role of GAMYB elsewhere in the plant is not well understood. To investigate the molecular function of GAMYB outside of the aleurone cells, we isolated loss-of-function mutants from a panel of rice mutants produced by the insertion of a retrotransposon, *Tos17*. Through PCR screening using primers for rice GAMYB *(OsGAMYB)* and *Tos17*, we isolated three independent mutant alleles that contained Tos17 inserted in the exon region. No alpha-amylase expression in the endosperm was induced in these mutants in response to GA treatment, indicating that the *Tos17* insertion had knocked out *OsGAMYB* function. We found no significant defects in the growth and development of the mutants at the vegetative stage. After the phase transition to the reproductive stage, however, shortened internodes and defects in floral organ development, especially a defect in pollen development, were observed. On the other hand, no difference was detected in flowering time. High-level *OsGAMYB* expression was detected in the aleurone cells, inflorescence shoot apical region, stamen primordia, and tapetum cells of the anther, but only low-level expression occurred in organs at the vegetative stage or in the elongating stem. These results demonstrate that, in addition to its role in the induction of alpha-amylase in aleurone, OsGAMYB also is important for floral organ development and essential for pollen development.

Publications

Papers

1. Miyao, A., Tanaka, K., Murata, K., Sawaki, H., Takeda, S., Abe, K., Shinozuka, Y., Onosato, K. and Hirochika, H. (2003) Target site specificity of the *Tos17* retrotransposon shows a preference for insertion within genes and against insertion in retrotransposon-rich regions of the genome. Plant Cell **15**: 1771-1780

2. Tanaka, K., Murata, K. Y., Onosato, K., Miyao, A. and Hirochika, H. (2003) Three distinct rice cellulose synthase catalytic subunit genes required for cellulose synthesis in the secondary wall. Plant Physiol **133**: 73-83

3. Sugimoto, K., Takeda, S. and Hirochika, H. (2003) Transcriptional activation mediated by binding of a plant GATA-type zinc finger protein AGP1 to the AG-motif (AGATCCAA) of the wound-inducible Myb gene NtMyb2. Plant J. **35**: 550-564.
4. Hong, Jung., Junghe, Hur., Choong-Hwan, Ryu., Young-Joo, Choi., Yong-Yoon, Chung., Akio, Miyao., Hirohiko, Hirochika. and Gynheung, An. (2003) Characterization of a Rice Chlorophyll-Deficient Mutant Using the T-DNA Gene-Trap System. Plant Cell Physiol **44**: 463-472.
5. Nonomura, K., Miyoshi, K., Eiguchi, M., Suzuki, T., Miyao, A., Hirochika, H. and Kurata, N. (2003) The *MSP1* gene is necessary to restrict the number of cells entering into male and female sporogenesis and to initiate anther wall formation in rice. Plant Cell **15**: 1728-1739.
6. Kaneko, M., Inukai, Y., Ueguchi-Tanaka, M., Itoh, H., Izawa, T., Kobayashi, Y., Hattori, T., Miyao, A., Hirochika, H., Ashikari, M. and Matsuoka, M. (2003). Loss-of-function mutations of the rice *GAMYB* gene impair the α-amylase expression in aleurone and flower development. Plant Cell **16**: 33-44.

E-d. Division of Applied Genetics Kunio Shiota Group

RESEARCH ACTIVITIES

(1) DNA methylation profiles of CpG islands in normal tissues

Jun Ohgane[1], Naka Hattori[1], Satoshi Tanaka[1] and Kunio Shiota[1] ([1]Univ. Tokyo)

Methylation of DNA is involved in various biological phenomena through gene silencing, stabilizing chromosomal structure and suppressing the mobility of retrotransposons. Most CpGs are methylated in the mammalian genome; the value varies between 60-90% depending on the report. Overall levels of DNA methylation reflect the hypermethylation status of intergenic regions such as repeated sequences rather than gene encoding regions, because a large amount of the genome is composed of non-genic repetitive elements. Although most CpG islands were once considered to be unmethylated regions in normal tissue, there are many CpG islands with tissue-dependently and differentially methylated regions (T-DMRs) in normal cells or tissues, and the methylation profile of the CpG islands is unique in each tissue or cell type[4] (Shiota *et al.*, Genes to Cells 7, 961-969, 2002). Abnormal DNA methylation profiles associate with various abnormal phenotypes[1].

(2) Preference of DNA methyltransferases for CpG islands in normal cells

Naka Hattori[1], Tetsuya Abe[1], Masako Suzuki[1], Tomoki Matsuyama[2], Shigeo Yoshida[2], En Li[3] and Kunio Shiota[1] ([1]Univ. Tokyo; [2]RIKEN; [3]Massachusets General Hospital)

In vitro studies indicated that DNA methyltransferase1 (Dnmt1) prefers hemimethylated DNA compared to unmethylated DNA, while Dnmt3a/3b methylate CpG dinucleotides without preference for hemimethylated or unmethylated DNA. Collectively, the fact that Dnmt1 is localized to DNA replication foci and associated with the methyl-CpG binding protein, MeCP2, which directs DNA methyltransferase activity to hemimethylated DNA[2], implies that Dnmt1 is involved in maintenance methylation *in vivo* to preserve methylation patterns in genomic DNA, and that Dnmt3a/3b function as *de novo* methyltransferases. However, a direct association between DNMTs was reported, and it was demonstrated that DNMT1 and DNMT3B function cooperatively for maintenance methylation in the human cancer cell line. Therefore, categorizing Dnmts into maintenance and de novo DNA methylation may not be appropriate when attempting to characterize *in vivo* mechanisms involved in determining or establishing DNA methylation profiles in the genome. To address the question how the T-DMR of CpG islands are regulated by Dnmts *in vivo*, we analyzed the genome-wide DNA methylation pattern focussing on CpG islands and found that each Dnmt has target preferences depending the genomic component (in preparation).

(3) Methyl-CpG-binding protein, MeCP2, is a target molecule for maintenance DNA methyltransferase, Dnmt1

Hiromichi Kimura[1] and Kunio Shiota[1] ([1]Univ. Tokyo)

Dnmt1 preferentially methylates hemimethylated DNA. During mammalian cell division, DNA methyla-

tion patterns are transferred accurately to the newly synthesized DNA strand. This depends on maintenance DNA methyltransferase activity. Methyl-CpG binding proteins (MBDs) bind methylated CpG dinucleotides. Dnmt1 does not contain a methyl-CpG binding domain common to the MBD family, raising the question how this enzyme is recruited to hemimethylated DNA and how its replicates the methylation pattern. We found that MeCP2 interacts directly with the maintenance DNA methyltransferase, Dnmt1. The region of MeCP2 that interacts with Dnmt1 corresponds to the transcription repressor domain, which can also recruit histone deacetylases (HDACs) via a corepressor, mSin3A. Although Dnmt1 can form complexes with HDACs as well as MeCP2, the MeCP2-Dnmt1 complex does not contain the HDAC1. Thus, Dnmt1 takes the place of the mSin3A-HDAC1 complex, indicating that the MeCP2-interacting Dnmt1 does not bind to HDAC1. Further, we demonstrated that MeCP2 could form a complex with hemimethylated as well as fully methylated DNA. Immunoprecipitated MeCP2 complexes show DNA methyltransferase activity to hemimethylated DNA. These results suggest that Dnmt1 associates with MeCP2 in order to perform maintenance methylation *in vivo*. We propose that genome-wide and/or -specific local DNA methylation may be maintained by the Dnmt1-MeCP2 complexes, bound to hemimethylated DNA. Dnmt1 may be recruited to targeted regions via multiple steps that may or may not involve histone deacetylases[2].

(4) Transcription of mouse DNA *methyltransferase 1 (Dnmt1)* is regulated by both E2F-Rb-HDAC-dependent and -independent pathways

Hiromichi Kimura[1], Takahisa Nakamura[1], Tomoya Ogawa[2], Satoshi Tanaka[1] and Kunio Shiota[1] ([1]Univ. Tokyo; [2]RIKEN)

Abnormal expression of Dnmt1 *in vivo* induces cellular alterations such as transformation, and an increase in *Dnmt1* mRNA plays a causal role in c-fos-, ras- and SV40 large T antigen-induced transformation of fibroblasts *in vitro*. Thus, Dnmt1 expression must be tightly regulated during normal cell growth. The expression of many replication-related genes, such as *PCNA, cycline E, polα, E2F-1 and cdc6*, is regulated by E2F family of transcription factors. For each target gene, the pathways regulating transcription are classified as either HDAC dependent or independent. We have investigated the regulation of *Dnmt1* transcription and identified the promoter region and major transcription start sites of mouse *Dnmt1* and found two important *cis*-elements within the core promoter region. One is an E2F binding site, and the other is a binding site for an as yet unidentified factor. Point mutations in the two cis-elements decreased promoter activity in both non-transformed and transformed cells. Thus, both sites play a critical role in regulation of *Dnmt1* transcription in proliferating cells. Treatment with trichostatin A, a specific inhibitor of histone deacetylase, increased *Dnmt1* promoter activity in G0/G1-arrested NIH 3T3 cells. Furthermore, the decrease in promoter activity induced by expression of E2F-1 and Rb was reversed by trichostatin A treatment of Saos-2 cells. Taken together, these data indicate that transcription of *Dnmt1* is regulated in a complex fashion by E2F and other transcription factors through E2F-Rb-HDAC-dependent and -independent pathways. These findings suggest that *Dnmt1* is a target gene of these pathways in cell proliferation, cell transformation and tumorigenesis[3].

(5) The *Sall3* locus is an epigenetic hotspot of aberrant DNA methylation associated with placentomegaly of cloned mice

Jun Ohagane[1], Teruhiko Wakayama[2,3], Sho Senda[1], Yukiko Yamazaki[3], Kimiko Inoue[2], Atsuo Ogura[2], Joel Marh[3], Satoshi Tanaka[1], Ryuzo Yanagimachi[3] and Kunio Shiota[1] ([1]Univ. Tokyo; [2]RIKEN; [3]Hawaii Univ.)

Cloned offspring develop a variety of abnormal phenotypes such as increased body weight (large fetus syndrome), pulmonary hypertension, placental overgrowth, respiratory problems and early death. We had identified several aberrantly methylated loci in the tissues of full-term cloned fetuses. Interestingly, each cloned animal has a different DNA methylation pattern and the extent of hyper- or hypo-methylation varies among the individuals (Ohgane *et al.*, Genesis 30, 45-50, 2001). In contrast, overgrowth of the placenta is one of the commonly observed symptoms in all cloned mice regardless of the sex and strain of animal and the type of donor cell. Thus, there may be genomic loci associated with the abnormal placental development in cloned mice and the genomic loci frequently

associated with the epigenetic error have been explored in the cloned animals. We identified the Spalt-like gene 3 *(Sall3)* locus as a hypermethylated region in the placental genome of cloned mice. The *Sall3* locus has a CpG island containing a T-DMR. The T-DMR sequence is conserved in the human genome at the *SALL3* locus of chromosome 18q23, which has been suggested to be involved in the 18q deletion syndrome. Intriguingly, larger placentas were more heavily methylated at the *Sall3* locus in cloned mice. This epigenetic error was found in all cloned mice examined regardless of sex, mouse strain and the type of donor cells. In contrast, the placentas of in vitro fertilized (IVF) and intracytoplasmic sperm injected (ICSI) mice did not show such hypermethylation, suggesting that aberrant hypermethylation at the *Sall3* locus is associated with abnormal placental development caused by nuclear transfer of somatic cells. Thus the Sall3 locus is the area with frequent epigenetic errors in cloned mice. These data suggest that there exists at least genetic locus that is highly susceptible to epigenetic error caused by nuclear transfer[1]).

Publications

Papers

1. Ohgane, J., Wakayama, T., Senda, S., Yamazaki, Y., Inoue, K., Ogura, A., Marh, J., Tanaka, S., Yanagimachi, R. and Shiota, K. (2004). The *Sall3* locus is an epigenetic hotspot of aberrant DNA methylation associated with placentomegaly of cloned mice. Genes to Cells, **9,** 253-260.
2. Kimura, H. and Shiota, K. (2003). Methyl-CpG-binding protein, MeCP2, is a target molecule for maintenance DNA methyltransferase, Dnmt1. J. Biol. Chem. **278,** 4806-4812.
3. Kimura, H., Nakamura, T., Ogawa, T., Tanaka, S. and Shiota, K. (2003). Transcription of mouse DNA methyltransferase 1 (Dnmt1) is regulated by both E2F-Rb-HDAC-dependent and -independent pathways. Nucl. Acids Res. **31**, 3101-3113.

Reviews

4. Shiota, K. (2004) DNA methyaltion profiles of CpG islands for Cellular differentiation and development in mammals. Cytogenetic and Genome Res., In press.
5. 小田真由美，塩田邦郎(2003)．エピジェネティックスと個体発生・細胞分化．分子細胞治療 **2**，469-475.
6. 冨川順子，塩田邦郎(2003)．体細胞クローンはなぜ短命か，遺伝子スイッチ異常説．Animus第8巻20-25.
7. 西野光一郎，塩田邦郎(2003)．DNAメチル化による発生プログラム．実験医学増刊号 **21**，1499-1507.
8. 服部奈緒子，塩田邦郎(2003)．発生・生殖とエピジェネティックス．現代医療 **35**，977-984.

EDUCATION

1. Dr. K. Shiota gave a lecture at Tokyo University of Pharmacy and Life Science, Tokyo, May, 2003 (in Japanese).
2. Dr. K. Shiota was invited to give a seminar on "DNA methylation" at Yamaguchi University, Ube, August, 2003 (in Japanese).
3. Dr. K. Shiota was invited to give a seminar on "DNA methylation" at the National Livestock Breeding Center, Shirakawa, October, 2003 (in Japanese).
4. Dr. K. Shiota was invited to give a seminar on "Epigenetics and DNA methylation" at Yamagata University, Yamagata, October, 2003 (in Japanese).
5. Dr. K. Shiota gave a lecture at The University of Tokyo, Graduate School of Veterinary Medical Sciences, Tokyo, October, 2003 (in Japanese).
6. Dr. K. Shiota was invited to give a seminar on "DNA methylation of normal and cloned animal development" at Yunsei University, Seoul, October, 2003.

SOCIAL CONTRIBUTIONS AND OTHERS

1. 東京大学大学院農学生命科学研究科　教授
2. 東京大学大学院農学生命科学研究科応用動物科学専攻　専攻主任
3. 日本繁殖生物学会　理事
4. 生殖免疫学会　評議員
5. 日本獣医学会生理・生化学分科会　会長

F. GENETIC STRAINS RESEARCH CENTER

F-a. Mammalian Genetics Laboratory Toshihiko Shiroishi Group

RESEARCH ACTIVITIES

(1) Genetic Incompatibility between X-linked Loci and Two Autosomal Regions Causes Hybrid Breakdown between Two Mouse Subspecies

Ayako Oka, Akihiko Mita, Yoichi Mizushina, Noriko Yamatani, Hiromi Yamamoto, Kazuo Moriwaki[1] and Toshihiko Shiroishi ([1]RIKEN BioResource Center)

Hybrid breakdown is a type of reproductive failure that appears after F_2 generation of crosses between different species or subspecies. It is caused by incompatibility between alleles of interacting genes. We have studied the hybrid breakdown using a consomic strain, C57BL/6J-X^{MSM}, in which the X chromosome of C57BL/6J (derived mostly from *Mus musculus domesticus*) is substituted by the X chromosome of the MSM/Ms strain *(M. m. molossinus)*. Males of this consomic strain are sterile due to the abnormal sperm head morphology, whereas F_1 hybrids between C57BL/6J and MSM/Ms are completely fertile. This finding implies that incompatibility between X-linked gene(s) and other chromosomal gene(s) causes the hybrid breakdown observed in C57BL/6J-X^{MSM}. The previous our study revealed that at least three X-linked QTLs are responsible for the sperm head abnormality and the hybrid breakdown.

In this study, we conducted the whole-genome scan to detect autosomal and/or Y chromosomal regions, which interact with the X-linked QTLs and underlie the genetic incompatibility that results in the hybrid breakdown. The result suggested that Chromosome 1 and 11 interact with the X-linked QTLs for proper male reproduction in parental MSM/Ms strain, and disruption of this interaction causes the hybrid breakdown.

(2) Functional analysis of *GsdmA-3* using mutant mice with aberrant epidermal morphogenesis

Shigekazu Tanaka[1], Masaru Tamura, Aya Aoki[2] and Toshihiko Shiroishi ([1]Grad. Univ. Advanced Studies, [2]Juntendo Univ. Medical School/Tokyo Metropolitan Hiroo General Hospital)

Epidermis is maintained by the balance between proliferation of stem cells and cell death following terminal differentiation. Epidermal stem cells reside in both basal layer and upper region of hair follicles, which is known as bulge. While the stem cells in the basal layer differentiate into epidermal keratinocytes, those in the bulge give rise to not only hair follicles but also epidermal keratinocytes and sebocytes in the sebaceous gland. However, the molecular mechanism of epidermal stem cells growth and differentiation is poorly understood. To elucidate this mechanism, our laboratory has analyzed several mutant mice in skin.

Two dominant mouse skin mutants, Recombinant-induced mutation 3 *(Rim3)* and Rex denuded *(Re^{den})*, exhibit hyperkeratosis and alopecia, which arose spontaneously in independent strains. Both mutants have a mutation in GasderminA-3 *(GsdmA-3)*, which is a member of novel gene family, *Gsdm*. The *Gsdm* family has leucine-rich domain of C-terminus, but no known motif.

In this study, to characterize the function of *GsdmA-3* in the epidermal morphogenesis, we performed the expression analysis of *GsdmA-3* and more detailed analysis of *Rim3* phenotype. RT-PCR and *in situ* hybridization analysis showed that *GsdmA-3* is expressed in epidermis and/or hair follicles from postnatal stage. We conducted histological analysis to examine whether hair follicles are degenerated depending on the hair cycle in *Rim3* over several months of ages. As a result, developmental stages of all hair follicles were synchronized in wild type mouse over any month of the ages. By contrast, developmental stages were desynchronized in *Rim3* mice. This result indicates that the hair cycle is disturbed in *Rim3*. Furthermore, BrdU labeling and immunohistochemical analysis with epidermal cells-specific markers demonstrated hyperproliferation and misdifferentiation of the upper follicular epidermis in *Rim3*. All data suggest that *GsdmA3* controls

proliferation and differentiation of epidermal keratinocytes.

(3) Identification and characterization of novel gene family, Gasdermin

Tomoaki Fujii[1], Shigekazu Tanaka[1], Aya Aoki[2], Masaru Tamura and Toshihiko Shiroishi ([1]Grad. Univ. Advanced Studies, [2]Juntendo Univ. Medical School/ Tokyo Metropolitan Hiroo General Hospital)

We report a novel gene family, named Gasdermin *(Gsdm)*, which is composed of seven genes, *Gsdm A-1, A-2, A-3, C-1, C-2, C-3* and *GsdmD* in the mouse. GsdmA-3 was originally identified as a causative gene for the two mouse mutantations, *Rim3* and *Re*^{den}, which exhibit epidermal hyperplasia. *GsdmA* and *GsdmC* genes form a cluster in the mouse chromosome 11 and 15, respectively, and *GsdmD* was mapped to mouse chromosome 15. Comparison of amino acids sequences revealed that members of the *Gsdm* family share similar sequences in the N- and C-terminus. Especially, in the C-terminus, all members of the *Gsdm* family share a well-conserved novel leucine-rich motif.

Gsdm family genes were expressed mainly in the gastrointestinal tract, and some of genes were in the skin. Expression of *GsdmC-2, -3* and *GsdmD* were observed in the small intestine and colon, but not in upper gastric tract. By contraries, *GsdmA-1* and *GsdmA-2* were expressed in the upper gastrointestinal tract, but not in small intestine and colon. More detailed analysis of the upper gastric tract revealed that expression of *GsdmA-1* is specific to the squamous epithelium (esophagus and fore stomach) and that of *GsdmA-2* was in the glandular epithelium (glandular stomach).

In-depth characterization of mouse mutants, Rim3 and *Re*^{den}, and analysis of the gene expression patterns suggested that the members of the *Gsdm* family are involved in regulation of proliferation and differentiation of the epithelial cells in a tissue specific manner.

Publications

Papers

1. Kikkawa, Y., Shitara, H., Wakana, S., Kohara, Y., Takada, T., Okamoto, M., Taya, C., Kamiya, K., Yoshikawa, Y., Tokano, H., Kitamura, K., Shimizu, K., Wakabayashi, Y., Shiroishi, T., Kominami, R. and Yonekawa, H. Mutations in a new scaffold protein Sans cause deafness in Jackson shaker mice. Hum. Mol. Genet. **12**, 453-461, 2003.

2. Floyd, J., Gold, D., Concepcion, D., Poon, T., Wang, X., Keithley, E., Chen, D., Ward, E., Chinn, S.B., Friedman, R. A., Yu, H-T., Moriwaki, K., Shiroishi, T. and Hamilton, B. A. A natural allele of *Nxf1*/TAP suppresses retrovirus insertion mutations. Nature Genet. **35**, 221-228, 2003.

3. Kikkawa, Y., Oyama, A., Ishii, R., Miura, I., Amano, T., Ishii, Y., Yoshikawa, Y., Masuya, H., Wakana, S., Shiroishi, T., Taya, C. and Yonekawa, H. A Small Deletion Hotspot in the Type II Keratin Gene mK6irs1/ Krt2-6g on Mouse Chromosome 15, a Candidate for Causing the Wavy Hair of the Caracul (Ca) Mutation. Genetics **165**, 721-33, 2003.

4. Katoh, H., Watanabe, Y., Ebukuro, M., Muguruma, K., Takabayashi, S. and Shiroishi, T. Chromosome mapping of the peroneal muscular atropy (*pma*) gene in the mouse. Exp. Anim. **52**, 433-436, 2003.

5. Takeya, C., Esumi, M., Shiroishi, T. and Yamamoto, T. Multiple single-nucleotide polymorphism in the methylenetetrahydrofolate reductase and its truncated pseudogene of 23 inbred strains of mice. Biochem. Biophys. Res. Commun. **312**, 480-486, 2003.

6. Sagai, T., Masuya, H., Tamura, M., Shimizu, K., Yada, Y., Wakana, S., Gondo, Y., Noda, T. and Shiroishi, T. Phylogenic conservation of a cis-acting regulator that controls polarized expression of Sonic hedgehog (*Shh*) in limb buds. Mammal. Genome **15**, 23-34, 2004.

7. Oka, A., Mita, A., Sakurai-Yamatani, N., Yamamoto, H., Takagi, N., Takano-Shimizu, T., Toshimori, K., Moriwaki, K. and Shiroishi, T. Hybrid Breakdown Caused by Substitution of X Chromosome between Two Mouse Subspecies. Genetics **166**, 913-924, 2004.

F-b. Mammalian Developmental Laboratory Yumiko Saga Group

RESEARCH ACTIVITIES

(1) Molecular mechanism of somite segmentation

Yu Takahashi (National Institute of Health Sciences), Kaoru Mitsui, Mitsuru Morimoto and Yumiko Saga

The somite is the first morphologically distinct segmental unit formed in a vertebrate embryo and gives rise to metameric structures such as vertebrae, ribs and skeletal muscles. Each somite is subdivided into anterior (rostral) and posterior (caudal) compartments that differ in their properties and gene expression. The rostro-caudal polarity of a somite is established within the PSM prior to segmentation. Our aim is to understand the mechanisms underlying generation of a segmental pattern within the PSM. By the genetic analysis, we have shown that bHLH-type transcription factor, Mesp2 plays a critical role in establishment of rostro-caudal polariy of a somite by regulating *Dll1* expression via Notch signaling pathways. We have shown that there are two Notch signaling cascades; one is Psen1-dependent Dll1-Notch pathway and results in activation of *Dll1* and the other is Psen1−independent Dll3-Notch pathway to antagonize the Dll1-Notch pathway to suppress *Dll1* expression[3]. Mesp2 is also a strong suppressor for *Dll1* expression. However, Mesp2 acts as like a transcriptional activator for rostral genes, *Cer1* and *EphA4*. To understand the mechanism of Mesp2 function and to identify direct targets of Mesp2, we started to analyze molecular nature of Mesp2. Since *Mesp2* expression is temporally restricted, we have analyzed the stability of Mesp2 protein and found that Mesp2 could be regulated by a proteosome-dependent pathway. By analyzing a series of deletion constructs, we have identified amino acid sequences required for the instability of Mesp2. Furthermore, Mesp2 is heavily phosphorylated. We have constructed mutant Mesp2 molecules and are planning to knock in these constructs in Mesp2 locus to know in vivo function of these sequences.

(2) Regulation of *Mesp1* and *Mesp2* expression

Yukuto Yasuhiko (National Institute of Health Sciences), Masayuki Oginuma and Yumiko Saga

Mesp1 and *Mesp2* are both expressed in the early mesoderm and presomitic mesoderm (PSM) just before segmentation. We have previously identified an enhancer required for *Mesp2* expression in the PSM. Using yeast one-hybrid screening, we have found that Tbx6 is a factor that specifically binds to the enhancer sequences. Actually the enhancer region contains consensus sequence known as a T-box binding motif. Using in vitro binding assay, we confirmed the specific binding of Tbx6 on the enhancer. *Tbx6* is expressed in PSM and partially overlapped with the *Mesp2* expression domain. However, the expression domain of *Tbx6* is much wider than that of *Mesp2*. Thus it is unlikely that the Tbx6 is an only factor to regulate *Mesp2* expression. For the further analysis, we are planning to generate transgenic mouse expressing Flag-tagged Tbx6.

In order to analyze global transcriptional regulation of both *Mesp1* and *Mesp2*, we use BAC transgenic strategy. *Mesp1* and *Mesp2* are located in head to head orientation and separated only by 16 kb. Since the expression pattern is very similar, we need to use different reporters for each gene. To introduce *LacZ* and *ALP* genes, we have constructed targeting vectors for homologous recombination in bacteria. So far *LacZ* gene has been introduced either *Mesp1* or *Mesp2* locus by homologous recombination.

(3) Heart morphogenesis and Notch signaling

Hiroki Kokubo, Yusuke Watanabe, Yoshiaki Okamura, Wataru Saito and Yumiko Saga

Notch signaling is crucial for the cardiovascular development. We have cloned three *hesr (hairy and enhancer of split related)* transcription factors, hesr1, hesr2 and hesr3 as possible downstream targets of Notch signaling. We have shown that *hesr2* mutant mice are highly lethal in early postnatal period with congenital heart defects, featured by the enlarged hearts. Although *hesr1* gene is also expressed in the developing cardiovascular system, *hesr1* single knockout mouse does not show any abnormality. However, mice lacking both *hesr1* and *hesr2* die at embryonic day (E) 11.5 and showed not only cardiac defect but also vascular malformations. The *hesr1* and *hesr2* double homozygous hearts exhibit hypoplastic development of myocardial trabeculation of the ventricle due to apoptosis, and few of the cardiac endothelial cells in the atrio-ventricular cushion undergo epithelial-mesenchymal transformation. Our results highlight the importance of *hesr1* and *hesr2* in the developing cardiac and vascular system.

We are also studying Notch function in heart morphogenesis using transgenic mouse, which has activated Notch1 after floxed CAT gene under the control of CAG promoter. We can achieve forced Notch

activation by intercrossing the transgenic mouse with a Cre-expressing mouse. Since *Mesp1* is expressed in the heart precursor cells, we can drive Notch activation only in *Mesp1*-lineage using *Mesp1*-cre mouse. The trans-heterozygous mouse exhibits heart abnormality, which is characterized by abnormal myocardial trabeculation. When we compared Notch activation between wild-type and the transgenic mouse, ectopic Notch activation was observed in myocardium in addition to endocardium in which Notch is normally activated. Furthermore, *hesr1* expression was abnormally induced in the myocardium. This system may provide a key to elucidate hesr1 function in heart morphogenesis.

(4) Gene hunting possibly involved in somitogenesis

Satoshi Kitajima (National Institute of Health Sciences), Aki Ishikawa and Yumiko Saga

In order to obtain possible downstream target genes of Mesp2, and to get more information about molecular mechanism underlying the formation and maturation of PSM cells required for the somite segmentation, we generated two subtractive cDNA libraries and screened them by in situ hybridization screening method. After screening, we identified more than 30 clones that showed the expression in somite and/or PSM region. Among them, we selected 4 genes for further expression and functional analyses. One of them, nkd1 that is a negative regulator for Wnt signaling, exhibits interesting expression pattern during somitogenesis. The expression showed oscillation in the PSM. Several genes are known to be controlled by a molecular clock. The expression of such genes changes during somitogenesis; it starts from tail bud and the expression domain progressively moves to the anterior region. The one cycle is perfectly matched with a cycle for somite formation. Obviously Notch signaling is oscillating, since expressions of many gene involved in Notch signal cascade are oscillating. In addition, Wnt signal might be also oscillating. So far only one oscillating gene, *axin2* has been reported. So *nkd1* is a second gene found to be oscillating. Interestingly, the oscillation phase between *axin2* and *nkd1* is not synchronized, indicating under the different regulation. To know the function of nkd1 during somitogenesis, we are generating the gene knockout mouse.

(5) Functional analysis of mouse nanos genes

Masayuki Tsuda, Yumiko Sasaoka, Makoto Kiso and Yumiko Saga

Previously we have isolated three mouse *nanos* genes *(nanos1, nanos2 and nanos3)*[2]. Among them, we focus on functions of nanos2 and nanos3 since these are specifically expressed and play important roles on germ cell development[4].

Studies on Zebrafish and medaka nanos have indicated that the 3' UTR of nanos gene is important for their localization and own translation. Accordingly, we have generated two types of knock-in genes for *nanos2* locus; one is *LacZ* gene with SV40 poly A signal and the other is *LacZ* with own *nanos2* 3'-UTR. When we compared the expression pattern between them, we noticed ß-gal activity is much higher in the mouse with endogenous nanos2 3'-UTR. However, the expression level of their transcripts is unchanged, indicating different translational efficacy. In contrast, maternal expression of nanos2 is lower in the mouse with endogenous nanos2 3'-UTR. These observations indicate that the expression of nanos protein is regulated both in the transcription and translation and 3'-UTR plays an important role on the translationl control.

