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GENERAL STATEMENT

Our institute was established 46 years ago as a center for comprehensive genetics research. Outstanding accomplishments in the advancement of research in genetics, in particular population genetics, plant genetics and molecular genetics, by members of our institute have made it the premier center for genetic study in our country and a major institution with world-wide recognition. In 1984 the institute was reorganized into a National Inter-university Research Institute to promote research activities through cooperation with other institutions. Together with seven inter-university research institutes, we formed the Graduate University for Advanced Studies, in 1988. Our institute admits graduate students to the Department of Genetics of the Graduate School of Life Science. We have 32 such students at present and in addition, about ten special research students from other universities, including those from foreign countries. I consider it vital for our research activities to have a steady flow of young scientists.

Recent rapid progress in the field of genetics using newly developed approaches has greatly shifted the course of study in biology. I consider it natural that research in our institute has recently focused more on molecular studies of various aspects of genetics, without ignoring the importance of more traditional approaches to genetics. Our institute is uniquely suited for pursuing cooperative work with scientists of various disciplines. Through interactions among various research disciplines, our institute will flourish.

We have been carrying out several research related services. The DNA Data Bank of Japan (DDBJ) is one of the three central banks in the world that gather, annotate, store and distribute information on DNA sequences. Our institute also stores and distributes various organisms with genetically characterized traits. Among them, services with mice, rice and *Escherichia coli* are particularly significant. These service activities will continue to develop in the coming years.

I hope that with guidance from people in and outside this institute and further support from governmental and private sources, I will be able to lead the institute into a more successful future.

In the past year, we have had an important shift in the organization according to the demands of the times. Separated from the DNA Research

Center, laboratories concerned with information biology were set up in a new Center for Information Biology which consists of four laboratories. The past year also saw a number of changes in the staff of the institute. Promoted as professor, Yoshio Sano was transferred to the Hokkaido University, Fumio Tajima to the University of Tokyo, and Tetsuya Toyoda to the Kurume University, respectively, and Masahiro Yamagishi to the Aichi Cancer Center Research Institute as senior researcher. The following eight people joined us: as professor, Tomoko Ogawa in the Division of Cytogenetics, Ken Nishikawa in the newly established Laboratory for Gene-Product Informatics; as associate professor, Yukiko Yamazaki in the Genetic Resources Laboratory, Yasuo Shirakihara in the DNA Synthesis Laboratory. Shigeo Tanaka joined the Division of Cytogenetics, Tsuyoshi Koide the Mammalian Genetics Laboratory, Satoshi Goto the Invertebrate Genetics Laboratory, Yukihiro Ito the Plant Genetics Laboratory.

It is a pleasure to note that the following three were honored with awards: Takashi Gojobori by the Kihara Memorial Yokohama Foundation for the Advancement of Life Sciences, Naruya Saitou by the Genetics Society of Japan and Nobuo Shimamoto by the Industry Applications Society of the U.S.A. Jun-ichi Tomizawa was elected a foreign associate of the National Academy of Sciences of the U.S.A.

Junichi Tomizawa

STAFF (as of December 31, 1995)

Director-General

TOMIZAWA, Jun-ichi, Ph. D.

Vice-Director

SENO, Takeshi, D. Sc.

Members

1. *Department of Molecular Genetics*

ISHIHAMA, Akira, D. Sc., Head of the Department

Division of Molecular Genetics

ISHIHAMA, Akira, D. Sc., Professor

FUJITA, Nobuyuki, D. Sc.

TOYODA, Tetsuya, D. Med.

Division of Mutagenesis

SENO TAKESHI, D. Sc., Professor

YAMAO, Fumiaki, D. Sc., Associate professor

KISHI, Tsutomu, D. Eng.

SEINO, Hiroaki, D. Sc.

Division of Nucleic Acid Chemistry (Guest member)

MORIKAWA, Kosuki, D. Sc., Professor

YAMAGISHI, Masahiro, D. Sc.

2. *Department of Cell Genetics*

HORIUCHI, Kensuke, D. Sc., Head of the Department

Division of Cytogenetics

OGAWA, Tomoko, Ph. D., Professor

IMAI, Hirotami, T., D. Sc., Associate professor

TANAKA, Shigeo, D. Med.

GOTO, Hideo, D. Ag.