For nanos3, we tried to determine the time for initial expression. By collaborating with Dr. Saito (CDB, Kobe), we have successfully detected the nanos3 expression in presumptive PGC cells in 7.25 dpc embryos. The expression was accompanied with expressions of other PGC markers, *stella* and *fragillis*. Importantly, no expression was observed in any somatic cells. Therefore it is formally proved that *nanos3* is an earliest gene that is activated in PGC and plays an important role.

Publications

Papers

1. Rhee, J., Takahashi, Y., Saga, Y., Wilson-Rawls, J. and Rawls, A. (2003) The protocadherin papc is involved in the organization of the epithelium along the segmental border during mouse somitogenesis. Dev Biol. **254**, 248-261.

2. Haraguchi, S., Tsuda, M., Kitajima, S., Sasaoka, Y., Nomura-Kitabayashi, A., Kurokawa, K. and Saga, Y. (2003). nanos1: a mouse nanos gene expressed in the

central nervous system is dispensable for normal development. Mech Dev. **120**, 721-731.

3. Takahashi, Y., Inoue, T., Gossler, A. and Saga, Y. (2003). Feedback loops comprising Dll1, Dll3 and Mesp2, and differential involvement of Psen1 are essential for rostrocaudal patterning of somites. Development **130**, 4259-4268.

4. Tsuda, T., Sasaoka, Y., Kiso, M., Abe, K., Haraguchi, S., Kobayashi, S. and Saga, Y. (2003). Conserved role of nanos proteins in germ cell development. Science **301**, 1239-1241.

5. Mitsui, K., Tokuzawa, Y., Segawa, K., Murakami, M., Itoh, H., Takahashi, K., Maeda, M. and Yamanaka, S. (2003). A novel homeobox gene ECAT4 is required and sufficient for self−renewal of mouse pluripotent cells. Cell **113**, 631-642.

6. Takahashi, K., Mitsui, K. and Yamanaka, Y. (2003). Important role of E-Ras in tumor-like properties of mouse embryonic stem cells. Nature **423**, 541-545.

7. Tokuzawa, Y., Kaiho, E., Maruyama, M., Takahashi, K., Mitsui, K., Maeda, M., Niwa, H. and Yamanaka, S. (2003). Fbx15 is a novel target of Oct3/4 but is dispensable for ES cell self-renewal and mouse development. Mol Cell Biol. **23**, 2699-2708.

8. Okazaki, N., Kikuno, R., Ohara, R., Inamoto, S., Koseki, H., Hiraoka, S., Saga, Y., Nagase, T., Ohara, O. and Koga, H. (2003). Prediction of the coding sequences of mouse homologues of KIAA gene: Ⅲ. the complete nucleotide sequences of 500 mouse KIAA-homologous cDNAs identified by screening of terminal sequences of cDNA clones randomly sampled from size-fractionated libraries. DNA Res. **10**, 167-80.

Books

9. 小林悟，相賀裕美子．ショウジョウバエとマウスの生殖細胞形成過程におけるNanosの機能，細胞工学，**22**，1065-1068，2003.

10. 相賀裕美子，松居靖久．生殖細胞形成，「発生生物学がわかる」わかる実験医学シリーズ，pp.24-29，2003.

EDUCATION

1. Dr. Y. Saga gave a lecture at the University of Tokyo, March, 2003 (in Japanese).

2. Dr. Y. Saga gave a seminar on "Molecular mechanism leading to the establishment of rostro-caudal polarity of somites" at UCLA, USA, August, 2003.

3. Dr. K. Mitsui was invited to give a seminar on "Maintenance of pluripotency in mouse embryonic stem cell" at the Institute of Life Science, Kurume University, Fukuoka, July, 2003 (in Japanese).

F-c. Mouse Genomics Resource Laboratory Tsuyoshi Koide Group

RESEARCH ACTIVITIES

(1) Quantitative traits analyses of sensitivities to heat evoked pain assayed by tests of hot plate and tail flick

Tamio Furuse, Toshihiko Shiroishi[1)] and Tsuyoshi Koide ([1]Mammalian Genetics Laboratory)

In animal, heat stimulation is transmitted to central nervous system (CNS) via sensory neuron, and is recognized as heat or pain. Various levels of heat sensations activate the multi functional pain receptors, TRPV channels that expressed in sensory ganglia. Many behavioral tests had been established to measure the pain sensitivity in rodents by investigating behavioral response from the aversive pain stimuli. Hot plate test displays the ability of pain recognition system or analgesic system based on upper CNS. The tail flick test displays the pain sensitivity based on nociceptive reflex. In the present study, we conducted QTL analyses to characterize genetic loci involved in the different sensitivity to heat evoked pain between two mouse strains, C57BL/6 and KJR. In the study, we applied hot plate test and tail flick test to assess the two types of pain sensitivity of BKF2 progeny generated form F1 progeny of C57BL/6 and KJR. In the analyses, we detected three significant loci for hot plate test, *Hpq1*, *Hpq2* and *Hpq3* and two significant loci for tail flick test, *Tfq1* and *Tfq2*. The pain associated quantitative traits loci, *Hpq1*, *Hpq2*, *Hpq3*, *Tfq1*, and *Tfq2* were located on the chromosomes, 2, 10, 18, 8 and 10, respectively. Hpq2 and Tfq1 were overlapped on the same region of chromosome 10. These data suggested that genetic mechanism for strain difference of pain sensitivities between C57BL/6 and KJR that mediated by upper CNS and reflection system are mostly different but partly shared.

(2) QTL analysis of home-cage spontaneous activity using hyper- and hypoactive mouse strains

Juzoh Umemori, Tamio Furuse, Toshihiko Shiroishi[1)] and Tsuyoshi Koide ([1]Mammalian Genetics Laboratory)

It has been previously reported that mice move spontaneously in the home cage according to a light/dark rhythm, and most mice are active during the dark period. This behavior is called as spontaneous activity, which is quite different among various mouse strains including wild-derived and laboratory strains. Among these strains, activity of KJR strain were significantly high comparing to that of BLG2 and C57BL/6J. The F1 females from the cross between KJR and either BLG2 or C57BL/6J exhibited the similar levels of spontaneous activity as KJR mice strain. We have conducted genetic analyses to elucidate the genetic basis of the spontaneous activity in the home cage. To obtain QTLs involved in spontaneous locomotive activity, we conducted QTL analysis by using F2 populations (BKF2), which were made by intercross of F1 mice between KJR and C57BL/6J. In the QTL analysis of BKF2, we found that two QTLs, positioned on chromosomes 2 and 10, are associated with the different level of spontaneous activity of KJR and C57BL/6. These loci are different to previously identified QTLs, loco1 and loco2, which are associated with different level of activity between KJR and BLG2. These findings suggested existence of complex genetic mechanism for controlling spontaneous activities among strains.

(3) Different effect of apomorphine, dopamine agonist, on open-field activities in wild derived mouse strains

Akinori Nishi, Toshihiko Shiroishi[1)] and Tsuyoshi Koide ([1]Mammalian Genetics Laboratory)

It is known that the dopaminergic system play important roles in controlling locomotor activity of mice. Apomorphine hydrochloride is known as a mixed D1/D2 dopamine receptor agonist, which causes behavior changes in locomotor activity and climbing behavior, and frequently induces stereotypic behavior in rodents. In this study, we investigated and compared the effect of apomorphine on open-field behavior in wild derived mouse strains, NJL/Ms, KJR/Ms and MSM/Ms, and common laboratory inbred strain, C57BL/6JJcl. Different dose of apomorphine, 0.2, 1.0, 2.0, 5.0mg/kg, were administrated to these mice strains. We found that sensitivities to apomorphine were significantly different among these strains. In case of the vehicle administration, the locomotor and rearing activities of NJL and MSM are significantly lower than that of KJR and C57BL/6. In contrast to the behavioral response of locomotion and rearing, number of grooming of NJL and MSM was significantly higher than that of NJL and KJR. The activities and behavioral traits of these strains were changed by apomorphine injection. NJL indicated high locomotor activity at 2mg/kg of apomorphine injection, while 0.2-1.0mg/kg of apomorphine injection exhibited no remarkable effect on this strain. Comparing to the vehicle injection, administration of 0.2mg/kg of apomorphine reduced locomotor activity of KJR and elevated grooming behavior. The locomotor activity of this strain was increased with further dose of apomorphine injection (1-5mg/kg). Locomotion of MSM was increased from 1mg/kg of apomorphine injection while grooming behavior was decreased with increasing dose of apomorphine. Comparing to wild derived strains, C57BL/6 exhibited striking reduction of locomotor activity and rearing behavior by apomorphine injection (1-5mg/kg). As a result of this experiment, we showed that dopaminergic systems are different among these strains.

(4) Descriptive analysis of the open-field behavior in wild mice strains

Aki Takahashi, Toshihiko Shiroishi[1)] and Tsuyoshi Koide ([1]Mammalian Genetics Laboratory)

Open-field is one of the apparatuses for measuring emotionality. The open-field behavior was believed to be influenced by genetic factors. Recent genetic studies have reported that some QTLs for open-field emotionality involved in the behavior. Although most of those study have used ambulatory movement and defecation as the open-field measurements, mice actually show more various behavior in the open-field such as scout and rear to look around, groom their body, or run in the field. We therefore described detail of open-field behavior with 12 behavioral patterns (sniffing, locomotion, stretching, leaning, rearing, grooming, face-washing, digging, gnawing, jumping, pausing, freezing) by counting presence of those behaviors in

every 5 seconds. Behavioral frequency of each pattern is calculated in every 1 min for 10 min test period. We used females and males of 10 wild mouse strains (Mishima battery), a laboratory strain C57BL/6, and a fancy mouse JF1. The 12 strains of mice showed apparent difference in the temporal changes of those behavioral patterns without any significant sex difference. We then applied the principal component analysis for those behavioral patterns to find the structure of the factors lurk behind of the open-field behavior. Four independent components explaining 66.8% of the total variance were found. Component 1 (PC1) acquired high loading from variables related to activity (locomotion, leaning and sniffing for positive, freezing for negative). Variables not related to locomotion (stretching and sniffing for positive, rearing and grooming for negative) loaded highly on Component 2 (PC2), might relate to exploration or habituation. The PC1 and PC2 were thought to be major components for open-field behaviors because they had large variance. We therefore used PC1 and PC2 to compare the 12 mouse strains. In the analysis, common temporal changes were observed; they first exhibited high score for both PC1 and PC2 (stretching, freezing), but both reduced with time (locomotion, leaning, rearing, grooming). KJR and SWN, belonging to same subspecies, showed exactly same characters in the scores and the temporal change patterns in PC1 and PC2. However, most strains showed various characters in PC1 and PC2. These findings indicated that PC1 and PC2 were influenced by multiple genetic mechanisms. Further analysis for characterizing these genetic factors will provide useful information for understanding mechanism of regulating open-field behavior in mice.

Publications

Papers

Furuse, T., Miura, Y., Yagasaki, Y., Shiroyishi, T. and Koide, T. (2003). Identification of QTLs for differential capsaicin sensitivity between mouse strains KJR and C57BL/6. Pain **105**, 169-175.

EDUCATION

Dr. T. Koide gave a lecture at Faculty of Science, University of Tokyo, April, 2003 (in Japanese).

F-d. Plant Genetics Laboratory Nori Kurata Group

RESEARCH ACTIVITIES

(1) Characterization of sporogenesis defects in sterile rice mutants

Ken-ichi Nonomura, Mutsuko Nakano, Mitsugu Eiguchi, Akio Miyao*, Hirohiko Hirochika* and Nori Kurata (*Natl. Inst. Agrobiol. Resour.)

This study aims to dissect genetic machinery for controlling sporogenesis of higher plants. We screened insertion mutant lines tagged by the *Tos17*, an endogenous retrotransposon of rice, and succeeded to isolate three sterile mutants: *pair1*, *pair2* and *msp1*. The *PAIR1 (HOMOLOGOUS PAIRING ABERRATION IN RICE MEIOSIS 1)* gene encodes a putative nuclear protein of 492 amino acids with the coiled-coil structure, and expresses in both male and female meiocytes (submitted). The *PAIR2* encodes a protein of 610 amino acids, homologous to the yeast *HOP1* and Arabidopsis *ASY1*, both of those play an important role in establishing the synaptonemal complex between homologous chromosomes. The loss-of-function mutation of *PAIR1* and *PAIR2* genes resulted in the defect of homologous chromosome pairing and division. The *MSP1 (MULTIPLE SPOROCYTES 1)* gene encodes a putative LRR receptor-like protein kinase of 1294 amino acids and expresses in the nurse cells surrounding the male and female sporocytes. The loss-of-function mutation resulted in aberrant increase of sporocyte numbers, suggesting a major function of *MSP1* to be the restriction of surrounding cells not entering into sporogenous pathway.

(2) Structural and functional analysis of rice *OsHAP3* genes

Kazumaru Miyoshi, Thiruvengadam Thirumurugan, Yukihiro Ito and Nori Kurata

We identified 11 genes *(OsHAP3A to OsHAP3K)* which encode a HAP3/NF-YB subunit of CCAAT-box binding complex in rice by cDNA screening and a database search. We showed that three genes, *OsHAP3A*, *OsHAP3B* and *OsHAP3C*, are involved in

chloroplast biogenesis by an antisense and RNAi approach[1]. Considering that *LEC1* and *L1L*, *Arabidopsis* members of the HAP3/NF-YB subunit, are critical regulators of embryogenesis, plant HAP3/NF-YB genes may have diverse functions ranging from chloroplast biogenesis to embryogenesis depending on each gene. We examined the function of other two members, *OsHAP3E* and *OsHAP3D*, which are most closely related to *LEC1* and *L1L*, respectively, by *in situ* hybridization and by generating transgenic rice plants of overexpression or knock down. The overexpressing transgenic plants showed various developmental abnormalities such as dwarfism, small erected leaves and malformed flowers. RNAi, antisense or repressor-mediated knock down plants are awaiting for their phenotypes.

(3) Functional analysis of *PLASTOCHRON1* in rice

Kazumaru Miyoshi, Yukihiro Ito and Nori Kurata

Rice plants with loss-of-function mutations of *PLASTOCHRON1 (PLA1)* show pleiotropic effects on the rice development, giving short plastochron (rapid emergence of leaves), small erected leaves, dwarfism and elongation of vegetative development. We showed that *PLA1* encodes a member of a cytochrome P450 family and is expressed in young leaves, but not in the SAM[2]. In plants, cytochrome P450s are involved in various metabolic pathways including phytohormone biosynthesis such as auxin, gibberellins and brassinosteroids.

To understand a molecular function of *PLA1*, we generated transgenic rice plants overexpressing the *PLA1*. Regenerated shoots showed severe dwarfism with twisted leaves. Initiation and/or elongation of roots were also inhibited. Metabolome analysis showed that the synthesis of auxin- and gibberellin-related compounds were affected in the overexpressing plants. RT-PCR analysis showed that *OsDWARF* and *OsBRI1*, which encode a brassinosteroid biosynthesis enzyme and a brassinosteroid receptor, respectively, and are targets of a negative feedback control, were down-regulated in the overexpressing plants. These results suggest that *PLA1* is involved in phytohormone synthesis.

(4) Regulation of expression of *KNOX* family class 1 homeobox genes of rice

Yukihiro Ito and Nori Kurata

KNOX family class1 homeobox genes play a vital function for shoot apical meristem (SAM) formation and maintenance. We found that cytokinin, which is necessary for shoot regeneration, induced the expression of *OSH1*, a member of the *KNOX* family, in the callus upon regeneration. It was assumed that the cytokinin-induced gene expression is under contol of a two-component system which consists of a histidine kinase, a phosphotransmitter protein and a response regulator, like as revealed in *Arabidopsis*. To examine this possibility, we searched cytokinin signal transducers in rice and examined one of histidine kinase homologues named *COS3*. We generated *COS3*-overexpressing rice calli and detected *OSH1* expression in it, even on a medium lacking the cytokinin. This suggests that the two-component system is involved in the cytokinin-induced *OSH1* expression in the callus. We have also prepared an examination of *OSH1* expression induced by several possible dominant negative mutations of *COS3*.

(5) Search for nuclear protein genes having regulatory activity in rice development

Kazuki Moriguchi, Mitsugu Eiguchi and Nori Kurata

We applied NTT (nuclear transportation trap) system to collect rice nuclear protein genes from three developmental stages; young panicle, flowering panicle and regenerating calli. We identified over 500 genes including many novel proteins and many transcription factors (submitted). A subset of those genes were examined for their nuclear localization by introducing the genes fused with GFP into onion epidermal cells. A variety of protein localization in the cell; for instance, nuclear peripheral, foci-like, speckle-like, chromatin-associated and matrix-associated distribution, were observed (submitted). Some of these genes were revealed to express at specific stages of rice development by RT-PCR and *in situ* hybridization. To characterize the unknown gene function, generation of transgenic rice which overexpress or being knocked down 14FPB077 protein is in progress.

(6) Introduction of rice artificial chromosomes into rice

Tadzunu Suzuki, Ken-ichi Nonomura and Nori Kurata

Since the transfer methods of huge size DNAs into rice cells have not been established, two methods of lipofection and particle bombardment system were examined for transferring the candidate DNAs into rice. Though lipofection can reduce damages of DNAs of huge size, it had rarely been used for plant cell transformation. This method was applied for rice protoplasts isolated from rice calli. Results indicated the effectiveness of the lipofection method for rice protoplasts, at least with a small size DNAs. Another attempt to introduce larger size BACs possessing rice centromeric sequences into rice by particle bombardment could generate a few lines of calli and regenerated plants. RT-PCR analysis showed that they expressed the marker gene. However, Southern analysis indicated that the tranfered DNAs should be integrated into the genome in the transformants.

(7) Generation and screening of enhancer trap lines of rice

Yukihiro Ito and Nori Kurata

To isolate tissue-specific enhancers and valuable mutants defective in various steps of rice development and to clone the corresponding genes, we are generating enhancer trap lines of rice. We employed an enhancer trap system based on the *Ac/Ds* two-element system and the GUS reporter gene. We already have established the system by which *Ds-GUS* transposants are efficiently identified in rice (in press).

This year we screened about 1,000 transposed lines and identified 62 lines with tissue-specific GUS activity. GUS expression was observed in various organs including embryo, leaf, root, flower, tiller and shoot apex. Some lines expressed the GUS protein upon wounding. We cloned a trapped gene by a *Ds-GUS* insertion in a line P2503, which expressed the GUS in the wounded portion of a leaf, as an example. The *Ds-GUS* was shown inserted between two genes, one was *OsLEA3* and the other was an unknown gene. *OsLEA3* is a member of a late embryogenesis abundant protein gene family and is expressed in roots by drought or ABA treatment. To examine which enhancer of the two genes is trapped by the *Ds-GUS*, we examined the expression of these genes by RT-PCR. *OsLEA3* was expressed transiently in the leaf after wounding, while the unknown gene was expressed without wounding. This indicates that the enhancer of OsLEA3 was trapped in P2503. Cloning of other trapped enhancers is underway. These data will be opened through Oryzabase (http://www.shigen.nig.ac.jp/rice/oryzabase/top/top.jsp).

(8) Positional cloning of a segregation distortion gene detected in a progeny of a cross between Japonica and Indica rice

Yoshiaki Harushima and Nori Kurata

The aim of this study is isolation of the most prominent barrier on chromosome 3 detected in the F_2 of Nipponbare-Kasalath hybrid by positional cloning, and elucidation of the molecular nature of the individual reproductive barrier. We have clarified the aimed gene was a male gametophyte gene that interact with a maternal locus on chromosome 6. In other words, the pollen with Kasalath genotype at the gametophyte gene preferentially takes place in fertilization of eggs by 94% probability in the maternal plant that is heterozygote or Kasalath homozygote at the interactive locus on chromosome 6.

For fine mapping the gametophyte gene, we have selected plants with recombination in the candidate region from 5691 F_2 and 473 backcross plants. The genotypes of the target gene in the recombinants were determined by segregation analyses of selfed progenies showing heterozygous genotype for the interactive region. In case the target gene locus of a recombinant is heterozygous, segregation of heterozygous marker in selfed progeny is 0:1:1 for Nipponbare homozygote (N): heterozygote (H): Kasalath homozygote (K). In case of homozygous of the gene, segregation would be 1:2:1 as Mendelian expectation. Progeny of one of the selected plants that recombined Kasalath homozygote to heterozygote at the vicinity of an *Argonaute* gene showed unexpected segregation, 1:3:2 for N: H: K. One possible explanation is that the causal cistron in this recombinant is chimera that has half ability to fertilize comparing to Kasalath homogeneous cistron and the *Argonaute* gene is the causal gene. Expression of this *Argonaute* gene in both Nipponbare pollen and Kasalath pollen was confirmed. RT-PCR and micro-array analysis and showed there was no difference in

the expression level. However, 14 single-base changes between Nipponbare and Kasalath genome sequences were detected in the 6.9 kb *Argonaute* region. Nine out of the 14 changes were in the peptide-coding region; one and eight were synonymous and nonsynonymous substitutions, respectively. High frequency of non-synonymous substitutions suggests rapid evolution that is common feature of reproductive barriers. We are preparing complementation test for this barrier.

Publications

Papers

1. Miyoshi, K., Ito, Y., Serizawa, A. and Kurata, N. (2003). *OsHAP3* genes regulate chloroplast biogenesis in rice. *Plant J.* **36**, 532-540.
2. Miyoshi, K., Ahn, B-O., Kawakatsu, T., Ito, Y., Itoh, J-I., Nagato, Y. and Kurata, N. (2004). *PLASTOCHRON1*, a timekeeper of leaf initiation in rice, encodes cytochrome P450. *Proc. Natl. Acad. Sci. USA* **101**, 875-880.
3. Nonomura, K.I., Miyoshi, K., Eiguchi, M., Suzuki, T., Miyao, A., Hirochika, H. and Kurata, N. (2003). The *MSP1* gene is necessary to restrict the number of cells entering into male and female sporogenesis and to initiate anther wall formation in rice. *Plant Cell* **15**, 1728-1739.
4. Suzuki, T., Nonomura, K-I., Takeda, N., Murayama, Y. and Kurata, N. (2003). Application of enhanced lipofection to rice transformation. *Rice Genet, Newslet.* **20**, 116-118.
5. Nonomura, K.I., Nakano, M., Murata, K., Miyoshi, K., Eiguchi, M., Miyao, A., Hirochika, H. and Kurata, N. (2003). The insertional mutation of rice *PAIR2* gene, the ortholog of *Arabidopsis ASY1*, caused a defect in homologous chromosome pairing in meiosis. *Mol. Genet. Genomics*, **271**, 121-129.
6. Nonomura, K-I., Nakano, M., Fukuda, T., Eiguchi, M., Miyao, A., Hirochika, H. and Kurata, N. (2004). The novel gene *HOMOLOGOUS PAIRING ABERRATION IN RICE MEIOSIS1* of rice encodes a putative coiled-coil protein nequired for homologous chromosome pairing in meiosis. Plant Cell **16**, 1008-1020.
7. Ito, Y., Chujo, A., Eiguchi, M. and Kurata, N. (2004) Radial axis differentiation in a globular embryo is marked by HAZ, a PHD-finger homeobox gene of rice. *Gene* **331**, 9-15
8. Ito, Y., Eiguchi, M. and Kurata, N. Establishment of an enhancer trap system with Ds and GUS for functional genomics in rice. Mol. Genet. Genomics: in press.

Book

9. Kurata, N. and Fukui, K. (2003). Chromosome research in genus Oryza. *'Monograph in genus Oryza.'* Nanda JS ed. Science Publisher. pp 213-261.

EDUCATION

1. 倉田のり「イネ科植物を例としたゲノムと進化の話」遺伝学講演会，遺伝学研究所一般公開，2004年４月
2. 倉田のり「ゲノムから見える植物の生存戦略」三島市図書館講座，三島市生涯学習センター，2004年11月

SOCIAL CONTRIBUTIONS AND OTHERS

特許

1. 出願番号：2004-017246，発明の名称：種子重量の増加したトランスジェニック植物とその利用，発明者：倉田のり・三好一丸・安　炳玉・伊藤幸博，出願人：国立遺伝学研究所長

学会活動

倉田のり（学術会議育種学研究連絡委員会委員，日本育種学会幹事，日本育種学会学会賞選考委員，Breeding Science 編集委員，Rice Genetics Newsletter Editor，農林水産省評価専門委員，生物遺伝資源イネ小委員会委員長）

F-e. Microbial Genetics Laboratory
Akiko Nishimura Group

RESEARCH ACTIVITIES

(1) Demonstration of native dynamics of FtsZ in the growing *Escherichia coli* single cell by on-chip microculture

Ippei Inouye, Kenji Yasuda and Akiko Nishimura

FtsZ, first identified genetically by Lutkenhaus is essential key protein acting at an early stage of cytokinesis, structural and functional homologue of eukaryotic tubulin, and highly conserved over probably all prokaryotes including archaea, chloroplasts, and some mitochondria. Precise timing of the FtsZ-ring dynamics in a cell cycle has become one of the important problems to make clear since Bi and

Lutkenhaus demonstrated that FtsZs are scattered through the cytoplasm during most of the cell cycle but assemble into a microtuble-like cytokinetic ring on the inner membrane of the potential divisionsite in the early stages of septum apparatus constriction in *E. coli*. Despite the exhilarating advances in role of FtsZ in bacterial cell division, it is not well known when and how FtsZs change their location in a growing cell cycle. We have succeeded to demonstrate the timing of native dynamics of FtsZ such as assembly, constriction, and disassembly in the exponentially growing single cells of E. coli, using an on-chip microculture system. In the system, single bacterium was grown in an arrayed microchamber arranged on glass slide and separated by semi-permeable membrane from cover chamber with circulating medium, and the real time dynamics of FtsZ-GFP localization was observed by fluorescent microscopy. Fluorescence intensity of FtsZ-GFP was measured by NIH image. Constructed strain expressed FtsZ-GFP, with long flexible linker to minimize the obstruction of GFP, under the control of native ftsZ promoters on chromosomal DNA, and divided/grew completely similar as the normal tube cultures. As the results, we proposed new model for dynamics of FtsZ and timing of cell division (in preparation).

(2) A complete set of *E. coli* genes/ORFs on a mobile plasmid: successful use in the isolation of all cell division mutants

Kimiko Saka, Ken Nishikawa, Hideaki Sugawara, Noriko Matsumoto, Hiroshi Fujishima and Akiko Nishimura

To facilitate genetic studies of *Escherichia coli*, we constructed a complete set of mobile plasmid clones of W3110 open reading frames (ORFs). The plasmid could be transferred from F^+ to F^- strains and carries a multi-cloning site as well as ampicillin resistance gene. Expression of the cloned ORF is strictly controlled by Ptac/$lacI^q$. The plasmids carrying each ORF were introduced in an F^+ *recA* strain and stored in 96-well microtiter plates. In this way, 96 clones of the plasmid can be transferred concurrently to F^- bacteria using the conjugative system and without making competent cells. This provides a convenient procedure for screening the ability of a cloned ORF to suppress or complement *E. coli* mutations, and, thus, for the systematic identification of mutant genes. Further, to minimize the difficulties in identifying a positive clone, we evaluated pools of 48 clones, which we found worked well for complementation studies. We created two types of clone sets: the original set stored as individual clones in 45 microtiter plates, the second set made up of pools of 48 clones stored in a single microtiter plate. Using these two kinds of clone sets, we have identified 388 genes that can correct the temperature sensitive defect in cell division (fts). We have further analyzed these genes systematically and identified up to hundred novel genes involved in the division process. We also demonstrated the global network coordinating cellular events in a cell cycle through cell division.

Publications

Papers

1. Makinoshima. H., Aizawa. S., Hayashi. H., Miki. T., Nishimura. A. and Ishihama. A. (2003). Growth phase-coupled alterations in cell structure and function of *Escherichia coli*. J. Bacteriol, **185**: 1338-1345.

Database

http://www.shigen.nig.ac.jp/ecoli/strain/top.jsp

Social contributions and others

特許

1. 出願番号：2003-359534, 発明の名称：マルチウェルプレート, 発明者：西村昭子・梶谷隆文, 出願人：国立遺伝学研究所長, ㈲ガジックストレーディング
2. 出願番号：2003-405263, 発明の名称：マルチウェルプレート, 発明者：西村昭子・梶谷隆文, 出願人：国立遺伝学研究所長, ㈲ガジックストレーディング

学会活動

1. Drs. Y. Kohara, A. Nishimura, and Y. Yamazaki organized a satellite poster session "Bio-resource" at 26th Annual Meeting of the Molecular Biology Society of Japan, Kobe, December, 2003 (in Japanese).
2. Dr. A. Nishimura was appointed for a member of editorial board of "Microbiology and Culture Collections".

F-f. Invertebrate Genetics Laboratory
Ryu Ueda Group

RESEARCH ACTIVITIES

(1) RNAi mutant fly bank for comprehensive analyses of gene function in *Drosophila*

Ryu Ueda, Misako Taniguchi, Kaoru Saigo[1] and Kuniaki Takahashi ([1]Graduate School of Science, University of Tokyo)

Genome sequencing projects have revealed the number of genes for several model organisms for genetics. The small worm *Caenolabditis elegans*, which is composed of only 959 cells, has 19,000 genes in its genome. On the other hand, *Drosophila melanogaster*, which has a long and sophisticated alimentary canal, a tubular heart that circulates hemolymph, and a large brain composed of over 10^4 cells, harbors only 13,500 genes. Considering there is such a small number of fly genes, each one of them may have an essential function in fly development and behavior. In other words, it may be easy to detect and analyze gene function in the fly by reverse genetics because the abnormal phenotype will frequently appear when knocking down a target gene whose function is unknown. We are planning to investigate the function of fly genes comprehensively as a suitable model for studying the functional genomics of multicellular organisms.

How does one investigate the function of all 13,500 protein-coding genes in the fly? We use RNA interference (RNAi) to knock down the activity of the target gene. RNAi is one of the emerging technologies with which to investigate gene function in multicellular organisms. When introduced into the cell, double stranded RNA (dsRNA) works as a specific mutagen for each gene. That is, dsRNA recognizes host mRNA and digests it in a sequence-specific manner, and consequently brings a loss-of-function mutation phenotype to the host cell. The detailed mechanism of this RNAi phenomenon has not yet been elucidated, but it works efficiently in many multicellular organisms, including humans.

We coupled the RNAi with the GAL4-UAS gene expression system to induce a conditional loss-of-function mutation in the fly. The GAL4-UAS system is a binary system for inducing transgene expression, in which two fly lines are used. One is the GAL4 driver fly line, which expresses yeast transcription factor, GAL4, in a specific cell/tissue or at a specific developmental stage in favor of the GAL4 transgene. The other fly line harbors a transgene on the chromosome, in which an appropriate gene to be expressed is fused to the UAS promoter, the GAL4 target. When these two fly lines are crossed with each other, we can observe in the fly progeny that the GAL4 protein induces target transgene expression in a driver-specific conditional fashion. In this GAL4-UAS system, when we use a UAS-transgene having an inverted repeat (IR) sequence, the transcribed RNA may form a dsRNA in the cell and induce a loss-of-function mutation by the RNAi mechanism. Such inducible RNAi caused by the transcription of an IR sequence was first successfully adopted to gene function analysis in *C. elegans*. It was then also found to be effective in fly genetics. By making a UAS-transformation vector containing an IR sequence of the gene predicted by the fly genome project, and by introducing it into a fly line (IR fly), a mutant phenotype of the gene can be easily observed in any cell or at any developmental stage of the progeny, whenever the IR fly is crossed to an appropriate GAL4 driver fly.

We are expanding this inducible RNAi to the whole genome of the fly. This process involves two major procedures.

1) *in vitro* construction of transformation vectors containing an IR sequence from each of the 13,500 predicted genes.

2) Transformation of IR vectors by injecting them into fly eggs and establishment of IR fly lines by traditional genetic methods.

As of the end of 2003, over 4000 transformation vectors had been constructed, 3600 of which have been successfully introduced into the fly. Considering the pace of this work, the construction of vectors for over 11,000 genes (accounting for 80% of the entire genome) should be accomplished during the next fiscal year. Completion of the transformation will require about one additional year. Clearly then, we will be able to obtain a powerful tool with which to determine the function of the entire fly genome in the near future.

Along with the establishment of IR fly lines, basic characterization of the target genes is conducted using these fly lines. All of the IR fly lines are crossed to the

Act5C-GAL4 fly. The Act5C-GAL4 induces the UAS-transgene in all cells at all developmental stages. Thus, if the gene targeted by RNAi has functions that are indispensable for fly development, the progeny of IR and GAL4 flies should die before the adult flies emerge. Among the 1954 genes tested, 51.1% of the fly lines showed lethality. This value is rather high compared to that obtained by classical genetics (25%), while the fact that many of the genes tested here were considered to have important functions in various aspects of fly development by our collaborators may bring about such a high score. Detailed analyses on known genes and greater accumulation of data are necessary. We are currently collaborating with 40 groups. The usefulness of inducible RNAi for investigating gene function in *Drosophila* is being revealed in many aspects. We published 2 papers using RNAi flies[2),3)] and 1 paper on RNAi mechanism[1)] in 2003.

This work was supported in part by financial assistance to Dr. Ueda from the Mitsubishi Kagaku Institute of Life Sciences (MITILS).

Publications

Papers

1. Doi, N., Zenno, S., Ueda, R., Ohki-Hamazaki, H., Ui-Tei, K. and Saigo, K. (2003). Requirement of Dicer and eIF2C translation initiation factors for short-interfering-RNA-mediated gene silencing in mammalian cells. Curr. Biol. **13**, 41-46.

2. Kamiyama, S., Suda, T., Ueda, R., Suzuki, M., Okubo, R., Kikuchi, N., Chiba, Y., Goto, S., Toyoda, T., Saigo, K., Watanabe, M., Narimatsu, H., Jigami, Y. and Nishihara, S. (2003). Molecular cloning and identification of 3'-phosphoadenosine 5'-phosphosulfate transporter. J. Biol. Chem., **278**, 25958-25963.

3. Takemae, H., Ueda, R., Ohkubo, R., Nakato, H., Izumi, S., Saigo, K. and Nishihara, S. (2003). Proteoglycan UDP-galactose:beta-xylose beta1,4 galactosyltransferase I is essential for viability in *Drosophila melanogaster*. J. Biol. Chem., **278**, 15571-15578.

Reviews

6. 西原祥子，上田龍(2003)「ショウジョウバエの糖鎖生物学」蛋白質核酸酵素2003年6月号増刊，「糖鎖機能」，谷口直之・川嵜敏祐・古川鋼一・木全弘治・鈴木明身編，1064-1071頁，共立出版，東京.

EDUCATION

1. R. Ueda gave a seminar on RNAi mutant fly bank at the ZoeGene Corporation on February, 2003.
2. R. Ueda gave a lecture on RNAi at the Mitsubishi Pharma Corporation on April, 2003.
3. R. Ueda gave a seminar on a database of RNAi mutant fly bank at the Mitsubishi Chemical Corporation on October, 2003.

SOCIAL CONTRIBUTIONS AND OTHERS

1. R. Ueda was invited to the Mitsubishi Kagaku Institute of Life Sciences as a Visiting Researcher and lead mutant fly bank project in collaboration with NIG lab.
2. R. Ueda joined organizing committee for biennial meeting of Japan Drosophila Research Conference held at Tokyo on July.

F-g. Laboratory for Frontier Research Takako Isshiki Group

RESEARCH ACTIVITIES

(1) Search for the factors involved in temporal specification within Drosophila neuroblast lineage

Kusano Ayumi and Takako Isshiki

During development, neural progenitors often generate diverse cell types in an invariant order, changing their property over time. Yet we know relatively little about the molecular mechanisms of how different cell fates are generated in order, especially in vertebrate, because of the lack of any particular vertebrate neural progenitor lineage which can be reproducibly identified in vivo. The *Drosophila* central nervous system (CNS) is an excellent model system to study temporal specification of cell fates within a lineage. All of the progenitors of the *Drosophila* embryonic CNS, called neuroblasts, can be easily identified, and each has a unique and invariant lineage. Moreover, we have shown that *Drosophila* neuroblasts sequentially express the transcription factors Hunchback, Krüppel, Pdm and Castor over time. The identification of these transcription factors enables us to investigate the molecular mechanisms of neuroblast

lineage development in detail.

To identify yet unrevealed factors involved in temporal specification within neuroblast lineage, we are searching a public dataset of the expression profiles of the *Drosophila* gene transcripts. So far, we found that several *Drosophila* homologs of the *C.elegans* heterochronic genes are expressed widely in the CNS in temporally regulated manner. The heterochronic genes control developmental timing in *C.elegans* larvae, and mutations in these genes cause defects in temporal specification within certain cell lineages. Recent studies have shown that *C.elegans hunchback* homolog *(hbl-1)* is actually one of the heterochronic genes.

We have been focusing on studying the three *Drosophila* homologs of heterochronic genes, *lin-28, lin-41,* and *lin-29. lin-28* encodes a putative well-conserved RNA binding protein, although its molecular function is largely unknown. We have revealed that *lin-28* mRNA and Lin-28 protein are expressed only in most, if not all, Hunchback positive neural precursors and neurons, and absent from neuroblasts. Furthermore, misexpression of *hunchback* leads the ectopic expression of Lin-28 in hunchback mis-expressed precursors and neurons. Thus, lin-28 is a very good candidate of differentiated cell specific target of Hunchback. Asymmetric division of neuroblasts generates cell fate difference between neural precursor and neuroblast. We are interested in investigating how asymmetric division and Hunchback coordinately regulate Lin-28 expressions in the future.

Lin-41 is also a RNA binding protein, but its molecular functions are barely understood even in *C.elegans. Drosophila lin-41* mRNA is expressed in neuroblasts from the early to mid stage of embryogenesis. Genetic analysis of *Drosophila lin-41* function is now underway.

(2) Neuroblast lineage development at late period in embryogenesis.

Takako Isshiki and Kusano Ayumi

Another heterochronic gene homolog, lin-29, encodes a Krüppel type zinc finger protein. We have found that Lin-29 is expressed in the CNS from the late period of embryogenesis. In addition, we have found that Krüppel and Seven-up, which are once expressed and turned off at relatively early time point in neuroblast lineage, are re-expressed afterward in neuroblasts. We are trying to reveal the precise order of Lin-29, Krüppel, Seven-up, and Castor expressions in individual neuroblasts during late embryogenesis. This analysis should provide the basic knowledge for elucidating the molecular mechanisms for the late steps of lineage development which are not neuroblast understood well at present.

G. CENTER FOR GENETIC RESOURCE INFORMATION

G-a. Genetic Informatics Laboratory Yukiko Yamazaki Group

RESEARCH ACTIVITIES

SHIGEN project

(1) PEC

Takehiro Yamakawa, Junichi Kato and Yukiko Yamazaki

PEC (Profiling of Escherichia coli Chromosome) database is an integrated public resource database which provides an useful information that could help to characterize the gene function of Escherichia coli. A unique characteristic of the PEC is the gene classification based on essentiality for cell growth. All of the Escherichia coli genes were classified into three groups, (1) gene essential for cell growth (essential), (2) those dispensable for cell growth (non-essential), and (3) those unknown to be essential or non-essential (unknown), mainly using information from deletion mutants and journal articles. Another unique characteristic is the comparative gene analysis among bacterial genomes. PEC provides the list of numbers of homologues genes found in other bacteria genomes analyzed three times with different e-values, so that species-specific genes and/or orthologous genes are easily detected. In the database, each gene has link to the relevant strain stocks publicly available. The database is based on ObjectStore (Object Oriented Database Management System) and is released with a comprehensive interface.

PEC database can be accessed through the SHIGEN server (http://www.shigen.nig.ac.jp/ecoli/pec/).

(2) CARD R-DB

Takehiro Yamakawa, Hideki Kato, Naomi Nakagata, Kenichi Yamamura and Yukiko Yamazaki

CARD R-DB is a genetically engineered mice database of Center for Animal Resources and Development (CARD), Kumamoto University, established as a core center for production, cryopreservation and supply of those mice.

The database system has been implemented with a direct online data submission and validation system. Each entry belongs to at least one group and one entry consists of strain-specific and/or gene specific information. Although the data type of most items is free text type, strain and gene names strictly follow the internationally controlled vocabulary. We have developed data submission and validation tools by which user can easily submit their data through the internet and administrator can validate the information right after the submission and then automatically update the database on the same web screen. The database is managed in relational database system (Oracle) with Java servlet applications. The CARD R-DB is accessible at http://cardb.cc.kumamoto-u.ac.jp/transgenic/.

(3) JMSR

Takehiro Yamakawa and Yukiko Yamazaki

JMSR (Japan Mouse Strain Resource) database provides a common gateway of mouse genetic resources from different organizations. Current database compiles information from four organizations including Kumamoto University, National Institute of Genetics, Tokushima University and Japan Animal Strain Committee. Each data consists of minimal items such as (1) Strain name, (2) Strain group, (3) Stock type, (4) Genes, (5) Model disease name and/or application field and (6) URL address from where full information of the certain strain can be retrieval. We use the free PostgreSQL relational database in order to make the system freely available under an Apache open source license. JSMR expects new members to join in the near future. Data distribution in XML format with the associated document type definition (DTD) file is also ongoing project. JMSR provides a web access at http://www.shigen.nig.ac.jp/mouse/jmsr/.

(4) Oryzabase

Takehiro Yamakawa, Shingo Ueno, Nori Kurata, Atsushi Yoshimura, Hikaru Sato, Yasuo Nagato and

Yukiko Yamazaki

Oryzabase represents an integrated rice science database, containing.

(1) Rice Genetic Resources, (2) Mutant Phenotype Collection, (3) Gene Dictionary, (4) Genetic Maps, (5) Developmental stage and gene expression, (6) Basic Information and (7) Useful site information.

Current database compiles ca. 11000 strains maintained by 25 stock centers. Oryzabase also provide the "Core Collection of Wild Rice" to show representative wild rice accessions, which cover 9 different genomes (AA, BB, CC, BBCC, CCDD, EE, FF, GG, and HHJJ) and 18 different species. All collections have been characterized and presented with image data. Mutant phenotype collection is currently classified into 5 groups such as Coloration, Heterochrony, Reproductive organ, Seed, and Vegetative organ. Oryzabase also provide web-based data submission system by which users can upload the mutant phenotype image files and submit them to the database.

The gene information of Oryzabase is based on the report of the Committee on Gene symbolization, Nomenclature and Linkage Groups.

Ontology is one of powerful idea to specify a certain concept and useful especially to compare the concept common between different fields. Among many biological ontology projects recently established, the gene ontology (GO) project is most popular and widely spread because major databases such as Flybase, SGD, MGI, TAIR, WormBase, PomBase, RGD, EBI, TIGR have already joined. Recently Gramene database maintained by Cornell University has joined the GO (Gene Ontology) project with leading of Rice databases. Gramene database now proposes three rice ontologies, gene ontology (GO), traits ontology (TO) and plant ontology (PO) that defines the plant anatomy and/ or plant developmental stages. Coordinating the Gramene's efforts, all genes of the current dictionary were assigned to the above ontologies (GO/TO) in the Oryzabase. Assignment of GO for newly coming genes obtained from genomic sequences as well as PO assignment is ongoing project in collaboration with rice researchers and other rice databases. To make this collaboration easier, comprehensive viewer called "GOALL", that will give researchers overview of the current GO and help them understand how ontology works and how it is useful, was developed.

Oryzabase is available at http://www.shigen.nig.ac.jp/rice/oryzabase/.

(5) KOMUGI

Takehiro Yamakawa, Takashi Endo, Yasunari Ogihara, Hitoshi Tsujimoto, Taihachi Kawahara, Tetsuro Sasakuma and Yukiko Yamazaki

KOMUGI was originally established as a wheat genetic resource database by collecting strain stock information from several Japanese universities and grew up to an integrated wheat information database. Current version of KOMUGI database consists of (1) strain resource information, (2) EST and DNA resource information, (3) wheat gene catalogue, (4) composite maps, (5) wheat information service (WIS), and (6) DNA sequence collection of wheat (Triticeae, Avena, Secale and..). The gene catalogue is published every 5 years and only supplemental data is annually published. To make the up-to-date gene catalogue available through the Internet, we've developed the management system of the gene catalogue called "MacGene" by which the catalogue keeper can easily submit and/or update the data followed by opening the database to the public through the Internet automatically. KOMUGI database is available at http://www.shigen.nig.ac.jp/wheat/komugi/.

Publicatons

Papers

1. Ogihara, Y., Mochida, K., Nemoto, Y., Murai, K., Yamazaki, Y., Shin-I, T. and Kohara, Y. (2003). Correlated clustering and virtual display of gene expression patterns in the wheat life cycle by large-scale statistical analyses of expressed sequence tags. The Plant Journal. 33, 1001-1011.

2. Mochida, K., Yamazaki, Y. and Ogihara, Y. (2003). Discrimination of homologous gene expression in hexaploid wheat by SNP analysis of contigs grouped from a large number of expressed sequence tags. Mol. Gen. Genomics. **270**, 371-377.

Reviews

3. 山崎由紀子「疾患モデル動物データベース」医学のあゆみVol.204 No.6, 463-467 (2003).

Databases

4. Oryzabase http://www.shigen.nig.ac.jp/rice/oryzabase
5. KOMUGI http://www.shigen.nig.ac.jp/wheat/komugi/
6. PEC http://www.shigen.nig.ac.jp/eco/pec/
7. Barley EST http://www.shigen.nig.ac.jp/barley/
8. C. elegans http://www.shigen.nig.ac.jp/c.elegans/
9. WGR http://www.shigen.nig.ac.jp/shigen/wgr

EDUCATION

1. Y. Yamazaki gave a lecture at the Kyoto University, March, 2003 (in Japanese).
2. Y. Yamazaki was invited to give a seminar on "遺伝資源データベースの将来構想" at University, Kyoto工芸繊維大, September, 2003 (in Japanese).

SOCIAL CONTRIBUTIONS AND OTHERS

1. 学術会議遺伝資源研究連絡委員
2. NBRP運営委員：ミヤコグサ・ダイズ、コムギ、オオムギ、藻類、ES細胞、メダカ
3. 評価委員：生物資源研究所BRC
4. 理研BRCデータベース検討委員会委員：

G-b. Genome Biology Laboratory Yuji Kohara Group

RESEARCH ACTIVITIES

(1) NEXTDB: The nematode expression pattern map database

Tadasu Shin-I, Ikuko Sugiura-Muramatsu, Masumi Obara, Wakako Shimizu, Yuko Suzuki, Aya Hamakawa, Jean Thierry-Mieg[1], Danielle Thierry-Mieg[1], Sachiko Takahashi, Kyoko Nakata, Hiroko Uesugi, Takayo Hamanaka, Yasuko Sugiyama and Yuji Kohara ([1]NCBI, NIH)

We have developed a WWW-based database "NEXTDB" to integrate all the information of ESTs, gene expression patterns and gene functions of *C.elegans* which are being produced and analyzed in this laboratory.

In order to integrate NEXTDB, we applied a hierarchical model to arrange all the clones and cDNA groups; 1) chromosome, 2) cosmid clone, 3) CDS, 4) cDNA group, and 5) cDNA clone. The cosmid and CDS map which connects 1), 2) and 3) are constructed from the genome and cosmid data obtained from the Sanger Centre and the WormPep database. All the information, such as tag sequences, homologies of the cDNA, in situ images, and RNAi phenotypes, are arranged based on the corresponding cDNA clones. They are integrated with the genome map based on WWW, and the information of maps and their relations are displayed visually by use of JAVA applets.

The database contained the information of about 135,000 cDNA clones, which classified into about 14,000 groups, corresponding to about two-thirds of the total gene number. About 11,400 groups hit to the predicted CDSs in the genomic sequences, and about 2,600 groups hit to the non CDS regions. We compared some ESTs and the corresponding genomic sequences strictly and found the presence of alternative splicings and differential termination. We also found that some CDSs were predicted incorrectly and some genes were not predicted even there were the corresponding ESTs. Therefore, close comparison of ESTs and the genomic sequences is very important to identify genes precisely. NEXTDB also incorporates in situ images of about 9,200 cDNA groups, RNAi phenotype images of 300 groups. Most of in situ images have been annotated preliminarily.

The latest version is available through the following URL. http://nematode.lab.nig.ac.jp

Many collaborations based on NEXTDB have been conducted (See Ref.1)

(2) Systematic antibody raising and maternal gene expression analysis in *C.elegans*

Yumiko Ueta, Masahiro Ito, Hiroko Ochi, Chihiro Hijikata, Sachiko Takahashi, Keiko Hirono, Yoshinori Ukai[1], Midori Shinohara[1], Hisayoshi Torii[2], Emi Shimizu[2], Miki Takenaka[2], Yoshitaka Iba[3], Yoshikazu Kurosawa[3] and Yuji Kohara ([1]Inst. for Antibodies, [2]MBL, [3]Fujita Health Univ.)

The accumulation of mRNA expression pattern data of *C.elegans* that have been produced in this lab provided unique sets of genes that are expressed in specific developmental stages, cell-lineages, tissues and so on. We are particularly interested in early

embryogenesis. Thus, we focus on a set of genes whose mRNA are maternally supplied and disappear by early stage of gastrulation or localize to specific cells in early embryos, since developmentally important genes are enriched in the subset. We found 1107 (about 10.8%) such genes when the expression analysis of 10290 genes (more than the half of all the genes) was finished. We are analyzing the subset of genes systematically with respect to the distribution of the protein products since translation and localization are important for the understanding of maternal gene functions. We started to raise rat or mouse antibodies against the 880 maternal gene products using bacterially expressed partial proteins in a systematic manner. Until now, we obtained antisera against 282 gene products, and immuno-stained embryos and gonads using the antisera. As to a part of the antibodies, phage display forms were constructed, which easily lead to monoclonal forms. The spatial immunostaining patterns were as follows; nucleus (33%), cytoplasm (27%), plasma membrane (17%), nuclear membrane (11%) and P-granules (18%). The temporal staining patterns were as follows; the staining signals were first observed from the mitotic region in gonad (27%), oogenesis (11%), and from 2-cell stage (16%). We confirmed the specificity of some of the antibodies by testing if the staining signals disappeared in RNAi-treated specimen.

We are trying to extract consensus regulatory sequence for translation and cellular localization. We will integrate the results of the immunostaining with the results of functional analysis by RNAi, ultimately aiming at understanding of the gene network of early embryogenesis in *C.elegans*.

(3) Protein phosphatase 2A (PP2A), encoded by *let-92* and *paa-1*, is required for early cleavage of *C. elegans*

Ken-ichi Ogura, David L. Baillie[1], Sachiko Takahashi and Yuji Kohara ([1]Simon Fraser Univ.)

The serine/threonine protein phosphatase 2A (PP2A) is implicated in many cellular functions, including cell division and cell fate determination. Here we revealed that the catalytic subunit of PP2A (PP2A-C) of *C. elegans* is encoded by the gene *let-92*. We found that *let-92* embryos showed incorrect positioning of chromosomes, uncoordinated cytokinesis, and inhibition of chromosome duplication. Furthermore, *let-92* caused the disappearance of P granules in early embryos, indicating a role for PP2A in the proper organization of P granules. We found that the phenotype of *RNAi* of a PR65/regulatory A subunit of PP2A, named *paa-1*, was identical to that of *let-92* embryos, supporting the idea that PP2A-C and PP2A-A form the core complex of PP2A. LET-92/PP2A-C was abundant in the cytoplasm of the gonad and all the blastomeres. (See Ref.3)

(4) Systematic analysis of cell-specific enhancers in *C. elegans*

Hiroshi Kagoshima, Akiko Kamamoto and Yuji Kohara

There are so many promoter::GFP reporters showing cell-specific expression in particular set of cells in *C. elegans*. It would be reasonable to expect that some of the genes expressed in the same cell might be controlled by the same mechanism. To examine the mechanism of the cell-specific transcriptional regulation, we first collect many GFP reporter constructs expressed in a certain cell. We next construct a series of deletion promoter:: GFP reporters, and checked the expression pattern in the transgenic worms. Lastly we can obtain numbers of minimized enhancer for the cell-specific expression. Not all, but some of them sould share the common sequence in the size around 5~10 bp. Most probably it would be the binding site for upstream transcription factor.

We are currently analyzing AFD thermosensory neuron-specific promoters. Thus far, we could have narrowed down 7 promoters for the expression in the AFD neuron; for example, 50 bp sequence of *gcy-8* (guanylyl cyclase), 120 bp sequence of *nhr-38* (nuclear hormone receptor), etc. Interestingly, in these sequences we have found numbers of putative binding sites for the transcription factor OTX1, which has important roles for head development in fly and vertebrate. Eventually, it has been revealed that *ttx-1* (OTX1 homolog of *C. elegans*) regulates both *gcy-8* and *nhr-38* expression in AFD. We confirmed direct binding ability of TTX-1 to the 50 bp sequence in the minimized *gcy-8* promoter/enhancer by gel shift assay. We have recently obtained the data indicating that another transcription factor, LIM homeobox gene ceh-14, might involve in AFD specific expression.

(5) Synergistic effect of T-box transcription factor *tbx-9* and Hox cofactor *Hth/Meis* orthologue *unc-62* on *C. elegans* embryonic morphogenesis

Yoshiki Andachi

T-box transcription factors play important roles in embryonic development of metazoans. Aiming at understanding the regulation of gene expression in *C. elegans* embryogenesis, we have been studying two T-box genes, *tbx-9* and *tbx-8*, that are phylogenetically most related to each other among 20 T-box genes in this organism. *tbx-9* is expressed in a subset of embryonic cells that are precursors of the intestine, body-wall muscle, and hypodermis. The expression pattern of *tbx-8* is markedly similar to that of *tbx-9*. Both *tbx-9* mutants and *tbx-8* mutants show incomplete penetrant morphogenetic defects in embryogenesis, but the malformations of the *tbx-9* and *tbx-8* mutants are observed in different parts of their bodies. In embryos with both *tbx-9* and *tbx-8* inactivated, the body structure is severely disorganized, more so than the sum of the separate mutant phenotypes. Further analysis shows that the hypodermis and body-wall muscle show abnormalities at the site of morphogenetic defects of these mutants. Together, these data indicate that *tbx-9* and *tbx-8* do not only contribute individually to formation of the hypodermis and body-wall muscle, but also suggests functional redundancy between *tbx-9* and *tbx-8* in embryonic morphogenesis.

To find TBX-9 binding proteins, especially those implicated in gene expression along with TBX-9, we employed the yeast two-hybrid system. One of the genes isolated by the screening is *unc-62*, an orthologue of *Drosophila Homothorax (Hth)* and vertebrate *Meis* that encode cofactors of Hox transcription factors. *in vitro* pulldown assay confirmed weak binding between TBX-9 and UNC-62. The transcripts of both *tbx-9* and *unc-62* were detected in several cells of early-stage embryos. *unc-62* mutants also show defects in embryonic morphogenesis that result from abnormality in the hypodermis. *unc-62(e644)* is a weak allele: not more than half worms of this mutant show the phenotype of malformation. In *tbx-9(ms31); unc-62(e644)* the phenotype was observed in most of worms, and enhancement of the phenotype was also found in *tbx-8(RNAi); unc-62(e644)*. To know that the function of *unc-62* for embryonic morphogenesis is required in the cells that also express *tbx-9*, *unc-62* cDNA was induced by the *tbx-9* promoter. Partial suppression of the phenotype of malformation was observed in *tbx-9(ms31); unc-62(e644); Ex[tbx-9promoter::unc-62]*. These results suggest that the T-box factor and the Hox cofactor work collaboratively. It is speculated that these factors function as an enhanceosome complex in the same enhancer of their target genes or that the Hox cofactor functions as a cofactor for the T-box factor to regulate their target genes. (See Ref.12)

(6) Physical Modeling of Cellular Arrangement in Early Embryos of *C. elegans*

Atsushi Kajita, Masayuki Yamamura[1] and Yuji Kohara ([1]Tokyo Institute of Technology)

Cellular arrangements are important for development. In *C. elegans*, cell-cell interactions are essential for cell-fate determination in early embryos. The arrangement of cells is largely restricted by physical conditions including the force and the direction of cell division, the existence of a hard egg shell, and so on. We created a physical model for cellular arrangement as a first step towards a platform of virtual experiments. Currently this model is based solely on dynamics of cells and, therefore, includes neither chemical reaction networks nor gene-regulated networks within the cells, in order to make the model as simple as possible. The physical model of an embryo consisted of a constant size eggshell and viscoelastic cells. The cell was represented by a mesh surface of mass particles connected by springs and dampers. Cell division was implemented with the processes of elongation of a cell and contraction of a contractile ring.

Using this model we performed computer simulations of wild-type embryos up to the 4-cell stage. We found a range of parameters at which the simulations of embryos successfully generated the same cellular arrangement, called diamond-shape, as real embryos. We also found that the simulations satisfied the cellular arrangements during the process of early cleavage, by comparing of the characteristic values of the cellular arrangements with those of real embryos that were extracted from the 4D microscopic images.

Next, we performed simulations up to the 8-cell stage. Using the same parameters as above, the simulations also generated the same cellular arrange-

ments as real embryos at the 8-cell stage. However, a statistical bias was not observed in the simulations, while it was observed in real embryos at the 6-cell stage. The statistical bias is that the left-side granddaughter cells (ABxl) of AB cell are arranged at more anterior than the right-side ones (ABxr). This means that the model lacks something to show the statistical flexibility at the stage, although it reconstructed successfully the restriction of cell movements by the eggshell. We are currently improving our model by implementing other phenomena in early cleavages. (See Ref.2)

(7) SPI: A tool for incorporating gene expression data into a four-dimensional database of *C. elegans* embryogenesis

Yohei Minakuchi, Masahiro Ito and Yuji Kohara

A comprehensive gene expression database is essential for computer modeling and simulation of biological phenomena, including development. Development is a four-dimensional (4D; 3D structure and time course) phenomenon. We are constructing a 4D database of gene expression for the early embryogenesis of the nematode *C. elegans*. As a framework of the 4D database, we have constructed computer graphics (CG), into which will incorporate the expression data of a number of genes at the subcellular level. However, the assignment of 3D distribution of gene products (protein, mRNA) of embryos at various developmental stages is both difficult and tedious. We need to automate this process. For this purpose, we developed a new system, named SPI, for superimposing fluorescent confocal microscopic data onto a CG framework.

The scheme of this system comprises the following: (1) acquirement of serial sections (40 slices) of fluorescent confocal images of three colors (DAPI for nuclei, Cy-3 for the internal marker, which is a germline-specific protein POS-1 (Tabara *et al.*, 1999) and Cy-5 for the gene product to be examined); (2) identification of several features of the stained embryos, such as contour, developmental stage and position of the internal marker; (3) selection of CG images of the corresponding stage for template matching; (4) superimposition of serial sections onto the CG; (5) assignment of the position of superimposed gene products. The Snakes algorithm identified the embryo contour. The detection accuracy of embryo contours was 92.1% when applied to 2- to 28-cell-stage embryos. The accuracy of the developmental stage prediction method was 81.2% for 2-8-cell stage embryos. We manually judged only the later stage embryos because the accuracy for embryos at the later stages was unsatisfactory due to experimental noise effects. Finally, our system chose the optimal CG and performed the superposition and assignment of gene product distribution. We established an initial 4D gene expression database with 56 maternal gene products. (See Ref.11)

(8) Semi-automatic system for creation of cell shape model in *C. elegans* embryogenesis

Hideaki Hiraki and Yuji Kohara

Cells change their shapes and arrangements during embryogenesis. 4Dmicroscopy is useful to observe the process of *C. elegans*. Time-lapse recording of multiple optical sections with Nomarski DIC microscope and tools to analyse the results have been developed and used successfully, especially for cell lineage analysis because cell nuclei of C. elegans are evident in DIC images. Although cell shape models of very early embryo can be reconstructed from DIC images, delineating cell boundaries become harder as the cell number increases. Since cell boundaries can be directly visualized by confocal fluorescent microscopy with vital stain of plasma membranes, we have been developing a computer system to create cell shape models from a time series of confocal images of plasma membranes.

In the system, cell boundaries are automatically calculated by a seeded region growing algorithm from a 3D image and a set of seed point coordinates. The algorithm segment the image volume to enclosed regions one per seed. Here a cell boundary is detected as ridges of high fluorescent signal between two seed points. In many cases, the seed points can be automatically placed using results of another time point. But manual editing of the seed sets is necessary where signal to noise ratio is low. The system constructs a pseudo cell lineage from the overlay relationships among the segmented regions over the time course. The inconsistency in the pseudo lineage indicates where editing is required and the editing is assisted by the graphical user interface.