Division of Microbial Genetics

HORIUCHI, Kensuke, D. Sc., Professor

YASUDA, Seiichi, D. Sc., Associate professor

HARA, Hiroshi, D. Sc.

HIGASHITANI, Atsushi, D. Sc.

Division of Cytoplasmic Genetics (Guest member)

OHTSUBO, Eiichi, D. Sc., Professor

YAMAMURA, Ken-ichi, D. Med., Professor

3. Department of Ontogenetics

SUGIYAMA, Tsutomu, Ph. D., Head of the Department

Division of Developmental Genetics

SUGIYAMA, Tsutomu, Ph. D., Professor

FUJISAWA, Toshitaka, Ph. D., Associate professor

SHIMIZU, Hiroshi, D. Eng.

HATTA, Masayuki, D. Sc.

Division of Phenogenetics

HIROSE, Susumu, D. Sc., Professor

MURAKAMI, Akio, D. Ag., Sc., Associate professor

UEDA, Hitoshi, D. Ag.

YAMADA, Masa-aki, D. Sc.

MINATO, Kiyoshi, M. Sc.

Division of Physiological Genetics (Guest member)

HANDA, Hiroshi, D. Med., Professor

OKUMURA, Katsuzumi, D. Ag., Associate professor

4. Department of Population Genetics

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

Division of Population Genetics

OHTA, Tomoko, Ph. D., D. Sc., Professor

TAKANO, Toshiyuki, D. Sc.

INA, Yasuo, D. Sc.

Division of Evolutionary Genetics

IKEMURA, Toshimichi, D. Sc., Professor

SAITOU, Naruya, Ph. D., D. Sc. Associate professor

MATSUMOTO, Ken-ichi, D. Ag.

TENZEN, Toyoaki, D. Ag.

Division of Theoretical Genetics (Guest member)

TAKAHATA, Naoyuki, D. Sc., Professor

TACHIDA, Hidenori, D. Sc., Associate professor

5. *Department of Integrated Genetics*

IMAMURA, Takashi, D. Med., Head of the Department

Division of Human Genetics

IMAMURA, Takashi, D. Med., Professor

FUJIYAMA, Asao, D. Sc., Associate professor

HORAI, Satoshi, D. Med., Associate professor

IZUHARA, Kenji, D. Med.

Division of Agricultural Genetics

MORISHIMA, Hiroko, D. Ag., Professor

HIRANO, Hiro-Yuki, D. Ag.

Division of Applied Genetics (Guest member)

WATANABE, Takeshi, D. Med., Professor

SHIMAMOTO, Yoshiya, D. Ag., Professor

6. *Genetic Stock Research Center*

NAKATSUJI, Norio, D. Sc., Head of the Center

Mammalian Genetics Laboratory

SHIROISHI, Toshihiko, D. Sc., Associate professor

Invertebrate Genetics Laboratory

HAYASHI, Shigeo, D. Sc., Associate professor

GOTO, Satoshi, D. Sc.

Plant Genetics Laboratory

ITO, Yukihiro, D. Ag.

Microbial Genetics Laboratory

NISHIMURA, Akiko, D. Ag., Associate professor

KANAMARU, Kengo, D. Ag.

Genetic Resources Laboratory

YAMAZAKI, Yukiko, D. Sc., Associate professor

FUJITA, Masaya, D. Eng.

Mammalian Development Laboratory

NAKATSUJI, Norio, D. Sc., Professor

SHIRAYOSHI, Yasuaki, D. Sc.

*7. **DNA Research Center***

KATSURA, Isao, D. Sc., Head of the Center

DNA-Protein Interaction Laboratory

SHIMAMOTO, Nobuo, D. Sc., Associate professor

NAGAI, Hiroki, D. Sc.

Recombinant DNA Laboratory

KATSURA, Isao, D. Sc., Professor

ISHIHARA, Takeshi, D. Sc.

DNA Synthesis Laboratory

SHIRAKIHARA, Yasuo, D. Sc., Associate professor

Gene Library Laboratory

KOHARA, Yuji, D. Sc., Associate professor

ANDACHI, Yoshiki, D. Sc.

*8. **Center for Information Biology***

GOJOBORI, Takashi, D. Sc., Head of the Center

DNA Data Analysis Laboratory

GOJOBORI, Takashi, D. Sc., Professor

IKEO, Kazuho, D. Sc.