We have tested the system on the "dub" data set kindly provided by Bill Mohler, UW-Madison, that is of N2 embryo in 24-200 cell stages. Changes in physical parameters of cell shapes and arrangements, such as cell volumes and cell-to-cell contacting areas, could be estimated from the resulting shape model. We are planning to apply this system to compare mutant embryos and the embryos from other species closely related to *C. elegans*.

(9) Towards comparative genomics: Genome/EST sequencing

Kazuko Oishi, Shinobu Haga, Hisayo Nomoto, Masako Sano, Fumiko Ohta, Sachiko Miura, Tomomi Morishita, Tomoko Endo, Motoyo Tamiya, Keiko Nogata, Akiko Hase, Tomoharu Tandoh, Yoshiki Mochizuki, Hiromitsu Miyauchi, Noriko Hasegawa, Masumi Mizukoshi, Etsuko Yokoyama, Nanayo Ishihara, Junko Miyamoto, Shigeko Iiyama, Tadasu Shin-I, Toshinobu Ebata, Shigeru Saito, Masaki Itoh, Masayo Nakagawa, Kumiko Kawaguchi, Naoko Sakamoto, Yasuko Sugiyama and Yuji Kohara

As a core facility of the group grant "Genome" (supported by MEXT and consisted of about 300 labs), we have established a DNA sequencing center. Currently our capacity is 12 million reads per year. Thus far, we have performed the followings in collaboration with the members of the group grant (in the parenthesis) ;

(a) EST: the nematode *Caenorhabditis elegans* and *Diploscapter sp.* (Yuji Kohara, NIG), Ascidian *Ciona intestinalis* (Nori Satoh, Kyoto U.), Ascidian *Ciona savignyo* (Nori Satoh, Kyoto U.), Ascidian *Halocynthia roretzi* (Kazuhiro Makabe, Kyoto U.), Hagfish *Eptatretus burgeri* (Masanori Kasahara, the Graduate U. for Advanced Studies), Medaka fish *Oryzias latipes* (Hiroyuki Takeda, U. Tokyo and Akira Kudo, Tokyo Inst. Tech.), ciclid fish *Haplochromis chilotes* (Norihiro Okada, Tokyo Insti. Tech.), loach *Misgurnus anguillicaudatus* (Yasufumi Emori, U. Tokyo), frog *Xenopus laevies* (Makoto Motii, NIBB), slime mold *Dictyostelium discodeum* (Yoshimasa Tanaka, Tsukuba U.), red algae *Cyanidioschyzon merolae* (Tsuneyoshi Kuroiwa, U. Tokyo), moss *Physcomitrella patens subsp. patens* (Mitsuyasu Hasebe, NIBB), barley (Kazuhiro Satoh, Okayama U.), wheat *Triticim aestivum cv. Chinese Spring* (Yasunari Ogiwara, Yokohama City U.), Japanese morning glory (Eiji Nitasaka, Kyushu U.). High quality sequences were obtained with 70-90% of the reads (depending on the samples) and deposited in DDBJ. The analyzed cDNA clones are also distributed from this laboratory. The EST sequencing was also supported by the National BioResource Project.

(b) Genome (BAC, fosmid clones): nematode *Pristionchus pacifficus* (Yuji Kohara, NIG and Ralf Sommer, MPI, Germany), fruit fly *Drosophila sechelia Drosophila simulans, Drosophila parabipectinata, Drosophila virilis, Drosophila yakuba* (Toshiro Aigaki, Tokyo Metoropolitan U.), Acidian *Ciona intestinalis* (Nori Satoh, Kyoto U.), medaka fish *Oryzias latipes* (Hiroshi Hori, Nagoya U. and Hiroyuki Takeda, U. Tokyo), Chimpanzee (Naruya Saitou, NIG), Gorilla (Naruya Saitou, NIG), liverwort *Marchantia polymorpha* L Y-chromosome (Kanji Ohyama, Kyoto U.).

(c) Genome (whole genome): read algae *Cyanidioschyzon merolae* 16Mb (Tsuneyoshi Kuroiwa, U. Tokyo), Ascidian *Ciona intestinalis* 160Mb (Nori Satoh, Kyoto U.) The whole genome shotgun sequencing of Medaka fish *Oryzias latipes* (800Mb) is completing. (See Ref. 4, 5, 6, 7, 8, 9, 10)

Publication

Papers

1. Ichimiya, H., Hino, O., Kohara, Y. and Ishii, N. (2003). VBP-1 is necessary for morphogenesis in *Caenorhabditis elegans*. Oncology Reports **10**, 293-295.

2. Kajita, A., Yamamura, M. and Kohara, Y. (2003). Computer Simulation of the Cellular Arrangement in Early Cleavage of the Nematode *C. elegans*. Bioinformatics **19**, 704-716.

3. Ogura, K-I., Kishimoto, N., Mitani, S., Gengyo-Ando, K. and Kohara, Y. (2003) Translational control of maternal *glp-1* mRNA by POS-1 and its interacting protein SPN-4 in *Caenorhabditis elegans*. Development **130**, 2495-2503.

4. Ohta, N., Matsuzaki, M., Misumi, O., Miyagishima, S., Nozaki, H., Tanaka, K., Shin-I, T., Kohara, Y. and Kuroiwa, T. (2003). Complete sequence and analysis of the plastid genome of the unicellular red alga *Cyanidioschyzon merolae*. DNA Research **10**, 67-77.

5. Ogihara, Y., Mochida, K., Nemoto, Y., Murai, K., Yamazaki, Y., Shin-I, T. and Kohara, Y. (2003). Correlated clustering and virtual display of gene expression patterns in the wheat life cycle by

large-scale statistical analyses of expressed sequence tags. The Plant Journal **33**, 1001-1011.
6. Nishiyama, T., Fujita, T., Shin-I, T., Seki, M., Nishide, H., Uchiyama, I., Kamiya, A., Carninci, P., Hayashizaki, H., Shinozaki, K., Kohara, Y. and Hasebe, M. (2003). Comparative genomics of the *Physcomitrella* gametophytic transcriptome and *Arabidopsis* genome: Implication for the land plant evolution. Proc. Natl. Acad. Sci., USA **100**, 8007-8012.
7. Muramoto, T., Suzuki, K., Shimizu, H., Kohara, Y., Kohriki, E., Obara, S., Tanaka, Y. and Urushihara, H. (2003). Construction of a gamete-enriched gene pool and RNAi-mediated functional analysis in *Dictyostelium discoideum*. Mechanisms of Development **120**, 965-975.
8. Satou, Y., Imai, K.S., Levine, M., Kohara, Y., Rokhsar, D. and Satoh, N. (2003). A genomewide survey of developmentally relevant genes in *Ciona intestinalis* I. Genes for bHLH transcription factors. Development Genes and Evolution **213**, 213-221.
9. Wada, S., Tokuoka, M., Shoguchi, E., Kobayashi, K., Di Gregorio, A., Spagnuolo, A., Branno, M., Kohara, Y., Rokhsar, D., Levine, M., Saiga, H., Satoh, N., Satou, Y. (2003). A genomewide survey of developmentally relevant genes in *Ciona intestinalis* II. Genes for homeobox transcription factors. Development Genes and Evolution **213**, 222-234.
10. Yagi, K., Satou, Y., Mazet, F., Shimeld, S.M., Degnan, B., Rokhsar, D., Levine, M., Kohara, Y., Satoh, N. (2003). A genomewide survey of developmentally relevant genes in *Ciona intestinalis* III. Genes for Fox, ETS, nuclear receptors and NFkB. Development Genes and Evolution **213**, 235-244.
11. Minakuchi, Y., Ito, M. and Kohara, Y. (2004). SPI: A tool for incorporating gene expression data into a four-dimensional database of *C. elegans* embryogenesis. Bioinformatics **20**, 1097-1109.
12. Andachi, Y. (2004). *Caenorhabditis elegans* T-box genes *tbx-9* and *tbx-8* are required for formation of hypodermis and body-wall muscle in embryogenesis. Genes to Cells, **9**, 331-344.

Database

13. NEXTDB: URL. http://nematode.lab.nig.ac.jp/

G-c. Publicity and Intellectual Property Unit Munehiro Tomikawa Group

RESEARCH ACTIVITIES

2003 Annual Publicity and Intellectual Property Unit Report

This unit consisting of Professor Munehiro Tomikawa, Ms. Nozomi Yokoyama and Ms. Kiyomi Kuwabara makes various efforts to return the outcome of research generated by NIG to the industrial world and the public while respecting the scientists' priorities in terms of scientific ideas, partnership and publications.

To be more precise, what we have done is to establish scientific results as intellectual property, explain the property rights to the industrial world intelligibly, seek collaboration with commercial industries for actualizing those results and establish partnerships. This shows the importance of carrying out both public relations and the acquisition of intellectual property.

This unit was set up in March 2003. Since April 2003, a secretary, Ms. Nozomi Yokoyama has devoted all her energy to this work from starting up. From August to November, Ms. Mika Ogawa had assisted, especially by creating the MTA (Material Transfer Agreement) form and getting it off the ground. In December, Ms. Kiyomi Kuwabara took over from her. The results of our work in 2003 are shown as follows.

(1) Acquirement and utilization of Intellectual Property Rights

First of all, we were engaged in promoting a sense of entitlement in the scientists belonging to NIG by giving them speeches on why entitlement is necessary to the outcome of basic research. Also, in visiting laboratories, monitoring the scientific progress and thinking of each scientist, we have explained how intellectual property rights can be gained from their results. We have also established an efficient patenting procedure. We have prepared an invention exposition format to minimize the scientists' burden in informing our unit of their scientific results. Our unit helps the scientists avoid the troublesome task of acquiring of patent rights to the practical applications of their

inventions.

In terms of patent applications, we try to promote nationalization of the patent rights so the NIG scientist does not need to bear the costs necessary for the preparation of patent documents. At this time, eight government patents have been applied for and six patents are under preparation, of which 12 will be applied for as government patents (including joint applications). Also, two international patents have been applied for. One patent has already come into marketing as of November 2003.

Secondly, we have been involved in making guidelines for the Material Transfer Agreement which stipulates ownership, ethical codes and compensation for biological resources based on research results. At present, two kinds of agreements, depending on the positioning of biological resources as intellectual property, have been created and are in use. Each has a domestic version and an international version. Despite not having been authorized yet, a total of 30 agreements (seven are domestic and twenty three are international) have been signed.

In the near future, preparation of a third agreement format will be necessary to correspond to specific materials such as yeast, worms and flies.

In addition, we participated in the Shizuoka-TLO-Yaramaika (STLO) campaign, which facilitates putting patents owned by the NIG into practical use.

(2) Acceleration of public relations activities

Ideally, this kind of work should be developed over a broad spectrum, however, we have focused our attention on the following main points. We have given priority to our response to visitors, promotion at academic conferences, introduction of research results to the public, and the search for potential collaboration.

Firstly, we had 10 groups of visitors in 2003: technical school students, legal apprentices, high school students, schoolteacher interns, members of the judiciary, elderly community college students and others. An overview of this institute, genetic science and current laboratory experiments was given to the visitors.

Secondly, promotion at academic conferences has been carried out in order to obtain more excellent students and scientists. We introduced the current research results obtained from NIG scientists at the Japanese Cell Biology Meeting (in Ootsu), the Japanese Society for Developmental Biology meeting (in Sapporo), and the Japanese Molecular-Biology meeting (in Kobe).

Thirdly, we presented five specifically excellent research results in the Hot News column on the website as an introduction of research results to the public. We have also had three research results inserted in main-stream newspapers through cooperation with the media. Furthermore, we introduced two scientists to companies which were interested in their work, and fortunately, this effort led to further business-academia collaboration.

(3) Social Action Work

The scientists at NIG have played central roles in the struggle to create barrier-free color vision. Their work has been shifted to the Association for Propagation of Genetic Knowledge in an attempt to bring it to full fruition. So far, however, we have not gotten completely satisfactory results, as not all groups participating in the project are in full agreement with the objectives and methodology.

Recently, the participation of public facilities, such as universities and NIG, in regional revitalization is increasing. In December I attended a regional revitalization symposium as a panelist on behalf of NIG, which was held as part of the events commemorating the 80th anniversary of Numazu's incorporation as a city. It was quite meaningful to be able to develop relationships with those involved.

In conclusion, acquiring intellectual property and engaging in returning excellent technology from scientific results to the industrial world through public relations will be important factors in gaining wide public recognition of NIG. These efforts are expected to facilitate the management of NIG, after turning into inter-university research institute organization.

H. STRUCTURAL BIOLOGY CENTER

H-a. Biological Macromolecules Laboratory Makio Tokunaga Group

RESEARCH ACTIVITIES

(1) Single Molecule Imaging and Quantitative Analysis of Nuclear Transport in Cells using Highly Inclined and Laminated Optical sheet microscopy

Makio Tokunaga and Naoko Imamoto[1,2] ([1]Gene Network Laboratoy, [2]Riken)

What is a key to enter the nuclei through nuclear pores? This question has been answered by visualizing single molecules inside cells. Clear video images of single molecules translocating into the nuclei are obtained using novel fluorescence microscopy, Highly Inclined and Laminated Optical sheet (HILO) microscopy. Very little is known about the interactions between transport molecules and the assembled nuclear pore complex (NPC) because of its large supramolecular structure. Obtained single-molecule video images inside cells are very clear, therefore kinetic parameters of the molecular interactions in cells are obtained through quantitative analysis. We have discovered how many molecules interact, how strong the interactions are, and which molecular interaction is the open sesame to nuclear import. We have opened up a new way to quantify molecular dynamics and interactions at the single-cell level, which is 'open sesame' toward quantitative molecular and system biology.

(2) Visualization and quantitative analysis of dynamics of single lipid rafts in vivo

Kumiko Sakata-Sogawa[1], Sho Yamasaki[2], Takashi Saito[1,2] and Makio Tokunaga ([1]Research Center for Allergy and Immunology, RIKEN, [2]Dept. of Molecular Genetics, Chiba University Graduate School of Medicine)

Lipid rafts are submicroscopic sphingolipid- and cholesterol-rich domains in which many membrane proteins are assembled. This assembly is supposed to enhance the interaction between membrane proteins and increase of the efficiency of signal transduction cascades. Although accumulating evidence from microscopic studies shows the existence of lipid rafts, the important features of their dynamics still remain to be elucidated.

Aiming at visualization of the dynamics of lipid rafts in vivo, we constructed LAT/GFP, a chimeric protein of GFP and LAT, which is known as a raft-localized protein, and established transgenic mice expressing LAT/GFP ubiquitously. This chimeric protein retains the consensus raft localization signal sequence but not the functional tyrosine residues. Indeed, LAT/GFP localized mainly in biochemically-identified lipid rafts in various types of cells from the transgenic mice. We prepared bone marrow derived mast cells from the mice and observed them at 37°C C using a single-molecule TIRF (Totally internal reflection fluorescence) microscope. The lateral and transverse motions of the fluorescent clusters were imaged, reflecting the movements of single rafts. Single molecule images of LAT/GFP were visualized and their movements showed the same behavior as single rafts. The results of the quantitative analysis of the dynamics of lipid rafts confirmed the existence of lipid rafts and revealed their quick movement both lateral and transverse.

(3) RNG105: A Novel Regulatory Protein in Neuronal RNA Granules Responsible for Stimulation-Dependent Local Translation

Nobuyuki Shiina, Kazumi Shinkura and Makio Tokunaga

mRNA translocation and subsequent local translation in neuronal dendrites are important bases for long-term synaptic plasticity, but responsible molecules have not been fully identified. We previously identified RNG105 (RNA granule protein 105) as a component of RNA granules, which play central roles in the transport of mRNAs to the dendrites. The RNG105-localizing RNA granules contain mRNAs, such as CaM kinase II alpha, CREB and BDNF mRNAs, whose translational products play key roles

in synaptic plasticity.

In this year, we have found that RNG105 contains an RNA-binding motif, and shown that RNG105 is a regulatory protein for local translation in dendrites of hippocampal neurons. RNG105 has an ability to repress translation in vivo, consistent with the finding that the RNA granule is translationally arrested in the basal conditions. Dissociation of RNG105 from the RNA granules is induced by BDNF (brain-derived neurotrophic factor), a growth factor responsible for synaptic plasticity. The dissociation of RNG105 is significantly correlated with the induction of local translation of the mRNAs near the granules. These findings indicate that RNG105 is responsible for the local translational regulation in neuronal dendrites, and suggest its implication in the regulation of local synaptic plasticity in a stimulation-dependent manner.

(4) Single Hydrogen Bonds of DNA Base Pairs Detected in Unzipping Force by Intermolecular Force Microscopy

Michio Hiroshima and Makio Tokunaga

Single hydrogen bonds of DNA base pairs have been measured by unzipping double-stranded DNA oligomers with an intermolecular force microscope (IMF). To detect such ultrafine forces, high resolution of force as well as high accuracy in controlling the probe position is required. IFM has achieved the force resolution of subpico-newton using ultrasensitive cantilevers. The probe position is controlled with nanometer accuracy using a feedback system, which uses laser radiation pressure to reduce thermal fluctuation of the cantilever.

Force vs. extension curves showed repeated force peaks of 10-15pN. Auto- or cross-correlation analysis and averaging of force curves were carried out to reduce noises in the force curve. The previous studies showed that the force for separating poly(G-C) DNA was 1.5 to 2 times stronger than that for poly(A-T). However, no difference was found in the force between individual G-C and A-T base pairs. The force curve of individual G-C and A-T pairs showed three and two peaks, respectively, which are assigned to single hydrogen bonds. The force is variable but the work is constant. The work to break single hydrogen bonds is 1.3 k_BT, in other words, about 1.3-fold of the thermal energy. This is the first report to detect the force of single hydrogen bonds in biological macromolecules.

(5) A Novel in vitro Assay System of Nucleocytoplasmic Transport

Atsuhito Okonogi, Michio Hiroshima, Nobuyuki Shiina, Naoko Imamoto[1,2] and Makio Tokunaga ([1]Gene Network Laboratoy, [2]Riken)

We have developed a novel *in vitro* assay system of nucleocytoplasmic transport. We aim at application of the method to new single-molecule experiments, imaging and nano- or force-measurement.

Nuclear envelope was formed on a planar surface of a small agarose plate. At first, agarose plates were modified with glutation. Then glutation-agarose plates were coated with GST-RanGDP fusion protein. Nuclear envelope was formed onto the RanGDP-coated surface using extracts from *Xenopus laevis* frog eggs. Formation of Nuclear Pore Complexes was confirmed by observing import of fluorescently labeled proteins. This new cell-free system has marked advantages: 1) Solutions in both sides, pseudo-cytoplasmic and pseudo nucleoplasmic sides, can be replaced independently. 2) The shape of the nuclear envelope can be changed as one likes, for example, a vertical plane and a horizontal plane. 3) The system does' nt contain no organelle or cellular structures except the nuclear envelope. Using the *in vitro* system together with single molecule techniques or fluorescence resonance energy transfer should provide an innovative and powerful tool to investigate molecular mechanisms of transport.

(6) Molecular Imaging of translation initiation factors in neuronal dendrites

Hiraku Miyagi, Nobuyuki Shiina and Makio Tokunaga

Local protein synthesis in neuronal dendrites is gradually revealed to be required for synaptic plasticity. In order to investigate when and where the translation is initiated locally in the dendrites, we visualized eukaryotic translation initiation factors (eIF) 4E and eIF4G in dendrites of rat hippocampal neurons.

Binding of eIF4E and eIF4G is known to trigger translation initiation. Immunostaning of rat hippocampal primary neurons showed that most of eIF4E and eIF4G were localized in distinct granular structures, whereas some of them were colocalized in some granular structures in the dendrites. Immunostaning of rat hippocampal slices showed essentially the same

results as in the case of the primary cultures. Stimulation by brain-derived neurotrophic factor (BDNF) increased colocalization of eIF4E and eIF4G in rat hippocampal slices and hippocampal primary neurons. Immunostaning of rat hippocampal slices for eIF4G and PSD-95, a marker protein for postsynapses, showed that colocalization of eIF4G and PSD-95 was increased by BDNF stimulation. These results indicated that translation in the neuronal dendrites is inhibited by separating spatially eIF4E and eIF4G, and translation is induced by BDNF stimulation at postsynaptic areas.

eIF4E and eIF4G were fused to GFP, CFP and YFP proteins to be used for real time imaging in living cells. These fusion proteins were bound to endogenous eIF4G and eIF4E respectively in CHO cells, indicating that these fusion proteins are functional in the cells. In cultured hippocampal neurons, eIF4E-CFP and YFP-eIF4G were observed as distinct granular structures under the thin-layer illumination fluorescence microscopy. We are trying to observe changes in dynamics of the fusion proteins induced by BDNF stimulation.

Publications

Papers

1. Hirakawa, Y., Suzutoh, M., Ohnishi, H., Shingaki, T., Erying, E.M., Tokunaga, M. and Masujima, T. (2003). Observation and analysis of single DNA nano-kinetics by pin-fiber video scope. Analyst **128**, 676-680.

EDUCATION

他大学／研究機関での講義やセミナー

1. Dr. M. Tokunaga gave a lecture at the University of Tokyo, Faculty of Engineering, May, 2003 (in Japanese).

客員教授等

1. 東京大学大学院工学系研究科特定研究客員大講座(物理工学)，教授.

SOCIAL CONTRIBUTIONS AND OTHERS

特許

1. 出願番号：2003-166887，発明の名称：光学系の薄層斜光照明法，発明者：徳永万喜洋，出願人：科学技術振興事業団

各種委員

1. 徳永万喜洋，バイオテクノロジー開発技術研究組合，「細胞内ネットワークのダイナミズム解析技術開発」研究開発委員.
2. 徳永万喜洋，科学技術振興調整費研究評価部会，「細胞・生体システム研究評価WG」科学技術・学術審議会専門委員.

集会／シンポジウムの主宰

3. Dr. M. Tokunaga organized a symposium at the 16th Symposium on Bio-Analytical Sciences 2003, Fujiyoshida, August, 2003.

H-b. Molecular Biomechanism Laboratory Nobuo Shimamoto Group

RESEARCH ACTIVITIES

(1) The branched mechanism of transcription initiation in *E. coli*

Motoki Susa[1], Shouji Yagi[1] and Nobuo Shimamoto[1]

In elucidating of the mechanism of a biological process, one tends to assume a simple sequence of essential steps at the cost of ignoring steps with unknown roles. The mechanism of transcription initiation in prokaryotes provides an example. For several decades, the mechanism has been assumed to be a sequence of three essential steps: formation of a complex between RNA polymerase and a promoter (closed complex), formation of another complex with partially melted DNA duplex to form phosphodiester bonds (open complex and chemical reaction), and escape of RNA polymerase from the promoter associated with the progress of RNA elongation (promoter clearance). This ignores the process called "abortive initiation", which is an iterative synthesis and release of oligo-RNA molecules. This process has been observed in vitro with all prokaryotic and eukaryotic RNA polymerases so far isolated, but its role as well

as its occurrence in vivo has been unknown. The products of abortive initiation, abortive transcripts, are typically 2 to 15 nucleotides in length. On the assumption that these short transcripts are "unsuccessful precursors" of the full-length transcript, abortive synthesis has been considered to precede promoter clearance.

We have been clarifying for several years that this sequential mechanism is not the case and the initiation follows branched pathways, one of which contains the moribund complex, being defined as a complex that produces only abortive and no full-length transcripts. Followings are its characteristics. 1. The moribund complex, as well as the productive complex that synthesizes full-length product, are formed from the same homogeneous fraction of enzyme molecules, and dissociation of the molecules from the promoter DNA cancels any difference between them. 2. Structural differences between these complexes have been demonstrated. 3. At some promoters, a moribund complex is converted into a dead-end complex that still retains a short transcript but has no elongation activity. Therefore, the initiation pathway is branched into the conventional productive pathway and the abortive pathway that can lead towards a dead end. 4. The fates of a moribund complex are either inactivation as a dead-end complex, dissociation from the DNA, or direct conversion into a productive complex, and the rates of these reactions vary with the promoter. 5. There are factors that affect the fate of the moribund complex in a manner that depends on the promoter.

To examine the existence and significance of the branched pathway *in vivo*, we selected GreA and GreB for clues. At the λP_RAL promoter these factors enhance conversion of the moribund complex into the productive one, in the presence of high concentrations of initiating nucleoside triphosphate *in vitro*. If the branched mechanism exists *in vivo*, absence of the Gre factors should result in reduction of productive transcription from promoters at which the moribund complex is susceptible to these factors. We constructed a double-disruptant of *E.coli*, Δ *greA* Δ *greB*, and then arbitrarily selected 10 genes from among those whose levels of transcripts in the mutant strain were found to be lower than those in the parental $greA^+greB^+$ strain. Finally, the promoter for three of these genes, *atpC (uncC)*, *cspA*, and *rpsA*, passed a further conventional test which confirmed that they displayed a branched initiation pathway in a reconstituted transcription system composed of purified components. The results obtained prove that the branched initiation pathway exists *in vivo* and is utilized in regulation of transcription initiation from some promoters, through modulation of the fraction of polymerase-promoter complexes entering each branch of the pathway.

In this year, we examined various physiological conditions that affect on thew levels of the Gre factors. The determination of the level of GreB was difficult because of the excess amount of GreA that has a similar molecular weight and cross-react with anti-GreB. The level of GreA remained the same through the growth phase. It did not respond much to the richness of the culture media. However, it decreased into half in aerobic conditions, indicating that some genes are regulated by the branched pathway mechanism. We have tested the possibility of formation of dead-end complex in vivo. In the cell homogenate of the Δ *greA* Δ *greB* strain, but not its parental strain with the *gre* genes, we have found a significant fraction of RNA polymerase remain bounded to chromosomal DNA after washing with high salt buffer. Since this salt resistence is the feature of dead-end complex, this evidence is consistent with the existence of the branched pathway mechanism *in vivo*. Because of this fraction, the yield of RNA polymerase from of the Δ *greA* Δ *greB* strain is small. We have solved this problem by combining differential precipitation of DNA-RNA polymerase complex with sonication, establishing the purification procedure of RNA polymerase free from the Gre factors.

(2) Structural dynamics of σ^{70} and its extra roles in vivo

Shoji Yagi, Usha Padmanabhan[1], Hiroki Nagai[1], Taciana Kasciukovic[2], Richard S. Hayward[2], Yumiko Sato[1] and Nobuo Shimamoto[1] ([1]Nat. Inst. of Genet./ Grad. School of Adv. Stud., [2]Inst. Of Cell and Molec. Biol., Edinburgh Univ., Scotland)

Several years ago, we found that σ^{70}, the major σ factors of *E. coli*, forms aggregate in vivo and in vitro at a high temperature within physiological condition. The oligomeric σ^{70} was confirmed to have little transcriptional activity and the oligomerization in vitro showed a sharp temperature dependence. We have constructed a strain with a disrupted *rpoD* (σ^{70}), and plasmid born σ^{70} supports its growth. We observed a positive correlation between the intracellular concen-

tration of σ^{70} and upper limit of growing temperature, suggesting a possibility that σ^{70} is a molecular thermometer controlling the upper limit of the growth temperature.

The major σ factors of proteobacteria mostly have a big spacer region between the conserved regions 1 and 2 which is not conserved in eubacteria. In *E. coli* σ^{70} this region includes extensive acidic patches but its role was not known. The *rpoD*-disrupted strain was used for testing the roles of this region. The mutant σ^{70} lacking the spacer region of aa130-374 (ΔSR) complemented the disruption at 25~30 ℃, but not at 35℃ or above, proving that the region is not essential for growth at low temperature. At all tested temperature this protein predominantly exists as aggregate which is in equilibrium with a small fraction of monomer. Therefore, the role of the spacer region is the maintenance of active monomeric form.

The subunit σ^{70} of RNA polymerase has been evidenced in vitro to be dissociated during transcription initiation. However, this conclusion was challenged by two recent studies: one using FRET assay, claiming that σ^{70} is never dissociated, and the other biochemical study shows that σ^{70} gets dissociated at log phase but only partially at stationary phase. To examine what actually occurs in cells, we are developing an in vivo assay using fluorescent anisotropy that is more reliable than FRET. We have constructed a strain lacking the gene of σ^{70} on chromosome but the essential factor is expressed from the gene on a plasmid as a form fused with GFP. To eliminate the possibility of generating semi-intact σ^{70} by cleavage in cells, GFP is buried among the domains of σ^{70}.

(3) Applicability of thermodynamics to equilibria in biology

Nobuo Shimamoto and Jyun-ichi Tomizawa

Most DNA-binding proteins are biologically functional as a specific complex, one containing a special short DNA segment. Such a complex is usually assumed as a state tenable for thermodynamic analysis of binding equilibrium. Thus, forward and backward reactions should balance at equilibrium in every pathway, and the affinity should be independent of the length of DNA. However, we have found that the balance at equilibrium is broken for some proteins by their sliding along DNA during association but not dissociation and that their affinities for their specific sites dependent on the length of DNA harboring the sites. This seeming disagreement is explained by an indeliberate use of the state of specific complex in thermodynamics. In the presence of sliding, the state does not satisfy the second law (the ergodic condition) and thus is disqualified for thermodynamic analysis. A general treatment of binding equilibrium, while maintaining the specific complex as a distinct state, is proposed on the base of the master equation or chemical kinetics.

Publications

Papers

1. Shimamoto, N. (2003). Movements of RNA polymerase along DNA. In Methods Enzymology, Vol.371, RNA polymerases and associated facrors, Part C, pp. 50-70. Elsevier Science, San Diego.

Reviews

2. 嶋本伸雄. (2003). リボ核酸, RNAポリメラーゼ, 転写開始, エロンゲーション(転写の), メッセンジャーRNA. In分子生物学・免疫学キーワード辞典第2版(永田和宏, 長野敬, 宮坂信之, 宮坂昌之編集). 医学書院.
3. 嶋本伸雄編. (2003). ナノバイオマシン創製のための技術及び市場性に関する調査研究(財団法人バイオインダストリー協会調査委員会(嶋本伸雄委員長), 財団法人バイオインダストリー協会.

EDUCATION

他大学／研究機関での講義やセミナー

1. Shimamoto, N., Physiological Significance of protein sliding along DNA: A new general regulatory mechanism suggested by two bacterial repressors, June 27, Department of Biochemistry, University of Wisconsin-Madison, Madison, Wisconsin, USA.
2. 嶋本伸雄, ナノバイオテクノロジーとOJTスキルスタンダード, ナノバイオフォーラム, 7月16日, 早稲田大学.
3. Shimamoto, N., Physiological Significance of protein sliding along DNA: A new general regulatory mechanism suggested by two bacterial repressors, September 10, National Yang-Ming University, Tiwan.
4. Shimamoto, N., Nanobiomachines as the target of nanobiology and nano-biotechnology of Japan: an example of transcription complex, September 12, National Health Research Institutes, Taiwan.

5. 嶋本伸雄，ナノバイオロジー概論，10月24日，早稲田大学.

6. Shimamoto, N. A revision of the mechanism of transcription initiation: The branched pathway mechanism and a molecular memory in transcription complex of *E. coli.* December 9, Ecole Normale Supérieur de Cachan, Paris, France.

7. Shimamoto, N. Nanobiology of DNA-protein interaction: Protein sliding along DNA and its significance in biology and physics, December 12, Padova University, Padova, Italy.

市民講座，大学以外の学校での講演・活動

8. 嶋本伸雄，バイオテクノロジーの夢と現実，第6回富士山麓医療関連器機製造業者等交流会，1月23日，富士市.

9. 嶋本伸雄，気球フェスティバル30周年招待記念飛行(日本気球連盟)，8月8－10日，上士幌バルーンフェスティバル.

10. 嶋本伸雄，仁張育夫，イカロス記念招待飛行(日本気球連盟)，10月30日－11月3日佐賀バルーンフェスティバル2003，佐賀.

SOCIAL CONTRIBUTIONS AND OTHERS

特許

1. 出願番号：2003-417494，発明の名称：抗体固定法および抗体固定基板，発明者：嶋本伸雄・須佐太樹・福島和久，出願人：国立遺伝学研究所長，横河電機株式会社

2. 出願番号：2003-347072，発明の名称：生体高分子検出方法およびバイオチップにおける抗原抗体反応を利用したアドレッシング法，発明者：嶋本伸雄・須佐太樹・福島和久，出願人：国立遺伝学研究所長，横河電機株式会社

委員

1. 嶋本伸雄，財団法人バイオインダストリー協会ナノバイオマシン創製のための技術及び市場性に関する調査委員会　委員長.

2. 嶋本伸雄，日本分子生物学会　庶務幹事HP係.

3. 嶋本伸雄，財団法人未踏科学技術協会「生命を測る」組織幹事.

4. バイオテクノロジー開発技術研究組合「細胞内ネットワークのダイナシズム解析技術開発」推進委員.

5. NEDOバイオ人材育成システム開発事業バイオテクノロジーとナノテクノロジーの融合．プログラム開発委員.

H-c. Multicellular Organization Laboratory
Isao Katsura Group

RESEARCH ACTIVITIES

(1) Analysis of synthetic dauer-constitutive mutants in the nematode *Caenorhabditis elegans*

Tomoko Yabe, Kiyotaka Ohkura, Takeshi Ishihara and Isao Katsura

If newly hatched larvae of *C. elegans* are in a crowded state and given a limited food supply, they sense the environmental signal of pheromone and food with a head sensory organ called amphid, and deviate from the normal life cycle, ending in non-feeding larvae called dauer larvae. Since the assay of dauer formation is much less time-consuming than behavioral assays such as chemotaxis assays, we are analyzing the pathway of sensory signals in the amphid neural circuit by detecting dauer formation as the output. We found that mutations in more than 50 known genes show synthetic dauer-constitutive (Sdf-c) phenotypes, i.e., they induce dauer larva formation in certain mutant backgrounds, regardless of the environmental conditions. The synthetic nature of the phenotype, we think, is based on the pathway of sensory signals. Namely, the signals are transmitted through parallel routes, and therefore two mutations are required to block them. We are determining the combinations of mutations for the Sdf-c phenotype and the pattern of suppression of the Sdf-c phenotype by various suppressor mutations. In this way we hope to elucidate the pathway of sensory signals for dauer regulation.

Furthermore, to identify new genes required for the sensory signal transduction, we have isolated and mapped 44 mutations that show the Sdf-c phenotype in the *unc-31(e169)* background, where *unc-31* gene encodes CAPS protein, which acts in secretion from dense core vesicles. Eight of the mutations map in 4 known genes, but most of the remaining 36 mutations, which map at least in 13 genes, seem to be located in novel genes, which we named *sdf* genes. Some of these genes, *sdf-13*, *sdf-9*, and *sdf-14*, have been cloned and characterized at the molecular level, as shown below.

sdf-13 gene encodes a homologue of the mammalian Tbx2 and Tbx3 transcription factors. The two *sdf-13* mutants that we isolated are missense hypomorphs,

and show abnormality in adaptation to odorants sensed by AWC sensory neurons. This gene is expressed in AWB, AWC, ASJ and many pharyngeal neurons, but expression in AWC neurons is sufficient for normal adaptation. The AWC neurons in the mutants retain their characteristic morphology and many marker gene expressions, suggesting that the mutants are abnormal in neural functions rather than neuronal differentiation. The results imply that the mammalian Tbx2 and Tbx3 genes, which play important roles in embryonic development, may also control functions like neural plasticity in differentiated neurons.

sdf-9 gene encodes a tyrosine phosphatase-like molecule. When cholesterol is removed from the culture medium, the mutants form dauer-like larvae that resemble those of *daf-9* and *daf-12* dauer-constitutive mutants. Epistasis analyses revealed that *sdf-9* gene acts in the *daf-9/daf-12* steroid hormone pathway. SDF-9 is expressed in two head cells in which DAF-9 is expressed. By genetic mosaic experiments, we identified these cells as XXXL/R cells, which were known as embryonic hypodermal cells and whose function at later stages was unknown. Killing of XXXL/R cells in the wild type first-stage larva induces formation of the dauer-like larva. Thus, XXXL/R cells seem to play a key role in the metabolism or function of a steroid hormone that acts in dauer regulation.

sdf-14 gene is identical to *mrp-1* gene, which was formerly identified by its homology to multidrug resistance protein genes in other organisms. Thus, we discovered a function of *mrp-1* concerning dauer larva formation. Three different SDF-14 proteins are produced from *sdf-14* gene by alternative splicing, and two of the cDNAs, but not the third one, rescue the Sdf phenotype of the mutants when expressed under the control of the *sdf-14* promoter. An *sdf-14*::GFP fusion gene that can rescue the *sdf-14* mutant phenotype is expressed in neurons, pharyngeal muscles and intestinal cells. Expression of *sdf-14* gene in each of the three tissues using extrinsic promoters results in partial suppression of the Sdf phenotype, while expression in two of them results in more efficient suppression. It seems that expression in each of the three tissues contributes to the wild type phenotype.

In the year 2003, we found that SDF-14 protein can be replaced by human MRP1 protein in *C. elegans* without loosing the function. The synthetic dauer-constitutive phenotype of *sdf-14* mutants was rescued by the wild-type human MRP1 cDNA (but not the L0dm missense mutant cDNA of MRP1) driven by the *sdf-14* promoter. This rescue could be reversed by the addition of drugs that inhibit the transport activity of human MRP1. We are also investigating in which pathway *sdf-14* gene works. The *daf-2(e1370)* mutant produces no dauer larvae at 20°C, but the *daf-2;sdf-14* double mutant produces many dauer larvae at the same temperature. We are testing various dauer-defective mutations for the ability to suppress the latter phenotype. Furthermore, we found that *sdf-14* mutants are not hypersensitive to dauer pheromone. We are continuing such experiments to locate *sdf-14* gene in dauer regulatory pathways.

(2) Molecular genetic studies on sensory integration and behavioral plasticity in *C. elegans*

Takeshi Ishihara, Yuichi Iino[1], Akiko Mohri[2], Ikue Mori[2], Keiko Gengyo-Ando[3], Shohei Mitani[3] and Isao Katsura ([1]Molecular Genetics Laboratory, University of Tokyo, [2]Division of Biological Science, Nagoya University, [3]Department of Physiology, Tokyo Women's Medical University School of Medicine)

Animals receive environmental cues, select and integrate necessary information, and make proper responses, while all these steps can be modified by experience or memory. In *C.elegans*, many behavioral mutants defective in chemotaxis and thermotaxis, for instance, have been isolated and analyzed, and the molecular mechanisms of sensation have been elucidated. On this basis and as a next step, we are analyzing mutants that show abnormality in the learning and selection (evaluation) of sensory signals, to elucidate novel mechanisms of higher order sensory signal processing.

C. elegans shows avoidance of copper ion and chemotaxis to odorants by receiving these stimuli with different sensory neurons in the head. We developed a behavioral assay for the interaction of two sensory signals: aversive copper ion and attractive odorant, diacetyl. Wild-type animals change their preference between the responses, depending on the relative concentration of copper ion and odorants. On the basis of the *C. elegans* neural circuitry, the result suggests that the two sensory signals interact with each other in a neural circuit consisting of about 10 pairs of neurons. While well-fed animals are usually used for

this assay, we found that animals starved for 5 hours tend to prefer chemotaxis to odorants. The change is due to the desensitization of copper ion avoidance by starvation, and can be suppressed by serotonin, which mimics the effect of food. This desensitization is advantageous in natural environment, because starved animals can search for food over a wider area.

To elucidate the mechanism of sensory integration in the neuronal circuit, we are isolating and analyzing mutants that show abnormality in this assay. The *hen-1* mutants showed much weaker tendency to cross the Cu^{2+} barrier when migrating toward attractive odorants than the wild type, although that the *hen-1* mutants had defects neither in the chemotaxis toward the attractive odorant nor in the avoidance of Cu^{2+} ion per se.

To elucidate molecular mechanisms for the sensory integration, we cloned the *hen-1* gene and found that it encodes a secretory protein with an LDL receptor ligand binding domain, LDLa. This domain in HEN-1 is most similar to that domain of *Drosophila* signaling molecule Jeb, which regulates migration and differentiation of visceral mesodermal precursor cells. Immunostaining by using antibody against recombinant HEN-1 protein revealed that the gene product is localized in the axon and cell body of each one pair of sensory and inter-neurons. The localization in the axon was abolished in *unc-104* (kinesin KIF1A homologue) mutants, which show defects in the transport of synaptic vesicles. Expression studies with various promoters showed that this gene acts non-cell-autonomously in the mature nervous system.

The *hen-1* mutants also show abnormality in learning by paired presentation of starvation and NaCl (collaboration with Dr. Iino, University of Tokyo) and by paired presentation of starvation and temperature (collaboration with Ms Mohri and Dr. Mori, Nagoya University). Wild type animals show chemotaxis to NaCl under a well-fed condition, although they avoid NaCl after conditioned with starvation and NaCl. The *hen-1* mutants show a weaker behavioral change than the wild type after the conditioning, although they show normal chemotaxis to NaCl under a well-fed condition. Wild type animals prefer the cultivation temperature under a well-fed condition, while they avoid that temperature after conditioned in the absence of food at the same cultivation temperature. Although the *hen-1* mutants show normal thermotaxis under a well-fed condition, they do not avoid the cultivation temperature after conditioned in the absence of food. Since starvation was used to induce plasticity in both learning assays, we analyzed whether *hen-1* animals can sense starvation, but we could not find any abnormality in the behavior after simple starvation. These results indicate that the *hen-1* show defects in the behavioral plasticity after paired presentation of starvation and NaCl or starvation and temperature, although it responds normally to each of these stimuli.

Molecular genetic analyses of HEN-1 suggest that HEN-1 functions as a neuronal modulator for sensory integration and learning. To elucidate the molecular mechanisms of this neuromodulation, we started investigating the protein interacting with the HEN-1 protein. First, we developed a binding assay for identification of receptors for HEN-1. By using a HEN-1-alkaline phosphatase fusion protein as a ligand, which was expressed by HEK293 cells, we found that HEN-1 specifically binds a subpopulation of the primary culture cells in *C. elegans*, suggesting that receptors for HEN-1 exist in these cells.

In 2003, Jeb protein in *Drosophila* was reported to regulate development of visceral mesodermal cells through the tyrosine kinase receptor, DAlk. Since the LDLa domain in Jeb is similar to that of HEN-1, we started to analyze the *scd-2* gene of *C.elegans*, which encodes a receptor tyrosine kinase similar to DAlk. Expression analyses by using the *scd-2* promoter GFP fusion gene suggested that SCD-2 is expressed in several sensory neurons and interneurons. In addition, an *scd-2* mutant shows behavioral defects same as hen-1 mutants in paradigms for sensory integration and learning. These results suggest that SCD-2 is a candidate for the HEN-1 receptor.

(3) *C. elegans* mutants in the "associative" learning with odorants and food

Ichiro Torayama, Takeshi Ishihara and Isao Katsura

The nematode *C. elegans* provides a good system for the study of learning with a combination of two stimuli. However, the mechanism of this learning looks different from that of classical conditioning, because (a) the unconditioned stimulus is usually limited to food or starvation, and because (b) the learning is efficient, if the conditioned stimulus is presented at the same time as but not before the unconditioned stimulus. To elucidate the molecular mechanism of

such "associative" learning in *C. elegans*, we are isolating and characterizing mutants that show abnormality in the learning with butanone and food/stravation. It is known that butanone attracts wild type animals without conditioning. Conditioning with butanone and starvation decreased the efficiency of chemotaxis to butanone, while conditioning with butanone and food increased it. We isolated mutants in these behaviors, some of which decreased the efficiency of chemotaxis after conditioning with food and butanone, while others are attracted efficiently by butanone only after conditioning with food and butanone. Of those mutants, *ut305* and *ut306*, which belong to the former category, have been studied in detail, because they showed strongest abnormality. Interestingly, *ut305*, but not *ut306*, showed abnormality in adaptation to isoamyl alcohol and benzaldehyde, which are sensed by the same type of sensory neurons (AWC) as butanone. Mapping with SNP markers revealed that the *ut305* mutation is located in the right-hand part of the linkage group X, while *ut306* near the center of the linkage group V.

In the year 2003, we found that *ut305*, but not *ut306*, shows abnormality in chemotaxis to butanone at low concentration, although *ut305* shows normal chemotaxis at the concentration used in the learning assay. It is known that serotonin has similar effects as food in various behaviors in *C. elegans*. The *ut305* mutant showed normal responses in the inhibition of locomotion by food (dopamine-dependent), and in further inhibition of locomotion on food by pre-starvation (serotonin-dependent). Also in the learning assay with butanone and food, serotonin had an effect similar to food in both wild type and *ut305* animals. However, a deletion mutant in *tph-1* (tryptophan hydroxylase) gene, in which serotonin is not synthesized, was essentially normal in the learning, showing that the learning of wild type animals with butanone and food occurs via serotonin-dependent and independent pathways.

We cloned *ut305* gene, which encoded a novel protein containing predicted transmembrane domains and showing limited homology to the *Drosophila* Raw protein. A functional GFP fusion gene was expressed in AIA interneurons as well as many pharyngeal neurons. Laser killing of AIA neurons in wild type animals resulted in learning abnormality similar to but somewhat weaker than that of the *ut305* mutant. We are now carrying out rescue experiments using extrinsic promoters to learn the cells in which the expression of the wild type *ut305* gene is sufficient for normal learning.

(4) Behavioral analysis with "uncomfortable" stimuli of the nematode *Caenorhabditis elegans*

Kotaro Kimura and Isao Katsura

C. elegans as well as many other animals escape from uncomfortable stimuli, such as repulsive tastes and odorants, noxious heat, acid and mechanical stimuli, to seek for better conditions to grow and reproduce. However, what if the animals *cannot* escape from those stimuli? Long or repetitive exposure(s) to repulsive stimulus ("fear conditioning" for example) makes vertebrates and invertebrates in some cases to give up escaping from the stimulus, and causes strange behavior and/or unbalanced homeostasis. What about *C. elegans*?

We hypothesized that the mechanism to cause such behavior and/or status with the repulsive stimulus might be evolutionarily conserved, and that genetic analysis of *C. elegans* could reveal the genes involved in the mechanism.

C. elegans' avoidance behavior is reported to be induced by several volatile or soluble compounds, or nociceptive stimuli, which are sensed by 3 different classes of sensory neurons. However, they are not studied in detail enough compared to attractive stimuli, and not at all in terms of long exposure. To understand how long exposure to repulsive stimulus affects *C. elegans*' behavior, we are currently optimizing experimental conditions to analyze avoidance behavior induced by 1-octanol (sensed via ADL sensory neuron), 2-nonanone (AWB sensory neuron), or high osmolarity (ASH polymodal sensory neuron). We will first examine whether the avoidance behavior is altered with long exposure before the assay, and then examine effects on other behaviors.

(4) Fluoride-resistant mutants of the nematode *Caenorhabditis elegans*

Akane Oishi, Takeshi Ishihara and Isao Katsura

Fluoride-resistant *(flr)* mutations of *C. elegans* are recessive and grouped into two categories: class 1 mutations, which map in *flr-1*, *flr-3* and *flr-4*, and class

2 mutations, which map in *flr-2* and *flr-5* (Katsura, I. *et al.*: Genetics **136**, 145-154, 1994). Class 1 *flr* mutations show diverse phenotypes: slow growth, short defecation cycle periods, frequent skip of the expulsion step of defecation, and synthetic abnormality in dauer formation, besides strong resistance to fluoride ion. The *flr-1* gene encodes an ion channel belonging to the DEG/ENaC (*C. elegans* degenerins and mammalian amiloride-sensitive epithelial sodium channels) superfamily, while *flr-4* and *flr-3* code for a novel Ser/Thr protein kinase and a kinase-like molecule, respectively, both having a hydrophobic domain on the carboxyl-terminal side. A functional *flr-1*::GFP fusion gene is expressed only in the intestinal cells from the comma stage of embryos to the adult stage, while *flr-4*::GFP is expressed in the intestinal cells from the 1.5-fold stage, in the isthmus of the pharynx from the 3-fold stage and in a pair of head neurons called AUA from L1 larvae to adults. Moreover, the expression of various *flr-3::lacZ* and *flr-3*::GFP fusion genes is detected only in the intestine. We therefore think that class 1 *flr* genes constitute a regulatory system that acts in the intestine and that controls many food-related functions. Class 2 *flr* mutations, which confer weak resistance to fluoride ion, suppress the slow growth and dauer formation abnormality but not the defecation abnormality and strong fluoride-resistance of class 1 *flr* mutations. Hence, it seems that the regulatory pathway bifurcates after class 1 genes, and that class 2 genes control only one of the two branches. Of the class 2 genes, *flr-2* encodes a secretory protein belonging to the gremlin/DAN/cerberus family. A functional *flr-2*::GFP fusion gene was expressed in some neurons in the head and the tail as well as many pharyngeal neurons. On the basis of these results, we have made a model in which a signal controlled by class 1 genes is transmitted from the intestine and represses the action of class 2 gene products in the head nervous system.

In the year 2003, we obtained the following results.

1) Both *flr-2* and *flr-5* mutants had shorter average longevity than wild type animals by about one week. Since this abnormality in *flr-2* mutants is rescued by the wild type *flr-2* gene, we think the same abnormality in the *flr-5* mutant may be used for the cloning of *flr-5* gene.

2) Although a functional *flr-2*::GFP fusion gene was expressed in neurons, the expression of the wild type *flr-2* gene with *myo-3* or *ges-1* promoters, which drive expression in the body wall muscle and intestine, respectively, was sufficient for the rescue of the most obvious *flr-2* phenotype, namely, suppression of slow growth caused by *flr-1* mutations. In contrast, rescue was not observed with H20, *tph-1*, and *che-2* promoters, which drive expression in all neurons, serotonergic neurons and ciliated sensory neurons, respectively.

3) To identify proteins that interact with FLR-2, we produced FLR-2::alkaline phosphatase fusion protein molecules in HEK293 cells, and added them to primary cell cultures of *C. elegans*. The results showed that the fusion protein molecules bound specifically to the intracellular compartment of a limited number of the culture cells as detected by alkaline phosphatase activity. We then screened for *C. elegans* cDNA clones whose expression in COS cells enables the cells to bind to FLR-2::alkaline phosphatase. By screening 264 pools of cDNA, where one pool consists of about 1000 clones, we obtained a single positive clone encoding a secretory protein, which we are now characterizing.

4) Since only a few alleles of class 2 *flr* mutants had been isolated, we carried out a new screen for the class 2 mutants using the phenotype of suppressing serotonin-hypersensitivity of the *flr-1(ut11)* mutant. Eight independent mutants were obtained by screening about 12,800 genomes. All the mutations also suppressed the slow growth of *flr-1(ut11)* mutant. By complementation tests, we found that four of them seem to map in *flr-2*, whereas the other four probably map in two new genes.

Publications

Papers

1. Ohkura, K., Suzuki, N., Ishihara, T. and Katsura, I. (2003). SDF-9, a protein tyrosine phosphatase-like molecule, regulates the L3/dauer developmental decision through hormonal signaling in *C. elegans*. Development **130**, 3237-3248.

2. Miyahara, K., Suzuki, N., Ishihara, T., Tsuchiya, E. and Katsura, I. (2003). TBX2/TBX3 transcriptional factor homologue controls olfactory adaptation in *Caenorhabditis elegans*. J. Neurobiol. Published Online: 3 Nov 2003.

Reviews

3. 石原健(2003).「線虫*C. elegans*における感覚情報の統合と学習に関わる分子メカニズム」生物物理 **43**, 287-290.

4. 石原健(2003).「高次脳機能の発現:線虫*C. elegans*における学習の分子機構」実験医学 **21**, 2399-2404.

Books

5. 桂　勲(2003).「環境に対する適応応答：耐性幼虫形成」飯野雄一／石井直明編「線虫　究極のモデル生物」シュプリンガー・フェアラーク東京　pp155-165.

EDUCATION

1. Dr. T. Ishihara was invited to give a seminar at the Center for Developmental Biology (Riken), February 2003 (in Japanese).
2. Dr. T. Ishihara gave a lecture at Kobe University Graduate School of Medicine, February 2003 (in Japanese).
3. Dr. I. Katsura gave a lecture at Kyoto University, Graduate School of Science, April 2003 (in Japanese).
4. Dr. I. Katsura gave a lecture at the University of Tokyo, Graduate School of Arts and Sciences, July 2003 (in Japanese).
5. Dr. I. Katsura gave a lecture at Gakushuin University, Graduate School of Science, July 2003 (in Japanese).
6. Dr. I. Katsura was invited to give a seminar on "Molecular biological analysis on the behavior of the nematode *C. elegans*" at Gakushuin University, Graduate School of Science, July 2003 (in Japanese).
7. Dr. T. Ishihara was invited to give a seminar at Tokyo Metropolitan Institute of Neuroscience, September 2003 (in Japanese).
8. Dr. I. Katsura gave a lecture at Kyoto University, Faculty of Medicine, November 2003 (in Japanese).

SOCIAL CONTRIBUTIONS AND OTHERS

1. 石原健「情報処理の仕組みを遺伝学を使って探る」文部科学教育通信2003.4.28号　pp28-29.
2. 桂　勲「動物行動とラクトースオペロンの類似性ー抑制された遺伝プログラムとその抑制解除ー」RNA Network Newsletter,（科研費特定領域RNA情報発現系の時空間ネットワーク）Vol. 2, No. 1, pp72-75, August, 2003.
3. 桂　勲「線虫で探る遺伝子と行動」総研大ジャーナル　総合研究大学院大学　No.4, pp6-9, 2003秋.
4. Dr. I. Katsura served as one of the associate editors of the journal "Genes to Cells.".

H-d. Biomolecular Structure Laboratory
Yasuo Shirakihara Group

RESEARCH ACTIVITIES

(1) Crystallographic Study of F1-ATPase

Yasuo Shirakihara and Aya Shiratori

F1-ATPase is the catalytic sector of ATP synthase that is responsible for ATP production in living cells. ATP synthase is buried in the energy conversion membrane, such as that of mitochondria, using Fo portion (subunit composition of ab2c8-12) and has F1 as a soluble and protruding portion. F1 has molecular mass of 380,000 dalton and a subunit composition of $\alpha_3\beta_3\gamma\delta\varepsilon$. The unique rotational catalysis mechanism of F1 includes rotation of the rod-like γ subunit, which is thought to control the conformations of the three catalytic β subunits in a cyclic manner by its rotation. We have moved to the structural study of the $\alpha_3\beta_3\gamma\varepsilon$ sub-assembly of thermophilic F1, after solution of the structure of the $\alpha_3\beta_3$ sub-assembly and committed attempts to crystallize the $\alpha_3\beta_3\gamma$-sub-assembly. Employing the $\alpha_3\beta_3\gamma\varepsilon$ sub-assembly that show catalytic properties very similar to those of F1, we aim to detect structural changes caused by different nucleotide occupancy, which should provide with structural basis for understanding the rotational catalysis mechanism. Last year, we solved the structure of the $\alpha_3\beta_3\gamma\varepsilon$ sub-assembly with Mg-free ADP bound to only one β-subunit and examined its biological significance[4].

The difficulty in interpreting the above structure resided in that the molecule has no bound Mg, which is essential for catalysis, and that little biochemical evidence on the Mg-free state was available. We therefore moved to the structure analysis of the Mg-bound form. Previous difficulties in dealing with Mg-bound form have gone as described last year, and a data set from a MgADP bound form was collected for the first time, to a resolution of about 5.5 Å at Spring8 BL44 (Protein Institute, Osaka Univ.). The MgADP bound form was crystallized in the presence of 25mM Mg and 0.5mM ADP. The structure was solved by molecular replacement. Among the various available models (with zero, one, two or three nucleotide bound β-subunits), one nucleotide bound form (i.e. the

PS3 $\alpha_3\beta_3\gamma\varepsilon$ sub-assembly with Mg-free ADP bound to only one β-subunit) fitted best with the lowest R-factor that is 5% lower than those for other models. The map, using the model correctly positioned in the crystal unit cell, was carefully examined paying attention to a possible problem of model bias. The resultant map was closely similar to that for the adopted model (single Mg free ADP bound form), and therefore suggests that Mg has no significant effect on the structure as far as the present low resolution structural features are concerned. However, at the same time, it raises an open question why there is only one nucleotide-bound β-subunit in the structure where at least two nucleotide-bound β-subunits are normally expected.

These structure studies were done in collaboration with Toshiharu Suzuki and Masasuke Yoshida, at Research Laboratory of Resource Utilization, Tokyo Institute of technology.

(2) Crystallization of ATP synthase

Yasuo Shirakihara and Aya Shiratori

ATP synthase, the mother complex of F1, is still a challenging target for crystallization, in view of only a few solved structures of the membrane proteins so far. We restarted the crystallization study by examining the purification procedure for the ATP synthase extracted with dodecylmaltoside from the PS3 membrane, and found that an array of Q-sepharose high performance column, Superdex prep grade 200 column and the second Q-sepharose high performance column yielded a preparation of crystallization grade. The preparation gave small crystals in screening experiments using polyethylene glycol as a precipitant and MgADP as an additive. Further refinement of the crystallization conditions is in progress. Also a number of detergents have been examined for good extraction capability in the preparation procedure, and in addition to dodecylmaltoside described above, both decylglucoside and dedcylmaltoside have been identified as promising candidates.

The crystallization study was done in collaboration with Satoshi Murakami at Institute of scientific and industrial research, Osaka University.

(3) Comprehensive Crystallographic Study of Transcription factors and Genome-partitioning Factors from *E.coli*

Yasuo Shirakihara, Aya Shiratori, Hisako Inoue and Megumi Seki

More than 160 transcription factors have been identified in *E.coli*, and they control transcription of their target gene(s) in either positive or negative manners, by binding to both their specific DNA sequence and the transcription apparatus. Sixty-five such transcription factors have been purified in one step Ni-NTA column chromatography using carefully constructed over-expression systems in Ishihama laboratory. In Niki laboratory, a number of novel proteins, that are judged to play roles in the genome and plasmid partitioning from their localization patterns, have been prepared using similarly constructed over-expression systems. Setting these transcription and genome partitioning factors as targets for structural study, we have started comprehensive structure determination in the Protein 3000 project (the sub-field of 'transcription and translation'). Last year, which was the first year of the project, we examined 34 proteins for their crystals but identified only two proteins that gave crystals although the crystals were not satisfactory.

Following the lessons from the unsatisfactory crystallization success rate in the last year, we initially tried to crystallize transcription factors in a form of complex with their cognate DNA. We examined six transcription factors (KdpE, Mlc, IlvY, RcsB, ModE, and SdiA) out of fifteen factors with known binding DNA sequence. Four transcription factors (KdpE, Mlc, IlvY and RcsB) crystallized in the form of such complex, thus with much higher success rate than that for protein alone crystallization. However, the best co crystal that was obtained from KdpE showed diffraction patterns of just 5 Å resolution at a Spring 8 beam line BL38B1. Partly due to high cost for large amount of DNA oligomer, we have suspended the co crystal approach.

We continued protein-alone crystallization conditions search against both the old targets provided last year and new targets (this year) comprising fifteen transcription factors (ModE, Hns, YedW, b2381, ArsR, CadC, CheY, CrI, DicA, PutA, Usg, YfgB, YafC, YagI, HupA, YigT, YebG) and seven partioning factors (MukF, PutA, Malk, FadD, 269#3). Among the new

targets, only CadC and HupA gave microcrystalline aggregates. Among the old targets, Mlc were retried after finding that the provided turbid preparation, which had looked unsuitable to a crystallization experiment, gave fairly high protein concentration in the supernatant after centrifuge. Mlc gave tiny crystals from Ammonium sulfate solution in the screening stage, and larger but aggregate-looking crystals in the refinement stage. The aggregating tendency was reduced by changing pH (pH7 -〉 pH5.8) or temperature (25° C -〉25°).

We did some biochemistry on the partioning factors. In examination with a gel filtration column, MukF, MalK and RecA appeared exclusively in the void, which may be consistent with failure of crystallization of these proteins due to preformed aggregates. We purified further the provided FadD preparation, which is composed of the intact protein and the two fragments; all three species bound to a Ni-NTA resin. Hydrophobic, ion exchange and hydroxyapatite columns worked in separation of those, but the separated components were not soluble which hindered the crystallization study.

This work has been done in collaboration with Akira Ishihama, Emi Kanda (Nippon Institute for Biological Science), Hironori Niki, Rie Inaba, Katsynori Yata, Yasushi Ogata (isotope center).

(4) Crystallographic Study of Transcription factors from *Pseudomonas aeruginosa*

Yasuo Shirakihara, Aya Shiratori, Hisako Inoue and Megumi Seki

Pseudomonas aeruginosa is the well-known opportunistic bacterial pathogen, and a number of transcription factors responsible for the pathogenicity have been identified. Among those, we chose PtxR, PtxS, PhzR, and PA3547 and have started their structure biology.

Initially, over expression system was constructed by incorporating the provided expression plasmids, which were designed to have 6 His-tags at The C-terminus, into host cells (BL21 (DE3), B834 (DE3), JM109 (DE3)) and by examining which conditions, characterized by temperature (37℃, 25℃) and inducer presence (plus, minus), produce the target protein in a soluble fraction. Fortunately good conditions were found for all the proteins. Three proteins out of the four were purified by the Ni-NTA column and an extra column, of which resin was chosen empirically depending upon the protein. PhzR was an exception, ands was purified with Q-sepharose high performance, Butyl-toyopearl and hydroxyapatite columns, because the protein did not bind to a Ni-NTA column. A gel filtration analysis and the subsequent x-ray crystal analysis showed that the PhzR molecules form a tetramer, suggesting that the His-tag attached C-terminus of the molecule may be buried in the tetramer thus explaining no attachment of PhzR to Ni-NTA.

The preparations were then subjected to crystallization study. All proteins gave signs of crystals in the screening stage, however, the diffraction grade crystals were obtained only from PhzR so far. Phz crystals were obtained from polyethylene glycol at pH 4.7, and with a typical size of 0.2 x 0.2 x 0.05 mm. They diffracted to about 3.0 A resolution in the Spring8 BL38 beam. MAD data were collected from HgCl2 derivative using the same beam line as above. The data were processed with MOSFLM and CCP4 program suite. The unit cell is a=74.4A b=242.9A c=129.3A (space group C222), and its asymmetric unit contains 4 molecules of PhzR. MAD analysis using programs SOLVE and Sharp is in progress, revealing a novel mode of tetramer assembly of the PhzR.

This work has been done in collaboration with Hironori ARAMAKI (Daiichi Pharmaceutical College).

(5) Crystallization of TRAF6, a transducer in CD40 signaling pathway

Yasuo Shirakihara, Megumi Seki and Aya Shiratori

CD40 signalings play crucial roles in B-cell function. Signals through CD40 rescue B cells from apoptosis induced by cross-linking of the surface immunoglobulin complex and also induce B cells to differentiate. In the down stream, the signal transducers, TRAF6 (TNF receptor associated factor 6) and TRAF5, interact with a cytoplasmic tail of CD40. We tried to crystallize the TRAF6 in order to understand the mechanism of signal transduction employing CD40 and TRAF6. TRAF6 was expressed in insect cells as His-tagged protein, and was purified with Ni-NTA and ion exchange columns. The crystallization condition search was done using the recently developed method for comprehensive crystallization of transcription factors as described above. Small crystals were obtained from MPD, but further study may be

necessary for its establishment.

The crystallization study was done in collaboration with Jun-ichiro Inoue, Ayaka Moriya at the Institute of medical science, the university of Tokyo.

(6) Structural analysis of glutaminase from *Micrococcus luteus K-3*

Yasuo Shirakihara and Aya Shiratori

Glutaminase (EC 3.5.1.2) catalyzes the hydrolytic degradation of L-glutamine to L-glutamic acid. The salt-tolerant glutaminase from marine *Micrococcus luteus* K-3 has an unusual property that its maximum activity is observed at about 2M salt, and also has industrial relevance that this enzyme may be more efficient than the conventional ones in the soy sauce fermentation that is carried out in the high-salt environments. Previously MAD data using the Se-Met derivatives for the intact form (2.6 Å resolution) and the truncated form (2.4 Å resolution) had been collected[1] and a structural analysis of the truncated form was attempted.

The structure of the truncated form (2.4 Å resolution) of the enzyme was refined successfully this year, with free R factor of 23% and satisfactory geometry. The structure solution of the intact enzyme has been attempted by molecular replacement using the structure of the truncated form as a model. There has been a difficulty in such calculation, because the asymmetric unit in the intact crystal contains more than one molecule of the enzyme.

The structural study was done in collaboration with Kazuaki Yoshimune and Mitsuaki Moriguchi at Oita University, and Mamoru Wakayama at Ritsumeikan University.

Publications

Papers

1. Chantawannakul, P., Yoshimune, K., Shirakihara, Y., Shiratori, A., Wakayama, M. and Moriguchi, M. (2003). Crystallization and preliminary X-ray crystallographic studies of salt-tolerant glutaminase from *Micrococcus luteus K-3*, Acta Crystallogr D **59**, 566-568.
2. Hosaka, T., Meguro, T., Yamato, I. and Shirakihara, Y. (2003). Crystal Structure of *Enterococcus hirae* enolase at 2.8 Å Resolution. J. Biochem (Tokyo), **133** 817-823.
3. Shiroishi, M., Tsumoto, K., Amano, K., Shirakihara, Y., Colonna, M., Braud, V. M., Allan, D. S. J., Azure, S., Makadzange, Jones. R., Willcox, B., Jones, E. Y., van der Merwe, P. A., Kumagai, I. and Maenaka, K. (2003). Human inhibitory receptors Ig-like transcript 2 (ILT2) and ILT4 compete with CD8 for MHC class I binding and bind preferentially to HLA-G. Proc. Natl. Acad. Sci. **100**: 8856-8861.
4. Suzuki, T., Murakami, T., Iino, R., Suzuki, J., Ono, S., Shirakihara, Y. and Yoshida, (2003). F0-F1-ATP ase/Synthase is geared to the synthesis mode by conformational rearrangement of e subunit in response to proton motive force and ATP/ADP balance. J. Biol. Chem. **278**, 46840-46846.

EDUCATION

1. Y. Shirakihara was invited to give a seminar on "Strucrural analysis of F1-ATPase to reveal its rotational catalysis mechanism" at the Protein Institute of Osaka University., Osaka, Janauary 2003 (in Japanese).
2. Y. Shirakihara was invited to give a seminar on "Structural analysis of F1-ATPase to reveal its rotational catalysis mechanism" at Tokyo University, Tokyo, July 2003 (in Japanese).
3. Y. Shirakihara was invited to give a seminar on "Structural analysis of F1-ATPase to reveal its rotational catalysis mechanism" at Tokyo Institute of Technology, Yokohama, September 2003 (in Japanese).

I. CENTER FOR INFORMATION BIOLOGY AND DNA DATA BANK OF JAPAN

I-a. Laboratory for DNA Data Analysis Takashi Gojobori Group

RESEARCH ACTIVITIES

(1) The origin and evolution of porcine reproductive and respiratory syndrome viruses

Kousuke Hanada, Yoshiyuki Suzuki and Takashi Gojobori

Porcine reproductive and respiratory syndrome viruses (PRRSV) are divided into North American and European types, which show about 40% difference between the amino acid sequences. The divergence time of these two types has been considered to be around 1987 from the epidemiological data. It follows that PRRSV have evolved at a higher evolutionary rate (order of 10^{-2}/site/year) compared with other RNA viruses because the evolutionary rates of most RNA viruses are the orders of $10^{-3} - 10^{-5}$. Here, to test the evolutionary history of PRRSV speculated by the epidemiological background, we estimated the divergence time and evolutionary rate of PRRSV with molecular evolutionary analysis. As a result, the divergence time (1986±1.8) estimated corresponded well to that estimated by the epidemiological data, and the rate (7.7×10^{-3}) of PRRSV was indeed the highest among RNA viruses so far reported. Furthermore, to examine how PRRSV have adapted to swine since they emerged in 1987, we inferred important sites for the adaptation. The adaptive sites were located not only in the epitopes related to immunity, but also in the transmembrane regions including a signal peptide. In particular, the adaptive sites in the transmembrane regions were considered to affect compatibility to the host cell membrane. Therefore, we conclude that PRRSV have recently been transmitted from another host species to swine in the middle of the 1980's, adapting to swine by altering the transmembrane regions.

(2) The discovery of the genes relating to the evolution of an eyeless cave dwelling form from an eyed surface dwelling form of Mexican tetra, *Astyanax mexicanus*, by cDNA microarrays

Nobuhiko Tanaka, Shozo Yokoyama , Kazuho Ikeo and Takashi Gojobori

The understanding of the evolution of gene diversification should be simplified by studying a single species which is in the process of evolutionary diversification. Mexcan tetra *(Astyanax mexicanus)*, a single species showing eyed surface and eyeless cave dwelling forms, is a suitable model species to study the evolutionary diversification process. "How do cavefishes cope with the dark environment?" and "What types of genes are involved in these changes?" To solve these evolutionary problems, we tried to discover the genes relating to the evolution from the surface form to the cave form by cDNA microarrays. 3070 kinds of clones from an eyed adult fish were finally used for the construction of microarrays. As a result of hybridizations, crystalline genes were relatively highly expressed in surface fish, whereas transferrin genes were relatively highly expressed in cave fish. The transferrin proteins are related to the oxygen transportation in the blood. As the variation and quantity of phytoplanktons and plants in the dark (cave) environment must be less than those in the light (surface) environment, the concentration of oxygen in the water of the dark (cave) environment must be lower than that of light (surface) environment. Accordingly, to effectively transport oxygen in the blood of cavefish, transferrin genes must be required to have relatively highly expression in the cavefish.

(3) Evolutionary mechanisms of duplicated genes

Takashi Makino, Yoshiyuki Suzuki and Takashi Gojobori

It is proposed that gene duplication produces a gene with novel function along with the evolution of the asymmetrical copies. It is quite interesting in the perspective of understanding how gene duplication contributes to the evolution of novel functions. Therefore, we analyzed duplicated genes from evolutionary rate and

functional differentiation for understanding evolutionary mechanisms of that in *Saccharomyces cerevisiae*. When the genes of *S. cerevisiae* were categorized along with the fitness effects, the fitness effect pairs of duplicated genes were almost dispensability/ indispensability pairs or dispensability/ dispensability pairs, and rarely indispensability/ indispensability pairs. The evolutionary rate ratio of the duplicated gene pairs without common protein-protein-interaction partners or common functions was higher than that of others. In particular, the evolutionary rate ratio of dispensability/ indispensability pairs was higher than that of dispensability/ dispensability pairs. In the case of dispensability/ indispensability pairs, dispensable genes evolved faster and expressed higher than indispensable genes. In the case of dispensability/ dispensability pairs, however, there were not the differences observed in dispensability/ indispensability pairs. The results indicated that the asymmetric evolution of duplicated genes was specific in dispensability/ indispensability pairs.

(4) Study on conserve upstream noncoding sequences between human and mouse

Hisakazu Iwama and Takashi Gojobori

With the aim of elucidating what functional category of genes tend to be conserved in their upstream regions, we systematically estimated the degree of upstream sequence conservation of 4,074 genes along the 4-kb stretch and categorized the gene function according to the Gene Ontology. We found that the highly upstream-conserved genes are dominantly transcription factor (TF) genes. In particular, developmental process-related TF genes showed higher conservation of upstream sequences.

(5) Data analysis on Rice Genome Project

Hisakazu Iwama and Takashi Gojobori

We have participated in the Rice Genome Research Program in Japan and conducted computational analyses on the rice genome. We studied the functional classification of all the predicted proteins encoded in the rice genome.

(6) Biased biological functions of horizontally transferred genes on 324,653 open reading frames of 116 prokaryotic complete genomes

Yoji Nakamura, Takeshi Itoh, Hideo Matsuda and Takashi Gojobori

Horizontal gene transfer is one of the major mechanisms contributing to microbial genome diversification. To clarify the overall picture of interspecific gene flow among prokaryotes, we have developed a new method for detecting horizontally transferred genes (HT genes) and their possible donors by Bayesian inference. As a result, we discovered that 46,759 (14%) out of 324,653 open reading frames (ORFs) in 116 prokaryotic complete genomes have been subjected to horizontal transfer. Based on this dataset, for the first time, we quantitatively revealed that the biological functions of HT genes except mobile element genes are biased to three categories; cell surface, DNA binding, and pathogenicity-related functions. Thus, it suggests that transferability of genes depends heavily upon their function.

(7) Comparative complete genome sequence analysis of the amino acid replacements responsible for the thermostability of *Corynebacterium efficiens*

Yousuke Nishio, Yoji Nakamura, Yutaka Kawarabayasi, Yoshihiro Usuda, Eiichiro Kimura, Shinichi Sugimoto, Kazuhiko Matsui, Akihiko Yamagishi, Hisashi Kikuchi, Kazuho Ikeo and Takashi Gojobori

Corynebacterium efficiens is the closest relative of *C. glutamicum*, a species widely used for the industrial production of amino acids. *C. efficiens* but not *C. glutamicum* can grow above 40°C. We sequenced the complete *C. efficiens* genome to investigate the basis of its thermostability by comparing its genome with that of *C. glutamicum*. The difference in GC content between the species was reflected in codon usage and nucleotide substitutions. Our comparative genomic study clearly showed that there was tremendous bias in amino acid substitutions in all orthologous ORFs. Analysis of the direction of the amino acid substitutions suggested that three substitutions: from lysine to arginine, serine to alanine, and serine to threonine, are important for the stability of the *C. efficiens* proteins. Our results strongly suggest that the accu-

mulation of these three types of amino acid substitutions correlates with the acquisition of thermostability and is responsible for the greater GC content of *C. efficiens*[15].

(8) The genome stability in *Corynebacterium* species due to lack of the recombinational repair system

Yoji Nakamura, Yosuke Nishio, Kazuho Ikeo and Takashi Gojobori

Corynebacterium species are members of gram-positive bacteria closely related to *Mycobacterium* species, both of which are classified into the same taxonomic order Actinomycetales. Recently, three corynebacteria, *C. efficiens*, *C. glutamicum*, and *C. diphtheriae* have been sequenced independently. We found that the order of orthologous genes in these species has been highly conserved though it has been disrupted in *Mycobacterium* species. This synteny suggests that corynebacteria have rarely undergone extensive genome rearrangements and have maintained ancestral genome structures even after the divergence of corynebacteria and mycobacteria. This is the first report that the genome structures have been conserved in free-living bacteria such as *C. efficiens* and *C. glutamicum*, although it has been reported that obligate parasites such as *Mycoplasma* and *Chlamydia* have the stable genomes. The comparison of recombinational repair systems among the three corynebacteria and *M. tuberculosis* suggested that the absence of *recBCD* genes in corynebacteria be responsible for the suppression of genome shuffling in the species. The genome stability in *Corynebacterium* species will give us hints of the speciation mechanism with the non-shuffled genome, particularly the importance of horizontal gene transfer and nucleotide substitution in the genome[11].

(9) Identification of I-cell specific genes of hydra and its evolutionary implication

J. Shan Hwang, Chiemi Nishimiya-Fujisawa, Toshitaka Fujisawa, Kazuho Ikeo and Takashi Gojobori

Epithelial hydra, which is derived from *Hydra magnipapillata* (strain 105), possesses no interstitial stem cell and its subsequent differentiation products such as nerve cells and nematocytes, but maintains its abilities to grow, bud and regenerate into a complete polyp. Using cDNA microarray analysis, we compared the expression profiles between epithelial hydra normal hydra (strain 105). The cDNA microarray was constructed by a total number of 6,588 non-redundant clones which is generated from our Hydra EST project.

As a result, we have obtained 200 genes that are differentially expressed in the normal but not in epithelial hydra. To further study whether these genes are essentially specific for the interstitial stem cell lineage, we are now in process of performing whole mount *in situ* hybridization to localize their expression patterns in hydra. Our preliminary results indicated that the gene expressions were found specifically in either the nematocyte lineage or the nerve cell lineage. Given the fact that nematocyte is unique to the phylum Cnidarian, it is interesting to examine if the genetic network controlling nematocyte pathway is also exclusive to Cnidarian or it is evolutionarily conserved among metazoans. Moreover, the study of neuron-specific genes that were identified by microarray would address the question of what molecular basic is essential to form the origin of nervous system in metazoan.

(10) The evolutionary origin of long-crowing chicken based on the molecular phylogeny

Tomoyoshi Komiyama, Kazuho Ikeo and Takashi Gojobori

We tried to elucidate the origin and the domestication process of *Naganakidori* using the mitochondrial DNA D-loop region sequences. In Japan, three varieties of chickens can be found that have been specifically bred to develop an exceptionally long crow for over 20 seconds. Such characteristics of long-crowing chickens are also as popular as the fighting cock among breeders. The long crowing chicken has been mainly kept for the purpose of beauty and its long crow. It is not found only in Japan but also in other Asian countries such as Indonesia and China. However, the domestication process of long-crowing chicken is currently unclear. Our study is to elucidate this issue by using mitochondrial DNA D-loop region sequences. For this purpose, we collected blood sample of 9 long-crowing chickens and 74 other ornamental chickens from the 14 varieties of Japanese domesticated chickens. Then, we also obtained 2 DNA sequence data

of wild chicken from the International DNA databank (DDBJ/ EMBL/ GenBank) as the out-group. A phylogenetic tree was constructed by using both the neighbor-joining method(NJ)and the maximum likelihood method(ML). The results indicated that the 14 varieties of Japanese chickens were clearly separated into three different groups. One group contained the samples from Okinawan fighting cocks, the second group consisted of the long-crowing chickens, and third group is composed of the fighting cocks from Honshu and Kyushu in Japan. This result suggested that the long-crowing chicken was descened from the fighting cock, *Shamo* for cockfighting. In addition, we found that the three varieties of chickens share the common origin in spite of conspicuously different characters.

(11) The analysis to the evolutionary relationship between duplication and alternative splicing in terms of increasing functional diversity

Lihua Jin, Kazuho Ikeo, Yoshiyuki Suzuki and Takashi Gojobori

Living things increase their biological diversity by variable evolutionary mechanisms. Among these mechanisms, gene duplication and alternative splicing (A. S.) are the two major evolutionary routes that can bring the functional variation by increasing gene diversification. Our research interest is to study about the evolutionary relationship between the two different phenomena by utilizing available all data resources. The results of this study showed that the percentage of A.S. locus in the singleton gene group was less than that in the duplicated gene group and the duplicate genes tended to have more A. S. isoforms than singleton genes. In spite of these results, there was no apparent tendency of increasing isoform numbers of alternative splicing when family size increased in the group of duplicates genes. Our results suggested that gene duplication would induce more alternative splicing events on the duplicate genes than on singletons by reducing the functional constraint on those duplicates, and the degree of reduction of functional constraint was significantly large when singleton duplicated into two homologous genes.

(12) Comparative evolutionary study of the single cell-based gene expression profiles from the ascidinan blastmeres

Katsuhiko Mineta, Kazuho Ikeo, Hiroaki Yamamoto, Yuzuru Tanaka and Takashi Gojobori

Since the multicellular organism is composed of cells at the most basal portion, to understand the evolutionary diversification of organisms, we took an approach to examine the genes expressed at the various types of cells. As a first target, we focused on the cells of ascidians (Ciona intestinalis). The cell lineage of ascidians is well-documented, and their phylogenetic position is near the root of the Chordata, which is suitable for the analysis of the evolutionary process toward the vertebrates. Successfully, we could isolate and collect the cells (blastomeres) from the 1, 2, 4 and 8 cell early developmental stages of ascidians, and then cDNA libraries were constructed for each type of cells. We are sequencing ESTs from those libraries and comparing them with EST of the other cells sequenced. As a result, we sequenced, first, ~2,400 ESTs from six different types of blastomeres (A3 and B3 from 4-cell stage, A4.1, a4.2, B4.1 and b4.2 from the 8-cell stage) and then obtained 291~1266 non-redundant clones. We are also developing a 3D database for the intuitive management of the expression data. By use of the database and comparing genes identified at each single cell, we found that on average 7% of clones were commonly expressed between blastomeres. This result showed that the divergence of genes expressed at these cells was large, suggesting the characteristics of a cell as a whole might have already begun to be established at these stages.

(13) Molecular evolutionary analyses of host-parasite interactions

Yoshiyuki Suzuki

In order to identify and characterize genetic polymorphism of the swine major histocompatibility complex (Mhc: SLA) class I genes, RT-PCR products of the second and third exons of the three SLA classical class I genes, SLA-1, SLA-2 and SLA-3 were subjected to nucleotide determination. These analyses allowed the identification of four, eight and seven alleles at the SLA-1, SLA-2 and SLA-3 loci, respectively, from three

different breeds of miniature swine and one mixed breed. Among them, 12 alleles were novel. Construction of a phylogenetic tree using the nucleotide sequences of those 19 alleles indicated that the SLA-1 and -2 genes are more closely related to each other than to SLA-3. Selective forces operating at single amino acid sites of the SLA class I molecules were analyzed by the Adaptsite Package program. Ten positive selection sites were found at the putative antigen recognition sites (ARSs). Among the 14 positively selected sites observed in the human MHC (HLA) classical class I molecules, eight corresponding positions in the SLA class I molecules were inferred as positively selected. On the other hand, four amino acids at the putative ARSs were identified as negatively selected in the SLA class I molecules. These results suggest that selective forces operating in the SLA class I molecules are almost similar to those of the HLA class I molecules, although several functional sites for antigen and cytotoxic T-lymphocyte recognition by the SLA class I molecules may be different from those of the HLA class I molecules[1]. We also analyzed the evolution of feline immunodeficiency virus[12], and Haemophilus influenzae[20]. The methodologies for analyzing host-parasite interactions were also summarized[21) 22) 24)].

(14) Molecular basis of convergent evolution of camera eye between octopus and human

Atsushi Ogura, Kazuho Ikeo and Takashi Gojobori

It is well known that the camera eye of octopus is very similar to that of human. However, a phylogenetic consideration has suggested that their camera eyes have been acquired independently during evolution. Thus, the camera eye structures of octopus and human have been known as a typical example of convergent evolution. To examine if convergent evolution has really taken place at the molecular level, I conducted the comparative analysis of gene expression in the camera eye between human and octopus. In practice, I sequenced about 16,234 ESTs of an octopus eye, and compared these EST sequences with the complete genome sequences of nematode, fly and human as well as the gene expression data of human eye. As a result, I found that 729 genes were expressed commonly between the camera eye of human and octopus. Conducting comprehensive homology search using octopus ESTs with non-redundant protein database, I found that 933 genes had already existed at the common ancestor of Protostomes and Deuterostomes. I also found that 875 out of 1,019 genes were conserved between human and octopus, although nematode and fly are closely related to octopus than to human. Thus, genes expressed in the eye of octopus are highly shared with human. This suggests that the evolution of the camera eye of human and octopus is not subjected to the convergent evolution.

(15) Genomic diversification of bilaterian animals

Atsushi Ogura, Kazuho Ikeo and Takashi Gojobori

To understand the process of bilaterian evolution, we estimated ancestral gene sets at the divergence of bilaterian animals and the split of the plant-animal-fungus using the currently available gene sequence data. Using these ancestral gene sets, we examined how the number of gene members in the gene clusters has changed during evolution. As a result, the ancestral gene set of the bilaterian animals and the plant-animal-fungus were estimated to be 6,577 and 2,469 gene clusters, respectively. Thus, we found 2.5-fold increase in the number of gene clusters during the period of the time from the evolutionary split of the plant-animal-fungus to the divergence of bilaterian animals. Moreover, when we compared those numbers of the ancestral gene clusters with those the extant animals such as nematode, fly, mouse and human, we found that the extant bilaterian animals retained most of the ancestral gene set. It implies that diversification of bilaterian animals must have been attained by differently networking of the available gene set rather than producing a lot of functionally new genes.

Publications

Papers

1. Ando, A., Kawata, H., Shigenari, A., Anzai, T., Ota, M., Katsuyama, Y., Sada, M., Goto, R., Takeshima, S.-N., Aida, Y., Iwanaga, T., Fujimura, N., Suzuki, Y., Gojobori, T. and Inoko, H. (2003). Genetic polymorphism of the swine major histocompatibility complex (SLA) class I genes, SLA-1, -2 and -3. *Immunogenetics* **55**, 583-93.

2. Anzai, T., Shiina, T., Kimura, N., Yanagiya, K., Kohara, S., Shigenari, A., Yamagata, T., Kulski, J. K., Naruse, T. K., Fujimori, Y., Fukuzumi, Y., Yamazaki,

M., Tashiro, H., Iwamoto, C., Umehara, Y., Imanishi, T., Meyer, A., Ikeo, K., Gojobori, T., Bahram, S. and Inoko, H. (2003). Comparative sequencing of human and chimpanzee MHC class I regions unveils insertions/deletions as the major path to genomic divergence. *Proc. Natl. Acad. Sci. USA* **100**: 7708-13.

3. Hotta, K., Takahashi, H., Ueno, N. and Gojobori, T. (2003). A genome-wide survey of the genes for planar polarity signaling or convergent extension-related genes in Ciona intestinalis and phylogenetic comparisons of evolutionary conserved signaling Components. *Gene* **317**, 165-85.

4. Ikeo, K., Ishi-i, J., Tamura, T., Gojobori, T. and Tateno, Y. (2003). CIBEX: Center for Information Biology Gene Expression Database. *C.R. Biol.* **326**, 1079-82.

5. Ishibashi, T., Nakazawa, M., Ono, H., Satoh, N., Gojobori, T. and Fujiwara, S. (2003). Microarray analysis of embryonic reinoic acid target genes in the ascidian Ciona intestinalis. *Dev. Growth Differ* 249-259.

6. Komiyama, T., Ikeo, K. and Gojobori, T. (2003). Where is the origin of the Japanese gamecocks? *Gene* **317**: 195-202.

7. Mineta, K., Nakazawa, M., Cebria, F., Ikeo, K., Agata, K. and Gojobori, T. (2003). Origin and evolutionary process of CNS elucidated by comparative genomics analysis of planarian ESTs. *Proc. Natl. Acad. Sci. USA* **100**: 7666-7671.

8. Miyazaki, S., Sugawara, H., Ikeo, K., Gojobori, T. and Tateno, Y. (2003). DDBJ in the stream of various biological data. *Nucleic Acids Res.* **32**, D31-4.

9. Miyazaki, S., Sugawara, H., Gojobori, T. and Tateno, Y. (2003). DNA Data Bank of Japan (DDBJ) in XML. *Nucleic Acids Res.* **31(1)**: 13-6.

10. Nakamura, Y., Nishio, Y., Ikeo, K. and Gojobori, T. (2003). The genome stability in Corynebacterium species due to lack of the recombinational repair system. *Gene* **317**: 149-55.

11. Nakamura, K., Suzuki, Y., Ikeo, K., Ikeda, Y., Sato, E., Nguyen, N. T. P., Gojobori, T., Mikami, T. and Miyazawa, T. (2003). Phylogenetic analysis of Vietnamese isolates of feline immunodeficiency virus: genetic diversity of subtype C. *Arch. Virol.* **148** 783-91.

12. Nakazawa, M., Cebria, F., Mineta, K., Ikeo, K., Agata, K. and Gojobori, T. (2003). Search for the evolutionary origin of a brain.; Planarian brain characterized by microarray. *Mol. Biol. Evol.* **20(5)**: 784-791.

13. Niimura, Y., Terabe, M., Gojobori, T. and Miura, K.(2003). Comparative analysis of the base biases at the gene terminal portions in seven eukaryote genomes. *Nucleic Acids Res.* **31**: 5195-201.

14. Nishio, Y., Nakamura, Y., Kawarabayashi, Y., Usuda, Y., Kimura, E., Sugimoto, S., Matsui, K., Yamagishi, A., Kikuchi, H., Ikeo, K. and Gojobori, T. (2003). Comparative complete genome sequence analysis of the amino acid replacements responsible for the thermostability of Corynebacterium efficiens. *Genome Res.* **13**: 1572-1579.

15. Ohshima, K., Hattori, M., Yada, T., Gojobori, T., Sasaki, Y. and Okada, N. (2003). Whole-genome screening indicates a possible burst of formation of processed pseudogenes and Alu repeats by particular L1 subfamilies in ancestral primates. *Genome Biol.* **4**: R74.

16. Ota, K., Tateno, Y. and Gojobori, T. (2003). Highly differentiated and conserved sex chromosome in fish species (Aulopus japonicus: Teleostei, Aulopidae). *Gene* **317**: 187-93.

17. Pignatelli, S., Dal Monte, P., Rossini, G., Chou, S., Gojobori, T., Hanada, K., Guo, JJ., Rawlinson, W., Britt, W., Mack, M. and Landini, MP. (2003). Human cytomegalovirus glycoprotein N (gpUL73-gN) genomic variants: identification of a novel subgroup, geographical distribution and evidence of positive selective pressure. *J. Gen. Virol.* 2003 Mar; **84(Pt3)**, 647-655.

18. Yajima, I., Endo, K., Sato, S., Toyoda, R., Wada, H., Shibahara, S., Numakunai, T., Ikeo, K., Gojobori, T., Goding, C. R. and Yamamoto, H. (2003). Cloning and functional analysis of ascidian Mitf in vivo: insights into the origin of vertebrate pigment cells. *Mech Dev.* **120**: 1489-504.

Reviews

19. 中村洋路，鈴木善幸，五條堀　孝：ヘモフィルス属（インフルエンザ桿菌）．日本臨床，日本臨床社，2003, **61**, 716-721.

Books

20. Suzuki, Y. and Gojobori, T. (2003). Analysis of coding sequences. In The Phylogenetic Handbook: A Proctical Approach to DNA and Protein Phylogeny. Salemi, M., and Vandamme, A.-M. eds. (Cambridge, UK: Cambridge University Press), pp. 283-303.

21. 池尾一穂，鈴木善幸，五條堀　孝監訳(2003)「生命情報学キーノート」シュプリンガーフェアラーク東京.

22. 伊藤　剛，五條堀　孝(2003)「シリーズ・あなたにも役立つバイオインフォマティクスII，大規模解析に完

全長cDNAを活用する」『蛋白質核酸酵素』11月号：1962-1967.
23. 五條堀　孝編(2003)「生命情報学」シュプリンガーフェアラーク東京.
24. 五條堀　孝(2003)「ポストゲノムシーケンシング時代の生命情報科学」『学術月報』8月号.
25. 五條堀　孝(2003)「比較ゲノム学と機能ゲノム学への招待—統合生命科学へのプレリュード」羊土社：『実験医学』8月号.
26. 五條堀　孝(2003)「バイオインフォマティクス・情報生物学」『医学大事典』南山堂(印刷中).
27. 五條堀　孝編(2003)「ゲノムからみた生物の多様性と進化」シュプリンガー・フェアラーク東京.
28. 五條堀　孝(2003)「ヒトゲノム解読完了，その先は？私が考える未来：情報から」『科学』岩波書店：Vol.73 No.4, 364-365.
29. 新村芳人，五條堀　孝(2003)「理論生物学：バイオインフォマティクス」『シリーズ・ニューバイオフィジックスⅡ-10，生物物理学とはなにか』共立出版：125-140.

SOCIAL CONTRIBUTIONS AND OTHERS

1. ゲノムひろば2003 in 東京，「ゲノム研究勢ぞろい」出展.「洞窟魚(メキシカンテトラ)の地上型から洞窟型への進化に関わった遺伝子を探索する.」田中信彦，横山竦三，池尾一穂，五條堀孝.
2. 五條堀　孝　国立遺伝学研究所研究集会「人類集団における遺伝子レベルとゲノムレベルにおける多様性」，三島，1月，2003.
3. 五條堀　孝　総研大レクチャー／寺子屋「情報生物学」第5回バイオインフォマティクス：生命の多様性と進化に基づく生命科学の統合に向けて，三島，2月，2003.
4. 五條堀　孝　ポストゲノムシーケンシング時代の生命の多様性と進化の研究，三島，12月，2003.
5. 五條堀　孝　国立遺伝学研究所共同研究ヒトゲノム多様性研究集会，三島，12月，2003.

I-b. Laboratory for Gene-Product Informatics Ken Nishikawa Group

RESEARCH ACTIVITIES

At present the Nishikawa laboratory consist of 15 members: Professor Ken Nishikawa, Assistant Professors Satoshi Fukuchi and Akira Kinjo, three postdoctral research fellows (Drs. Homma,Nagashima and Minezaki), 8 technical assistants (Mses. Mimura, Ito, Yamamoto, Kuromaru, Abe, Hongo, Komatsubara and Suzuki), and a secretary (Ms. Sugiyama). We aim at theoretical work on protein molecules, such as prediction of three-dimensional structures from amino acid sequences, theoretical basis of protein stability, and molecular dynamics of proteins. Recently we have extended our research fields to genome analysis based on protein three-dimensional structures. We have developed two databases, Protein Mutant Database (PMD) and Genomes to Protein Structures and Functions (GTOP), and three computer tools for analysis of amino acid sequences and structures of proteins, LIBRA (Protein structure-sequence compatibility analysis), MATRAS (Protein tertiary structure comparison), and SSThread (Prediction of protein secondary structure using threading).

(1) Unique amino acid composition of proteins in halophilic bacteria

Satoshi Fukuchi, Kazuaki Yoshimune[1], Mitsuaki Moriguchi[1] and Ken Nishikawa ([1]Dept. Appl. Chem., Oita Univ.)

The amino acid compositions of proteins from halophilic archaea were compared with those from non-halophilic mesophiles and thermophiles, in terms of the protein surface and interior, on a genome-wide scale. As we previously reported for proteins from thermophiles, a biased amino acid composition also exists in halophiles, in which an abundance of acidic residues was found on the protein surface as compared to the interior. This general feature did not seem to depend on the individual protein structures, but was applicable to all proteins encoded within the entire genome. Unique protein surface compositions are common in both halophiles and thermophiles. Statistical tests have shown that significant surface compositional differences exist among halophiles, non-halophiles, and thermophiles, while the interior composition within each of the three types of organisms does not significantly differ. Although thermophilic proteins have an almost equal abundance of both acidic and basic residues, a large excess of acidic residues in halophilic proteins seems to be compensated by fewer basic residues. Aspartic acid, lysine, asparagine, alanine, and threonine significantly contributed to the compositional differences of halophiles from meso- and thermophiles. Among them, however, only aspartic acid deviated largely from the expected

amount estimated from the dinucleotide composition of the genomic DNA sequence of the halophile, which has an extremely high G+C content (68%). Thus, the other residues with large deviations (Lys, Ala, etc.) from their non-halophilic frequencies could have arisen merely as "dragging effects" caused by the compositional shift of the DNA, which would have changed to increase principally the fraction of aspartic acid alone. See Ref. 4 for details.

(2) Compositional changes in RNA, DNA and proteins for bacterial adaptation to higher and lower temperatures

Hiroshi Nakashima[1], Satoshi Fukuchi and Ken Nishikawa
([1]School of Health Science, Kanazawa Univ.)

It is known that in thermophiles the G+C content of ribosomal RNA linearly correlates with growth temperature, while that of genomic DNA does not. Although the G+C contents (singlet) of the genomic DNAs of thermophiles and methophiles do not differ significantly, the dinucleotide (doublet) compositions of the two bacterial groups clearly do. The average amino acid compositions of proteins of the two groups are also distinct. Based on these facts, we here analyzed the DNA and protein compositions of various bacteria in terms of the optimal growth temperature (OGT). Regression analyses of the sequence data for thermophilic, mesophilic and psychrophilic bacteria revealed good linear relationships between OGT and the dinucleotide compositions of DNA, and between OGT and the amino acid compositions of proteins. Together with the above-mentioned linear relationship between ribosomal RNA and OGT, the DNA and protein compositions can be regarded as thermostability measures for RNA, DNA and proteins, covering a wide range of temperatures. Both the DNA and proteins of psychrophiles apparently exhibit characteristics diametrically opposite to those of thermophiles. The physicochemical parameters of dinucleotides suggested that supercoiling of DNA is relevant to its thermostability. Protein stability in thermophiles is realized primarily through global changes that increase charged residues (i.e., Glu, Arg, and Lys) on the molecular surface of all proteins. This kind of global change is attainable through a change in the amino acid composition coupled with alterations in the DNA base composition. The general strategies of thermophiles and psychrophiles for adaptation to higher and lower temperatures, respectively, that are suggested by the present study are discussed. See Ref. 5 for details.

(3) Prediction of catalytic residues in enzymes based on known tertiary structure, stability profile, and sequence conservation

Motonori Ota[1], Kengo Kinoshita[2] and Ken Nishikawa
([1]Global Scientific Information and Computing Center, Tokyo Institute of Technology, [2]Graduate School of Integrated Science, Yokohama City Univ.)

The catalytic or functionally important residues of a protein are known to exist in evolutionarily constrained regions. However, the patterns of residue conservation alone are sometimes not very informative, depending on the homologous sequences available for a given query protein. Here, we present an integrated method to locate the catalytic residues in an enzyme from its sequence and structure. Mutations of functional residues usually decrease the activity, but concurrently often increase stability. Also, catalytic residues tend to occupy partially buried sites in holes or clefts on the molecular surface. After confirming these general tendencies by carrying out statistical analyses on 49 representative enzymes, these data together with amino acid conservation were evaluated. This novel method exhibited better sensitivity in the prediction accuracy than traditional methods that consider only the residue conservation. We applied it to some so-called "hypothetical" proteins, with known structures but undefined functions. The relationships among the catalytic, conserved, and destabilizing residues in enzymatic proteins are discussed. See Ref. 6 for details.

Publications

Papers

1. Fukami-Kobayashi, K., Tateno, Y. and Nishikawa, K. (2003). Parallel evolution of ligand specificity between LacI/GalR family repressors and periplasmic sugar-binding proteins. *Molecular Biology and Evolution*, **20**, 267-277.

2. Kinjo, A.R. and Takada, S. (2003). Competition between protein folding and aggregation with molecular chaperones in crowded solutions: Insight from mesoscopic simulations. Biophysical Journal, **85**, 3521-3531.

3. Kashiwagi, K., Shiba, K., Fukami-Kobayashi, K., Noda, T., Nishikawa, K. and Noguchi, H. (2003). Characterization of folding pathways of the type-1 and type-2 periplasmic binding proteins MglB and ArgT. *J. Biochem.* **133**, 371-376.
4. Fukuchi, S., Yoshimune, K., Wakayama, M., Moriguchi, M. and Nishikawa, K. (2003). Unique amino acid composition of proteins in halophilic bacteria. *J. Mol. Biol.*, **327**, 347-357.
5. Nakashima, H., Fukuchi, S. and Nishikawa, K. (2003). Compositional changes in RNA, DNA and proteins for bacterial adaptation to higher and lower temperatures. *J. Biochem.*, **133**, 507-513.
6. Ota, M., Kinoshita, K. and Nishikawa, K. (2003). Prediction of catalytic residues in enzymes based on known tertiary structure, stability profile, and sequence conservation. J. Mol. Biol., **327**, 1053-1064.
7. Kashiwagi, K., Fukami-Kobayashi, K., Shiba, K. and Nishikawa, K.: Construction and characterization of chimeric proteins composed of type-1 and type-2 periplasmic binding proteins MglB and ArgT. *Biosci. Biotec. Biochem.*, in press.

Reviews

8. 中島広志, 福地佐斗志, 西川建, 「好熱菌の熱安定性への戦略」, 『ゲノムからみた生物の多様性と進化』(五條堀孝編, シュプリンガー・フェアラーク東京)pp.41-49(2003).

I-c. Laboratory for Gene Function Research Yoshio Tateno Group

RESEARCH ACTIVITIES

(1) Parallel evolution of ligand specificity between LacI/GalR family repressors and periplasmic sugar-binding proteins

Kaoru Fukami-Kobayashi, Yoshio Tateno and Ken Nishikawa

The bacterial LacI/GalR family repressors such as lactose operon repressor (LacI), purine nucleotide synthesis repressor (PurR), and trehalose operon repressor (TreR) consist of not only the N-terminal helix-turn-helix DNA-binding domain but also the C-terminal ligand-binding domain that is structurally homologous to periplasmic sugar-binding proteins. These structural features imply that the repressor family evolved by acquiring the DNA-binding domain in the N-terminal of an ancestral periplasmic binding protein (PBP). Phylogenetic analysis of the LacI/GalR family repressors and their PBP homologues revealed that the acquisition of the DNA-binding domain occurred first in the family, and ligand specificity then evolved. The phylogenetic tree also indicates that the acquisition occurred only once before the divergence of the major lineages of eubacteria, and that the LacI/GalR and the PBP families have since undergone extensive gene duplication/loss independently along the evolutionary lineages. Multiple alignments of the repressors and PBPs furthermore revealed that repressors and PBPs with the same ligand specificity have the same or similar residues in their binding sites. This result, together with the phylogenetic relationship, demonstrates that the repressors and the PBPs individually acquired the same ligand specificity by homoplasious replacement, even though their genes are encoded in the same operon.[1]

(2) CIBEX: Center for Information Biology Gene Expression Database

Kazuho Ikeo, Jun Ishi-i, Takurou Tamura, Takashi Gojobori and Yoshio Tateno

We describe the current status of the gene expression database CIBEX (Center for Information Biology gene EXpression database, http://cibbex.nig.ac.jp), with a data retrieval system in compliance with MIAME, a standard that the MGED Society has developed for comparing and data produced in microarray experiments at different laboratories worldwide. CIBEX serves as a public repository for a wide range of high-throughput experimental data in gene expression research, including microarrayˆbased experiments measuring mRNA, serial analysis of gene expression (SAGE tags), and mass spectrometry proteomic data.[2]

(3) Highly differentiated and conserved sex chromosome in fish species (*Aulopus japonicus*: Teleostei, Aulopidae)

Kinya Ota, Yoshio Tateno and Takashi Gojobori

While highly differentiated and long-conserved sex chromosomes such as XY and ZW chromosomes are

observed, respectively, in mammalian and avian species, no counterparts to such chromosomes were observed in fish until we reported in the previous study that well-conserved and highly differentiated ZW sex chromosomes existed in the family of Synodontidae. Then, the problem was if the evolutionary history of the fish ZW chromosomes was long enough to be comparable to the mammalian and avian counterparts. To tackle the problem, we had to extend our finding of the fish sex chromosomes further than a family alone. For this purpose, we chose Aulopus japonicus that belonged to one of the related families to Synodontidae. Our cytogenetic and fluorescence in situ hybridization (FISH) analyses have clearly demonstrated that A. japonicus also has ZW chromosomes. We have also found that 5S rDNA clusters are located on the Z and W chromosomes in this species. Using nontranscribed intergenic sequences in the 5S rDNA clusters as PCR primers, we successfully amplified a 6-kb-long female-specific sequence on the W chromosome. The 6-kb-long sequence contained one transposable element and two tRNA sequences. The function of the sequence remains to be studied. Our Southern blot analysis confirmed that the 6-kb sequence was located only on the W chromosome.Therefore, it is now said that highly differentiated ZW chromosomes have been conserved over two fish families. As these families were reported to have been diverged 30-60 million years ago, the fish ZW chromosomes have an evolutionary history corresponding to the history of the families. This is perhaps the first case that fish sex chromosomes are shown to have such a long evolutionary lineage.[3]

(4) DNA Data Bank of Japan (DDBJ) in XML

Satoru Miyazaki, Hideaki Sugawara, Takashi Gojobori and Yoshio Tateno

The DNA Data Bank of Japan (DDBJ, http://www.ddbj.nig.ac.jp) has collected and released more entries and bases than last year. This is mainly due to large-scale submissions from Japanese sequencing teams on mouse, rice, chimpanzee, nematoda and other organisms. The contributions of DDBJ over the past year are 17.3% (entries) and 10.3% (bases) of the combined outputs of the International Nucleotide Sequence Databases (INSD). Our complete genome sequence database, Genome Information Broker (GIB), has been improved by incorporating XML. It is now possible to perform a more sophisticated database search against the new GIB than the ordinary BLAST or FASTA search.[4]

(5) Characterization of folding pathways of the type-1 and type-2 periplasmic binding proteins MglB and ArgT

Kenji Kashiwagi, Kiyataka Shiba, Kaoru Fukami-Kobayashi, Tetsuo Noda, Ken Nishikawa and Hiroshi Noguchi

The family of periplasmic binding proteins (PBPs) is believed to have arisen from a common ancestor and to have differentiated into two types. At first approximation, both types of PBPs have the same fold pattern, reflecting their common origin. However, the connection between the main chains of a type 2 PBP is more complicated than a type 1 PBP's. We have been interested in the possibility that such structural changes affect the folding of PBPs. In this study, we have characterized the folding pathways of MglB (a type 1 PBP) and ArgT (a type 2 PBP) by using urea gradient gel electrophoresis, fast protein size-exclusion liquid chromatography and hydrophobic dye ANS binding assay. We found a distinct difference in folding between these two proteins. The folding of MglB followed a simple two-state transition model, whereas the folding of ArgT was more complicated.[5]

(6) Standardization of Microarray Data

Brazma Alvis, Kazuho Ikeo and Yoshio Tateno

It is now a routine practice to conduct microarray experiments in order to study gene expression under various biological, chemical and physical conditions. However, there is a serious problem with microarray data produced at different laboratories. As no unified protocol for the microaray experiment exists, the data produced at one laboratory cannot be compared to that produced at another. The data are not like DNA sequence data. To alleviate this problem, some concerned researchers organized the Microarray Gene Expression Data Society (MGED) and have worked to formulate the Minimum Information About Microarray Experiments (MIAME) that makes it possible for one to compare two sets of the data produced at two different laboratories. We describe MIAME particularly to relevant Japanese researchers in the hope that

they understand it and cooperate with MGED.[6] (in Japanese)

(7) DNA Data Bank of Japan as an Indispensable Public Database

Satoru Miyazaki and Yoshio Tateno

The International Nucleotide Sequence Databases (INSD), which are composed of the DNA Data Bank of Japan (DDBJ), EMBL Bank, and GenBank, have served innumerable researchers worldwide as the public repository of DNA sequence data and the related information for more than 15 years. INSD have steadily been recognized and acknowledged, and become now indispensable for research in bioscience and biotechnology. To serve researchers in these fields more effectively and efficiently, DDBJ has developed a number of devices in collaboration with the two partners. One of them is to form an XML format for data presentation. The data in the traditional flat file format is straightforward so that one can easily glimpse the data contents. Recently, however, one entry has tended to contain more and more pieces of information. Therefore, it is often difficult for one to extract the pieces of information of one's interest from such entries. To make it easier to cope with such a situation, we adopted XML and developed the DDBJ-XML.[7]

Publications

Papers

1. Fukami-Kobayashi, K., Tateno, Y. and Nishikawa, K. (2003). Parallel evolution of ligand specificity between LacI/GalR family repressors and periplasmic sugar-binding proteins. Mol Biol Evol **20**, 267-277.

2. Ikeo, K., Ishi-i, J., Tamura, T., Gojobori, T. and Tateno, Y. (2003). CIBEX: Center for Information Biology Gene Expression Database. C.R.Biologies **326**, 1079-1082.

3. Ota, K., Tateno, Y. and Gojobori, T. (2003) Highly differentiated and conserved sex chromosomes in fish species (*Aulopus japonicus*: Teleostei, Aulopidae) Gene **317C**, 187-193.

4. Miyazaki, S., Sugawara, H., Gojobori, T. and Tateno, Y. (2003). DNA Data Bank of Japan (DDBJ) in XML, Nucleic Acids Res **31**, 13-16.

5. Kashiwagi, K., Shiba, K., Fukami-Kobayashi, K., Noda, T., Nishikawa, K. and Noguchi, H. (2003). Characterization of folding pathways of the type-1 and type-2 periplasmic binding proteins MglB and ArgT., Journal of Biochemistry **133**, 371-376.

6. アルヴィス＝ブラズマ，池尾一穂，舘野義男，マイクロアレイデータの標準化，蛋白質核酸酵素**48**：280-285 (2003).

Books

7. Miyazaki, S. and Tateno, Y. (2003) DNA Data Bank of Japan as an Indispensable Public Database, In: *Populations and Genetics*, B. M. Knoppers ed, pp115-121, Martinus Nijhoff Publishers, Leiden/Boston.

Database

8. DDBJ is operated by the DNA Data Analysis, Gene Function Research, Gene-Product Informatics, Research and Development of Biological Databases, and Gene Expression Laboratories and Division of Population Genetics. DDBJ collects, annotates and publishes DNA sequence data on the daily basis. In addition, it edits the data published by DDBJ, EMBL and GenBank together four times a year and publishes as a release. In 2003 DDBJ published four releases as follows.

Release 53	March, 03	23,250,813 entries
		29,711,299,332 bases
Release 54	June, 03	25,149,821 entries
		32,162,041,177 bases
Release 55	Sept., 03	27,753,140 entries
		34,280,225,489 bases
Release 56	Dec., 03	30,405,173 entries
		36,079,046,032 bases

9. Dr. Y. Tateno attended the 16th International Nucleotide Sequence Databases Collaborative Meeting, and the 14th International Nucleotide Sequence Databases Advisors Meeting held at NLM, NIH, Bethesda, U.S.A., May, 2003.

10. Dr. Y. Tateno attended the H-Invitational 2, Leaders' Meeting, Odaiba, Tokyo, November, 2003.

EDUCATION

1. Dr. Y. Tateno gave a lecture on "Genetics, Environments and Health" at the Mishima Welfare Association Meeting, Mishima, Shizuoka, February, 2003 (in Japanese).

2. Dr. Y. Tateno gave a lecture on "Population Genetics" at Tokyo Medical and Dental University, February, 2003 (in Japanese).

3. Dr. Y. Tateno was invited to give a seminar on

"Comparative genomics" for The 2nd Korea- Japan Bioinformatics Training Course at KAIST, Daejeon, Korea, August, 2003.

4. Dr. Y. Tateno gave a lecture on "Evolution of Genes and Proteins" at Chiba University, November, 2003 (in Japanese).

SOCIAL CONTRIBUTIONS AND OTHERS

1. Dr. Y. Tateno served the International Genetics Federation as Treasurer (1999-2003), and attended the 19th International Congress of Genetics, Melbourne, Australia, July, 2003.

2. Dr. Y. Tateno serves the Microarray Gene Expression Data Society as an Advisory Board Member (2001-), and attended the Board Member Meeting, Colorado, USA, February, 2003, and that, Axan-Provance, France, September, 2003.

3. Dr. Y. Tateno served the Society of Evolutionary Studies, Japan as Treasurer and General Secretary (2003).

4. Dr. Y. Tateno serves the Society of Evolutionary Studies as a Member of the Committee of Genetics under the Science Council of Japan.

5. Dr. Y. Tateno serves the Genetics Society of Japan as a Member of Editorial Board.

I-d. Laboratory for Research and Development of Biological Databases
Hideaki Sugawara Group

RESEARCH ACTIVITIES

(1) Information systems for molecular biology and its related disciplines

1) From Web services to a Bioportal

The publicly available bioinformatics resources, comprising databases[2,5] and analytical tools[1], have expanded in recent years. While the information environment for life sciences has gradually become more abounding, it is still difficult to combine multiple, heterogeneous bioinformatics resources for a specific research purpose. To set up and run an integrated system, it is often necessary to write and update custom programs. In addition, different research groups continually write programs that have overlapping functions. We need an information environment that is conducive to efficient and appropriate bioinformatics resource utilization for a wide range of users. Therefore, the Center for Information Biology and DNA Data Bank of Japan, in alliance with the National Institute of Informatics (NII) and the Mitsubisi Research Institute, Inc. (MRI) have started a three years long project, "Research and Development of the New Generation of Bio-portal", to enhance the information environment for the relevant user communities. In this project, the Laboratory for Research and Development of Biological Databases is responsible for the following tasks:

-Development of a meta-database by analyzing the specifications of major information resources that are listed in the database issue of the journal "Nucleic Acids Research".

-Development of an electronic workbench, by use of web services[3], a workflow description language, and GRID[4] technology.

-Development of a semantic web prototype, in collaboration with NII.

In the meantime, we recognized the need to process a long job and a large data file to cope with the tsunami of biological data. However, the SOAP server that we set up using tools in the public domain could not satisfy the requirements. Therefore, we have enhanced the SOAP server for the web services to process a long job and a large scale data[3].

2) Expansion of Genome Information Broker (GIB)

GIB was originally created for the retrieval and analysis of *E. coli* genomic information in a set. We implemented microbial genome data into GIB whenever genome sequencing was completed and the data is made open to the public. At the GIB Web page (http://gib.genes.nig.ac.jp/), key word search, homology search, links to DBGET, KEGG and GTOP and visualization of the data are available for more than 150 strains as of April 2004. We have utilized XML, CORBA and a distributed database in order to cope with the explosion of microbial genome information.

(2) Information systems on microbes

1) WFCC-MIRCEN World Data Centre for Microorganisms (WDCM)

WFCC and MIRCEN stand for World Federation for Culture Collections and Microbial Resource Centers network respectively. The laboratory is the host of

WDCM and maintains the World Directory of microbial resource centers. The on-line World Directory contains the detailed information of 469 centers in 62 countries and also the list of their holdings. Any culture collection is able to register, update and delete the information at http://www.wdcm.org/. We also used XML technology to organize the Web page. The WDCM Web site also provides a parallel search engine named AHMII[6].

2) Development of an e-Workbench for Biological Classification and Identification (InforBIO)

We continued the development of an e-Workbench named InforBIO by use of JAVA, XML and a relational database management system in the public domain. We have distributed InforBIO to several laboratories that study microbes and improved the utility and robustness of InforBIO based on the feedback from them.

3) An information system for pathogenic microorganisms

We participated in a national project for the resource center of pathogenic microorganisms. Our role is to develop an information system for pathogenic fungi and actinomycetes, and also a portal site for pathogenic microorganisms in general.

(*) The information system on pathogenic microorganisms has been supported by Special Coordination Funds for Promoting Science and Technology.

(3) Applications of IT to the International Nucleotide Sequence Database

1) Development of Open Annotation System

A number of the complete genome sequences have been submitted to INSD since 1995. The annotation information, however, is not consistent among genome sequencing teams. In addition, researchers outside of the team might have more information and knowledge on some genes and biological molecules. Therefore, it is quite important to develop the system which allows any expert to evaluate the annotation given by the team to attach more valuable information. As a new feature of INSD, we develop so-called "Open Annotation System (OASYS)" as an annotation editor in the distributed environment on the Internet.

(*) OASYS project has been supported by BIRD of Japan Science and Technology Agency (JST)

2) Exhaustive evaluation of microbial genome information by use of GRID

Tsunami of biological data and multiple views of the data analysis require an expandable and flexible information environment. GRID computing is expected to be the solution. We prepared a computational environment composed of 5 sites in OBIGrid and succeeded in analyzing horizontal gene transfer and clusters of ORFs of more than 120 microbial genomes that were stored in the Genome Information Broker as of May, 2003[4]. This scheme is being applied to more than 300 thousands ORFs of genomic sequences of 124 microbial species.

Publications

Papers

1. Chenna, R., Sugawara, H., Koike, T., Lopez, R., Gibson, T. J., Higgins, D. G. and Thompson, J. D. (2003). Multiple sequence alignment with the Clustal series of programs. Nucleic Acids Research **31(13)**, 3497-3500.

2. Miyazaki, S., Sugawara, H., Gojobori, T. and Tateno, Y. (2003). DNA Data Bank of Japan (DDBJ) in XML. Nucleic Acids Research31(1), 13-16.

3. Shigemoto, Y., Ymaguchi, M. Miyzazakim, S. and Sugawara, H. (2003). Enhancement of the SOAP Server in DDBJ Web Services to Process Tsunami of Biological Data. Genome Informatics **14**, 669-670.

4. Sugawara, H., Nakamura, Y., Ikeo, K., Miyazaki, S., Gojobori, T., Satou, K. and Konagaya, A. (2003). Analysis of Hrizontal Gene Transfer and Clustering of Microbial ORFs by Use of a GRID Environment. Genome Informatics **14**, 567-568.

5. Sugawara, H., Gojobori, T., Konno, T., Shigemoto, Y. and Ymaguchi, M.(2003). A Prototype of PubMed Central Japan.Genome Informatics **14**, 671-672.

6. Sugawara, H. and Miyazakim, S. (2003). AHMII: Agent to Help Microbial Information Integration. Nucleic Acids Research31(13), 3727-3728.

7. 菅原秀明. (2003). ヒトゲノムデータの迅速・無条件公開とそれをめぐる議論. 蛋白質核酸酵素48(13), 1857-1862.

Books

8. Satoru, Miyazaki. and Yoshio, Tateno. (2003). DNA Data Bank of Japan as an Indispensable Public Database, Bartha maria Knoppers eds. (Martinus Nijhoff Publishers), pp.115-121.

9. 菅原秀明. (2003). 生命科学における大規模システム：有馬朗人監修「これからの大学等研究施設　第２編　生命

科学編」. (科学新聞社), pp.10-3-10-9.
10. 菅原秀明(分担執筆). (2003). バイオ系のためのインターネット活用法(講談社), pp.
11. 菅原秀明. (2003). はじめに. バイオインフォマティクス研究会編「バイオ系のためのインターネット活用法」(講談社サイエンティフィク).
12. 菅原秀明(編集). (2003). バイオインフォマティクスがわかる(羊土社).
13. 宮崎　智. (2003). 配列の相同性：バイオインフォマティクスがわかる(菅原秀明編集), (羊土社), pp.20-26.

SOCIAL CONTRIBUTIONS AND OTHERS

1. Prof. H. Sugawara organized the International Conference on Interoperability, Tokyo, November, 2003.
2. Prof. H. Sugawara organized a symposium of the Frontier Studies and International Networking of Genetic Resources in Pathogenic Microorganisms, Tokyo, December, 2003.
3. Prof. H. Sugawara organized the conference of the Interoperability of Biological Data Resources, Mishima, December, 2003 (in Japanese).
4. Prof. H. Sugawara organized a symposium at the WFCC workshop of Commercial Use of Microbial Diversity, Melborne, December, 2003.
5. Task Force of Biological Resource Centers, OECD Working Party for Biotechnology (Vice-chair).
6. World Federation for Culture Collections, Executive board member and journal editor Genome Informatics 2004 (Program committee).
7. 極限微生物学会(評議員)
8. 日本情報知識学会(理事)

I-e. Laboratory for Gene-Expression Analysis Kousaku Okubo Group

RESEARCH ACTIVITIES

(1) Expression profiling of human genes

(1a) Data integration (BodyMapII): "Do you know how much of our genes have reliable expression patterns on the net?"

Osamu Ogasawara and Kousaku Okubo

Unexpectedly small difference in gene numbers among multicellular organisms, precisely determined though whole genome sequence supports the idea that the complexity in our body is evolutionary achieved by sophistication in expression controls of genes. The anatomically comprehensive genome-wide gene expression profile is key data to appreciate such sophistication coded in our genome. Moreover, availability of such data opened up opportunities to explore the dependence of constitutive expression patterns on other features of genes and genomes, which may eventually leads to the understanding of coding principles in our genome.

Despite the frequent use of the term 'genome-wide profiling' and wealth of expression data in the public domain, it is still not explicit what fraction of our genes are provided with anatomical expression patterns (COVERAGE) and to what extent different data set agrees in terms of tissue distribution (ACCURACY). In order for rational design of studies of human transcriptome as a whole, we started to integrate data from multiple different platforms on the framework of latest human genome. The preliminary data is opened to the public in collaboration with 'integrated database team at JBIRC. (https://www.jbirc.aist.go.jp/hinv/h-angel)

(1b) Data generation and analysis; "Are you satisfied by the present resolution of anatomical expression data?"

Makiko Otsuji and Koichi Itoh

In order to functionally relate genes in co-expression cluster or to deduce promoter sequences through alignment of upstream regions of them, the

resolution of expression pattern should be at the level of cells or homogeneous cell populations. The vast majority of the public data do not meet this criterion, except for those from induction experiments with cell lines. Moreover, the majority of the target genes for drug development such as receptors for signaling molecules and channels localize in the minority cells in the complex organs rather than in homogeneous cell population. We are taking several different approaches to generate such data in streamline in the organs with complex cell population such as brain and kidney.

(2) Knowledge encoding and computation with gene functions (BOB): "Are you confident in your massive data interpretation?"

Kousaku Okubo, Koichi Itoh and Osamu Ogasawara

With the advent in high throughput genome-wide measurement, hypothesis generation on gene functions by systematic and integrative interpretation of the accumulating data is anticipated. For the last few years, various statistical analysis techniques have been employed in extraction of global patterns from the massive data, in the forms of gene clusters and networks. However, at present, even with intensive use of web-based knowledge bases, human interpretation will not match machine-aided data production in neither speed nor scope. Automation of interpretation process, at least in part, appears essential for systematic and efficient hypothesis generation.

There are three steps in automation of interpretation process; (1) encode biomedical knowledge into computable form, (2) interpret data using encoded knowledge, and (3) represent the interpretation results. The pioneering approaches in the step (1) were all declarations (KEGG, GO). In general, some inherent limitations are known in declarative approach. First of all, continuous revising and updating efforts by experts are inevitable. Secondly, the manually declared structure has usually low dimension and representation power is poor. For example GO provides only three 'aspects' in the representation of functional relations in genes. And thirdly, experts' declaration is apt to be inconsistent when target domain becomes wider. Although such limitation may causes little problem in biochemistry and cell biology, they become serious in the field of medical biology where gene functions have been described by relating their roles with so many types of concepts from behavior to chemical compounds. These limitations make the effectiveness of declaration approach in the medical problems still elusive.

Rationale for our approach: A major process in biomedical interpretation of these data is to determine whether if there is a unique and common functional feature, within any part of data-driven structures. For this purpose, experts recall features of genes from every aspect. Such a process appears so dependent on experts' flexible recalling and thinking that machine cannot possibly do. In medical biology, however, we assumed that each aspect mostly represents a certain topic in biomedicine, such as 'pathogenesis of a disease' and 'molecular mechanism of an organs function.' If there is a book covering essentially all fundamental topics in medical biology, and if each page contains a list of all relevant genes, interpretation would be achieved by computing fitness of a given gene cluster to each page (meaningfulness) and by returning the title of the page that fits best as a meaning of the cluster.

We aimed to create such a book, 'BOB (Biomedical OminiBook)', by concept-based structuring of pages in biomedical textbooks and description of gene functions. We applied a concept based indexing technique, latent semantic indexing (LSI), for this purpose. LSI was developed to overcome the problems in term-matching-based-document-searching caused by the fact that similar concept may represented by texts with different term combinations and vice versa. In LSI, terms and documents are structured in a 'semantic' vector space, based on the global patterns in term-document association data, beforehand. In searching, users' queries are mapped onto this space as pseudo-documents. For each queries, the document vectors having supra-threshold cosine value to the query vector are returned.

In our application, we took textbooks in place of documents to be searched. Started from term-page association data, provided in the index sections of textbooks, we created a high-dimensional vector space wherein objects (term or page) are placed according to their relevance. Then we prepared a gene vector, that corresponds to a 'query vector' in original LSI, by counting occurrence of textbook terms in corresponding molecular database entry including abstracts for cited papers. Thus prepared gene vectors were mapped onto the textbook space similarly to the mapping of users queries. Resulted space contains vector repre-

sentation of three different classes of objects; term meanings, page contents, and gene functions, arranged according to their conceptual relevance. High dimensionality of this space is expected to allow discriminative representation of many aspects of relations without inconsistency. Using this space, we may scale the functional relevance of any pairs of genes, with which 'meaningfulness' of any given gene cluster can be calculated. In addition, the title for the page-vector nearest to the center of a gene cluster will explain the biomedical 'meaning' of the meaningful cluster.

Publications

Papers

1. Chiba, H., Michibata, H., Wakimoto, K., Seishima, M., Kawasaki, S., Okubo, K.,Mitsui, H., Torii, H. and Imai, Y. (2004). Cloning of a gene for a novel epithelium-specific cytosolic phospholipase A2, cPLA2delta, induced in psoriatic skin. J Biol Chem., **279**, 12890-12897.
2. Ogasawara, O., Kawamoto, S. and Okubo, K. (2003). Zipf's law and human transcriptomes: an explanation with an evolutionary model. C R Biol., **326**, 1097-1101.
3. Wakimoto, K., Chiba, H., Michibata, H., Seishima, M., Kawasaki, S., Okubo, K., Mitsui, H., Torii, H. and Imai, Y. (2003). A novel diacylglycerol acyltransferase (DGAT2) is decreased in human psoriatic skin and increased in diabetic mice. Biochemical and Biophysical Research Communications., **310**, 296-302.
4. Kawasaki, S., Kawamoto, S., Yokoi, N., Connon, C., Minesaki, Y., Kinoshita, S. and Okubo, K. (2003). Up-regulated gene expression in the conjunctival epithelium of patients with Sjogren's syndorome. Exp Eye Res., **77**, 17-26.
5. Kaimori, JY., Takenaka, M., Nakajima, H., Hamano, T., Horio, M., Sugaya, T., Ito, T., Hori, M., Okubo, K. and Imai, E. (2003). Induction of glia maturation factor-beta in proximal tubular cells leads to vulnerability to oxidative injury through the p38 pathway and changes in antioxidant enzyme activities. J. Biol. Chem., **278**, 33519-33527.

Books

6. Okubo, K. and Hishiki, T. (2003) Knowledge Discovery from the Human Transcriptome, In Introduction to Bioinformatics. Krawetz, SA. and Womble, D.D. ed. (Human Press).

SOCIAL CONTRIBUTIONS AND OTHERS

1. Databese: http://www.jbirc.aist.go.jp/hinv/h-angel
2. Databese: http://bodymap.ims.u-tokyo.ac.jp
3. ACL2003 (41st Annual Meeting of the Association for Computational Linguistics) Program committee
4. JST領域探索プログラム「ゲノムと言語」コオーガナイザー

J. RADIOISOTOPE CENTER

RESEARCH ACTIVITIES

(1) Identification of a cis-acting site for bipolar positioning of *oriC* on the *E. coli* chromosome

Yoshiharu Yamaichi, Katsunori Yata and Hironori Niki

We have already reveled subcellular localization of different DNA segments on the *E. coli* chromosome during the cell division cycle by using fluorescence in situ hybridization. These chromosome segments are located within the cell in the same order as a genetic map. A large chromosomal region including replication origin, *oriC* shows bipolar localization after replication, which we call the Ori domain. Then, if a cis-acting site is involved in bipolar positioning of *oriC*, the site will located within the Ori domain.

To identify the cis-acting site, we have constructed a new mutant, in which a circular chromosome was split into two circular chromosomes (Yamaichi and Niki, 2004). In the mutants, we were able to study an effect of a cis-acting segment on chromosome segregation. One of the chromosome-split mutants, in which 740 kb of the chromosome region [84.7-89.4 min] in the Ori domain is separated from the original chromosome, showed irregular localization of nucleoids and defective in bipolar migration of the chromosomal segment including *oriC*. These results suggest that the cis-acting site for bipolar positioning of *oriC* is located within this chromosomal segment. Furthermore, we have analyzed that nucleoid localization and *oriC* migration in a series of chromosome-split mutants, and deletion mutants. Finally, we identified the site that was responsible for bipolar migration of the *oriC* segment was a 25 bp sequence on 89.1 min of the *E. coli* map. In addition, multicopy of the sequence in cells affected on bipolar migration of the *oriC* segment on the chromosomes. A plasmid with the sequence was able to migrate from mid-cell to bipolar positions. We conclude that the 25 bp sequence with a stem-loop structure is involved in bipolar localization of *oriC*.

(2) Subcellular localization of *Escherichia coli* proteins in living cells

Toshiyuki Hatano, Yasuyuki Ogata and Hironori Niki

Escherichia coli genome has been fully sequenced and the genes of unknown function revealed. To estimate their function, we used a plasmid library, archive clone library, that expresses fusion genes containing open reading flame (ORF) of *E. coli* W3110 strain and green fluorescent protein (GFP) gene and we observed their subcellular localizations in the living *E. coli* cells. These proteins were classified into five categories: (i) at cell periphery, (ii) in whole cell, (iii) spot, (iv) filament, and (v) on nucleoid.

We subsequently investigated the localizations of the proteins of known function and found the correlations between subcellular localization and protein function. Most of the cell peripheral proteins have either trans membrane elements or signal peptides. Proteins with spots at cell quarter positions like a replication factory include replication machineries and recombination enzymes and proteins with several spots participate in transcription. Some proteins with a ring at mid cell are involved in cell division. Nucleoid proteins contain nucleoid constituents such as histone-like proteins and various enzymes involved in DNA-related events including DNA supercoiling, recombination, and repair.

We also found that the genes encoding the filamentous proteins contain a novel genes that contributes to cell division and the genes of unknown function.

(3) Stationary phase-induced illegitimate recombination in *Escherichia coli*

Yasuyuki Ogata and Hideo Ikeda (Institute of Molecular Genetics, Medinet Inc.)

In nature, bacterial cells are usually in stationary stages, in which the cells alter their genetic inheritance. Adaptive mutations are induced when *Escherichia coli* cells are exposed to conditions of stationary-phase starvation, resulting in a variety of genetic changes,

some of which allow the cells to survive under such conditions. Gene amplification of the *lac* operon of *E. coli* also occurs during stationary-phase starvation. Whether or not stationary-phase starvation induces chromosomal rearrangements has remained obscure.

We found that illegitimate recombination is induced when growth phase is changed from log to stationary phase. Illegitimate recombination occurred more frequently in early-stationary phase than in mid-log phase during formation of λ*bio*-transducing phage. Moreover, we found that illegitimate recombination increased in the *tag alkA1* double mutant in early-stationary phase, thereby implying that the alkylated lesion may be responsible for the stationary phase-induced illegitimate recombination.

When we did a quantitative analysis of deletion formation using a miniF-based plasmid, we found that the stationary phase-induced illegitimate recombination can be also detected in this assay system.

Since illegitimate recombination results in chromosomal rearrangements including deletion, duplication, insertion, or translocation, stationary phase may induce chromosomal rearrangements.

Publications

Paper

1. Yamaichi, Y. and Niki, H. (2004). *migS*, a cis-acting site that affects bipolar positioning of oriC on the Escherichia coli chromosome. *EMBO* J. **23**, 221−233, doi: 10.1038/sj.emboj.7600028 Published online 18 December 2003.

Reviews

2. 小方康至, 仁木宏典, 「バクテリアに於ける細胞周期に応じたタンパク質と核酸の動的な局在変化」生化学, 第75巻, 第2号, 2003年2月, 137-143.

3. Ikeda, H., Shiraishi, K. and Ogata, Y. (2004). Illegitimate recombination induced by double-strand break and end-joining. Adv. *Biophys.* in press.

EDUCATION

Dr. Y. Ogata gave a lecture at the University of Tokyo, Tokyo, June, 2003 (in Japanese).

K. EXPERIMENTAL FARM

RESEARCH ACTIVITIES

Experimaental Farm is responsible for preparation of all kinds of rice strains necessary for the studies of genetic resources and of functional genomics. All works in the Experimental Farm have been carried out as collaborative works with the Plant Genetics Lab. For details, see the reports of plant genetics lab. Only a preliminary topic in the wild rice research is shown here.

(1) Preparation of wild rice accessions for comparative genomics

Toshie Miyabayashi, Mitsugu Eiguchi and Nori Kurata

For making rice genetic stocks good resources, we developed core collection of wild rice. Out of 2,000 accessions composed of twenty-one wild rice species in nine genomes, several accessions from each species were selected to prepare core collection. About 300 selected lines had already sub-grouped to Rnak1, Rank2 and Rank3 categories and a part of them were characterized for their phenotypes to record them in the Oryzabase, a rice comprehensive database.

Comparative genomics among nine rice genomes will reveal important aspects on evolution and genetic diversity in rice. For finding out genome specific genes or far-related genes different from AA, comparison of BB and CC genome species with the cultivated AA species has been started. Searches for structural and functional diversity between genes of different genomes are performed by cDNA clone sequencing and by microarray expression profile analysis. Highly divergent genes between the genomes may explain the way of evolution and the gene function.

Publications and activities shown in the Plant Genetics Lab. were all done with the members of the Experimental Farm.

Publications

Papers

1. Miyoshi, K., Ito, Y., Serizawa, A. and Kurata, N. (2003). *OsHAP3* genes regulate chloroplast biogenesis in rice. *Plant J.* **36**, 532-540.

2. Miyoshi, K., Ahn, B-O., Kawakatsu, T., Ito, Y., Itoh, J-I., Nagato, Y. and Kurata, N. (2004). *PLASTOCHRON1*, a timekeeper of leaf initiation in rice, encodes cytochrome P450. *Proc. Natl. Acad. Sci. USA* **101**, 875-880.

3. Nonomura, K.I., Miyoshi, K., Eiguchi, M., Suzuki, T., Miyao, A., Hirochika, H. and Kurata, N. (2003). The *MSP1* gene is necessary to restrict the number of cells entering into male and female sporogenesis and to initiate anther wall formation in rice. *Plant Cell* **15**, 1728-1739.

4. Suzuki, T., Nonomura, K-I., Takeda, N., Murayama, Y. and Kurata, N. (2003). Application of enhanced lipofection to rice transformation. *Rice Genet, Newslet.* **20**, 116-118.

5. Nonomura, K.I., Nakano, M., Murata, K., Miyoshi, K., Eiguchi, M., Miyao, A., Hirochika, H. and Kurata, N. (2003). The insertional mutation of rice *PAIR2* gene, the ortholog of *Arabidopsis ASY1*, caused a defect in homologous chromosome pairing in meiosis. *Mol. Genet. Genomics*, **271**, 121-129.

6. Nonomura, K-I., Nakano, M., Fukuda, T., Eiguchi, M., Miyao, A., Hirochika, H. and Kurata, N. (2004). The novel gene *HOMOLOGOUS PAIRING ABERRATION IN RICE MEIOSIS1* of rice encodes a putative coiled-coil protein nequired for homologous chromosome pairing in meiosis. Plant Cell **16**, 1008-1020.

7. Ito, Y., Chujo, A., Eiguchi, M. and Kurata, N. (2004) Radial axis differentiation in a globular embryo is marked by HAZ, a PHD-finger homeobox gene of rice. *Gene* **331**, 9-15

8. Ito, Y., Eiguchi, M. and Kurata, N. Establishment of an enhancer trap system with Ds and GUS for functional genomics in rice. Mol. Genet. Genomics: in press.

Book

9. Kurata, N. and Fukui, K. (2003). Chromosome research in genus Oryza. '*Monograph in genus Oryza.*' Nanda JS ed. Science Publisher. pp 213-261.

L. TECHNICAL SECTION

The Technical section supports the research activity of NIG in many fields. For example, we support the Radioisotope center and Experimental farm, and the Genetic strain research center where genetic and transgenic strains of mice, rice, flies, and *E. coli* are produced, maintained, and distributed to other institutions. Members of the staff additionally support many types of experiments in various laboratories, such as developmental, cell, and molecular genetics. We continually update our technical services through attending the training and annual meetings for the technical officials.

Publications

Papers

1. Chihara, T., Kato, K., Taniguchi, M., Ng, J., and Hayashi, S. (2003). Rac promotes epithelial cell rearrangement during tracheal tubulogenesis in Drosophila. Development **130(7)**, 1419-1428.
2. Kato, M., Miura, A., Bender, J., Jacobsen, S.E., and Kakutani, T. (2003). Role of CG and non- CG methylation in immobilization of transposons in *Arabidopsis*. Current Biology **13,** 421-426.
3. Kinoshita, T., Miura, A., Choi, Y., Kinoshita, Y., Cao, X., Jacobsen, S.E., Fischer, R.L., and Kakutani, T. (online publication Nov. 20, 2003). One-way control of *FWA* imprinting in *Arabidopsis* endosperm by DNA methylation. Science, *in press.*
4. Miura, A., Kato, M., Watanabe, K., Kawabe, A., Kotani, H., and Kakutani, T. (online publication Nov. 8, 2003). Genomic localization of endogenous mobile CACTA family transposons in natural variants of *Arabidopsis thaliana.* MGG, *in press.*
5. Nonomura, K.I., Miyoshi, K., Eiguchi, M., Suzuki, T., Miyao, A., Hirochika, H., and Kurata, N. (2003). The *MSP1* gene is necessary to restrict the number of cells entering into male and female sporogenesis and to initiate anther wall formation in rice. Plant Cell **15,** 1728-1739.
6. Sugimura, K., Yamamoto, M., Niwa, R., Satoh, D., Goto, S., Taniguchi, M., Hayashi, S., and Uemura, T. (2003). Distinct developmental modes and lesion-induced reactions of dendrites of two classes of Drosophila sensory neurons. J. Neurosci. **23(9),** 3752-3760.
7. Takayama, Y., Kamimura, Y., Okawa, M., Muramatsu, S., Sugino, A., and Araki, H. (2003). GINS, a novel multiprotein complex required for chromosomal DNA replication in budding yeast. Genes Dev. **17(9),** 1153-1165.

BIOLOGICAL SYMPOSIUM 2003

Jan.7 バイオナノ磁性粒子を用いた遺伝子同定（Hideki Nakayama）
Jan.14 Segregation distortion caused by nuclear RanGAP activity in Drosophila（Ayumi Kusano）
Jan.15 細胞外因子と内在性プログラムによる神経幹細胞の分化制御（Kinichi Nakashima）
Jan.15 新技術融合が拓くDynamical Biology（Kenji Nagai）
Jan.15 生細胞におけるクロマチン転写機構の解明に向けて（Hiroshi Kimura）
Jan.17 巨大分子混みあい：蛋白質の安定性、折れたたみ、凝集への影響（理論）（Akira Kinjo）
Jan.20 知識の機械的利用によるゲノム科学データの統合と利用（Kousaku Okubo）
Jan.21 Molecular mechanism for signaling cytokinesis in animal cells（Maki Hori）
Jan.21 エンドソーム－ゴルジ膜動輸送系の生理機能とメカニズム（Masato Ohashi）
Jan.23 線虫C.elegansの神経系で機能するインスリン受容体相同遺伝子daf-2（Kotaro Kimura）
Jan.28 Germ/Soma Distinction in C.elegans.（Yingdee Unhavaithaya）
Jan.29 REGULATION OF p53-MEDIATED TRANSCRIPTION BY NONHISTONE CHROMATIN PROTEIN（Tapas K Kundu）
Jan.30 CDKによるDNA複製制御とゲノムの安定維持機構（Seiji Tanaka）
Feb.5 塩基除去DNA修復における蛋白質因子の相互作用の役割（Yoshihiro Matsumoto）
Feb.5 DNA損傷チェックポイントコントロールとそのDNA損傷認識機構（Katsunori Sugimoto）
Feb.5 Novel Y-family DNA polymerases and their roles in mutagenesis（Takehiko Noumi）
Feb.10 ES細胞の未分化性維持とECAT4（Kaoru Mitsui）
Feb.17 キイロショウジョウバエにおけるクチクラ炭化水素多型の分子機構とその進化（Aya Takahashi）
Feb.17 ハクサンハタザオを用いた分子集団遺伝学・細胞遺伝学的研究（Akira Kawabe）
Feb.19 Inductive Tissue Interactions: Looking through the eye into the heart（Yasuhide Furuta）
Feb.19 データベースからの知識抽出：情報圧縮の視点から（Ge Xijin）
Mar.5 Signature of balancing selection in Arabidopsis; Balancing selection and linkage disequilibrium of RPS5 presence/absence polymorphism in Arabidopsis thaliana（Hitoshi Araki）
Mar.6 "Gene Finding in DNA Sequences: from Viruses to High Eukaryotes"（Mark Borodovsky）
Mar.13 HAR1を介した共生と器官形成の全身的制御システム（Masayoshi Kawaguchi）
Mar.19 Study of DRB gene polymorphism in crab-eating monkey (Macaca fascicularis)（Antoine Blancher）
Mar.26 A Role for Ran-GTP and Crm1 in Blocking Re-Replication（Ryuji Yamaguchi）
Mar.27 Axon Guidance in the developing Xenopus visual system（Christine Holt）
Apr.23 細胞内メンブレントラフィックのダイナミクス（Akihiko Nakano）
Apr.25 細胞内小胞輸送系の構造生物学：ARFとの相互作用によるアダプタータンパク質GGAのTGN膜へのドッキング（Soichi Wakatsuki）
May.12 メダカ性決定遺伝子のポジショナルクローニング（Masaru Matsuda）
May.19 体節形成を支配する分子時計のメカニズム（Yasumasa Bessho）
May.26 生命機能に関する知識の抽出と表現（Toshihisa Takagi）
May.28 Xite, X-inactivation intergenic transcription elements that regulate the probability of choice（Yuya Ogawa）
May.28 M期キナーゼの機能と基質の探索（Katsuya Takenaka）
Jun.6 前立腺癌特異的抗原を利用したワクチン療法と、腫瘍特異的CTLの誘導による免疫遺伝子治療(Koichi Ito)
Jun.23 Building hearts: genetic perturbations, functional networks, and systems biology(Joseph H. Nedeau)
Jun.25 ショウジョウバエ精子形成におけるMyosin ＶＩの細胞内機能についての解析（Tatsuhiko Noguchi）
Jun.25 ゼブラフィッシュ網膜分化に関する突然変異体の単離と解析（Ichiro Masai）
Jul.10 細胞周期を制御するAurora-Aキナーゼの活性化機構（Takeshi Urano）
Jul.14 ユビキチンシステムの制御機構とその生理機能解析（Tomoki Chiba）
Jul.22 Targeted Expression of Temperature-Sensitive Dynamin to Study Neural Mechanisms of Complex Behavior in Drosophila（Toshihiro Kitamoto）
Jul.23 The Intimate Association of Transposable Elements and Genes（Thomas Bureau）

Aug.11 生物学としての構造生物学：構造・機能相関の解明に向けた試み（Hiroshi Ito）
Aug.19 細胞内共生菌の分子進化：進化速度上昇に伴うアミノ酸置換パターンの解析（Takeshi Ito）
Aug.22 ショウジョウバエ遺伝子量補正複合体の染色体認識機構（Yuji Kageyama）
Aug.25 感覚情報とcGMPシグナル経路による線虫の体長と行動の制御（Manabu Hujiwara）
Sep.1 Retargeting Retroviral Entry（Monica J. Roth）
Sep.10 SPB asymmetry and spindle positioning in yeast（Hiromi Maekawa）
Sep.10 DNA修復・複製に関与する分裂酵母*slr*遺伝子の単離とその解析（Yasuhiro Tsutsui）
Sep.24 The Nature Journals and the world of scientific publishing（Charles Jennings）
Sep.29 細胞内化学反応の揺らぎと細胞機能（Tatsuo Shibata）
Oct.1 Adaptive Mutation（Susan Rosenberg）
Oct.8 Microarray Applications in Developmental Toxicology（Thomas B. Knudsen）
Oct.9 Migrating cells can not only receive signals from their environment, but also deliver signals to it（Norbert Konig）
Oct.15 異常細胞を排除する方法としての非自律的細胞死（Takashi Adachi）
Oct.16 Epigenetic inheritance in mammals（Emma Whitelaw）
Oct.16 Sequence and analysis of the Dictyostelium genome（William F. Loomis）
Oct.20 MITOTIC MODIFICATION OF TOPOISOMERASEII BY SUMO-2（Yoshiaki Azuma）
Oct.22 Open reading frame categorization by cellular automata and genetic algorithms（Johannes Olson）
Oct.23 におい知覚のメカニズム：におい地図および行動実験からの視点（Naoshige Uchida）
Oct.23 Germline transgenesis of an ascidian *Ciona intestinalis* by a Tc1/*mariner* superfamily transposon *Minos*.（Yasunori Sasakura）
Oct.27 Genetic and genomic approaches to the Drosophila immune response（Bruno Lemaitre）
Oct.30 ゼブラフィッシュ雄生殖細胞培養系の確立とその培養系による精子ベクター（Noriyoshi Sakai）
Oct.30 ゼブラフィッシュを用いた脳内光受容体の解析（Daisuke Kojima）
Oct.31 細胞シグナリングの分子発生遺伝学（Atsushi Kawakami）
Oct.31 メダカを用いた変異体の体系的スクリーニングによる前後軸に沿った神経系の形態形成機構の解析（Makoto Furutani-Seiki）
Nov.5 脊椎動物の中枢神経系の進化；無顎類ヤツメウナギを用いた分子発生学的解析（Yasunori Murakami）
Nov.6 Individual variation in aggression of feral rodent strains: a standard for the genetics of aggression and violence?（Jaap M. Koolhaas）
Nov.7 Molecular mechanisms controlling the integrity of replicating chromosomes（Marco Foiani）
Nov.12 ショウジョウバエ神経系を用いた細胞機能の微細形態学的解析（Emiko Suzuki）
Nov.13 Redox regulation of germline and vulval development in Caenorhabditis elegans（Yukimasa Shibata）
Nov.13 Molecular mechanisms specifying muscle-identity and myotube number in the Drosophila adult（K. Vijay Raghavan）
Nov.20 Environmental factors, DNA at-risk motifs and genetic defects that severely challenge genome duplication.（Dmitry A. Gordenin）
Nov.26 Insulin signaling and growth control in Drosophila（Ernst Hafen）
Nov.28 DNA Replication Control and Genome Stability（John F.X. Diffley）
Dec.2 Natural Selection and Random Drift in Genome Evolution（Giorgio Bernardi）
Dec.2 C.elegansの個体の大きさの制御機構の解析（Yasumi Ohshima）
Dec.4 Controlling the events of the cell cycle（Tim Hunt）
Dec.5 転写因子Mybの標的遺伝子の同定と転写制御機構の解析（Masahiro Okada）
Dec.8 Studying the Cell Cycle by Functional Proteomics（Karim Labib）
Dec.15 Methylation crosstalk between histones and DNA in Neurospora（Hisashi Tamaru）
Dec.16 Forward Genetics of Vision-dependent Behaviors in Zebrafish（Akira Muto）
Dec.17 Transposable elements, RNA interference and the origin of heterochromatin（Robert Martienssen）
Dec.18 Sleeping Beauty: Evolution, Regulation and Genetic Applications of DNA Transposons in Vertebrates（Zsuzsanna Izsvak）

FOREIGN VISITORS IN 2003

Jan.21	Maki Hori	Department of Physiology, University of Massachusetts Medical School
Jan.28	Yingdee Unhavaithaya	Department of Cell Biology, University of Massachusetts Medical School,Worcester
Jan.29	Tapas K Kundu	Transcription and Disease Laboratory Jawaharlal Nehru Centre for Advanced Scientific Research ,Bangalore:560064 INDIA.
Jan.30	Seiji Tanaka	Cancer Reserach U.K.
Feb.5	Yoshihiro Matsumoto	Department of Pharmacology, Fox Chase Cancer Center,Philadelphia, PA, USA
Feb.19	Yasuhide Furuta	Department of Biochemistry & Molecular Biology University of Texas, M.D. Anderson Cancer Center, Houston, TX, USA
Mar.5	Hitoshi Araki	Department of Ecology and Evolution, University of Chicago
Mar.6	Mark Borodovsky	Schools of Biology and Biomedical Engineering, Georgia Institute of Technology, Atlanta, Georgia, USA
Mar.19	Antoine Blancher	Laboratoire d'Immunogenetique Moleculaire, Universite Paul Sabatier, Hopital Rangueil FRANCE
Mar.26	Ryuji Yamaguchi	University of California San Diego
Mar.27	Christine Holt	Department of Anatomy, University of Cambridge
May.28	Yuya Ogawa	Department of Molecular Biology,Massachusetts General Hospital,Harvard Medical School
Jun.23	Joseph H. Nedeau	Case Western Reserve University, Cleveland OH, USA
Jun.25	Tatsuhiko Noguchi	Miller lab, Department of Biology, Washington university in St. Louis, MO, USA
Jul.22	Toshihiro Kitamoto	Department of Anesthesia, Roy J. and Lucille A. Carver College of Medicine,University of Iowa
Jul.23	Thomas Bureau	Department of Biology, McGill University
Sep.1	Monica J. Roth	Department of Biochemistry & Molecular Biology, University of Medicine and Dentistry of New Jersey, Robert Wood Johnson Medical School, USA
Sep.10	Hiromi Maekawa	Paterson Institute for Cancer Research, UK
Sep.24	Charles Jennings	Executive Editor, Nature Research Journals
Oct.1	Susan Rosenberg	Department of Molecular and Human Genetics Baylor College of Medicine
Oct.8	Thomas B. Knudsen	Department of Pathology, Anatomy and Cell Biology, Thomas Jefferson University
Oct.9	Norbert Konig	EPHE and INSERM, University of Montpellier 2, France.
Oct.16	Emma Whitelaw	Department of Biochemistry, University of Sydney, Australia
Oct.16	William F. Loomis	Division of Biological Sciences, UCSD
Oct.20	Yoshiaki Azuma	LGRD/NICHD, USA
Oct.23	Naoshige Uchida	Cold Spring Harbor Laboratory
Oct.27	Bruno Lemaitre	Centre de Genetique Moleculaire, CNRS
Oct.30	Daisuke Kojima	The Biological Laboratories, Harvard University
Nov.6	Jaap M. Koolhaas	Department of Animal Physiology, Biological Center, University of Groningen, The Netherlands
Nov.7	Marco Foiani	F.I.R.C. Institute of Molecular Oncology, Milan, Italy
Nov.13	K. Vijay Raghavan	National Centre for Biological Sciences, Tata Institute of Fundamental Research
Nov.26	Ernst Hafen	Zoological Institute, University of Zurich
Nov.28	John F.X. Diffley	Cancer Research UK, London Research Institute, Clare Hall Laboratories

Dec.2	Giorgio Bernardi	President, Stazione Zoologica Anton Dohrn, Italy
Dec.4	Tim Hunt	Cancer Research UK London Research Institute Clare Hall Laboratories
Dec.8	Karim Labib	Cancer Research U.K.,Paterson Institute for Cancer Research
Dec.15	Hisashi Tamaru	University of Oregon, USA
Dec.16	Akira Muto	Department of Physiology, University of California, San Francisco
Dec.17	Robert Martienssen	Cold Spring Harbor Laboratory
Dec.18	Zsuzsanna Izsvak	Max Delbruck Center for Molecular Medicine, Berlin, Germany

Annual Report of the National Institute of Genetics. No.54

Issued by Director-General : Yoshiki Hotta, D.Med.
Research Organization of Information and Systems
National Institute of Genetics
Yata 1111 Mishima, Shizuoka-Ken, 411-8540 Japan
Tel. 055-981-6718
Fax. 055-981-6715
Edited by Ryu Ueda & Hironori Niki
Published by National Institute of Genetics