IMANISHI, Tadashi, D. Sc.

Laboratory of Gene Function Research

TATENO, Yoshio, Ph. D., D. Sc., Professor

Laboratory for Gene-Product Informatics

NISHIKAWA, Ken, D. Sc., Professor

9. ***Radioisotope Center***

SADAIE, Yoshito, D. Sc., Associate professor, Head of the Center

10. ***Experimental Farm***

MORISHIMA, Hiroko, D. Ag., Head of the Farm

11. ***Technical Section***

MITA, Akihiko, Chief of the Section

12. ***Department of Administration***

KONO, Kenji, Head of the Department

SATO, Shigeru, Chief of the General Affairs Section

TAMURA, Mituo, Chief of the Finance Section

COUNCIL (as of December 31, 1995)

Chairman

NAGAKURA, Saburo; Director, Kanagawa Academy of Science and Technology Foundation

Vice-chairman

SUGANO, Haruo; Director emeritus, Cancer Institute

Members (Alphabetical order)

HAMAI, Osamu; Professor, Graduate School of Humanities and Sociology. The University of Tokyo

HIRASA, Takao; President, Osaka Prefectural University

KATO, Nobuo; President, Nagoya University

KYOGOKU, Yoshimasa; Professor, Institute for Protein Research, Osaka University

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MIYAMOTO, Misako; President, Nihon Women's University

MOHRI, Hideo; Director-General, National Institute for Basic Biology

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OSAKI, Hitoshi; Director, Japan Society for the Promotion of Science

OSAWA, Shozo; Adviser, Biohistory Research Hall

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TAKANAMI, Mitsuru; Director, Kazusa DNA Research Institute

TAKEUCHI, Ikuo; President, Okazaki National Research Institutes

TANAKA, Ryuso; President, Hiroshima City University

TOYOSHIMA, Kumao; President, The Center for Adult Diseases, Osaka

YAMAGATA, Hirotada; Professor, Faculty of Bioscience and Technology, Kinki University

ADVISORY COMMITTEE (as of December 31, 1995)

Chairman

SENO, Takeshi; Professor, Division of Mutagenesis

Vice-chairman

OKADA, Masukichi; Professor emeritus, University of Tsukuba

Outside Members (Alphabetical order)

GO, Mitiko; Professor, Faculty of Science, Nagoya University

HANAOKA, Fumio; Professor, Institute for Molecular and Cellular Biology, Osaka University

HINATA, Kokichi; Professor, Faculty of Agriculture, Tohoku University

HOTTA, Yasuo; Professor, Graduate School of Biological Sciences, Nara Institute of Science and Technology

ISHIWA, Sadao; Professor, Faculty of Science, Ochanomizu Women's University

ISONO, Katsumi; Professor, Faculty of Science, Kobe University

OISHI, Michio; Director-General, National Institute of Bioscience and Human-Technology

TAKAGI, Nobuo; Professor, Graduate School of Environmental Science, Hokkaido University

TAKEBE, Hiraku; Professor, Faculty of Medicine, Kyoto University

Inside Members (Alphabetical order)

GOJOBORI, Takashi; Professor, Center for Information Biology

HIROSE, Susumu; Professor, Division of Phenogenetics

HORIUCHI, Kensuke; Professor, Division of Microbial Genetics

IKEMURA, Toshimichi; Professor, Division of Evolutionary Genetics

IMAMURA, Takashi; Professor, Division of Human Genetics

ISHIHAMA, Akira; Professor, Division of Molecular Genetics

MORISHIMA, Hiroko; Professor, Division of Agricultural Genetics

NAKATSUJI, Norio; Professor, Genetic Stock Research Center

OHTA, Tomoko; Professor, Division of Population Genetics

SUGIYAMA, Tsutomu; Professor, Division of Developmental Genetics

PROJECTS OF RESEARCH FOR 1995

1. DEPARTMENT OF MOLECULAR GENETICS

Division of Molecular Genetics

Regulatory mechanisms of gene transcription in prokaryotes (ISHIHAMA and FUJITA)
Molecular architecture of transcription apparatus from eukaryotes (ISHIHAMA)
Molecular mechanisms of transcription and replication of animal and plant viruses (ISHIHAMA and TOYODA)

Division of Mutagenesis

Multiple role of ubiquitin system in the cell-cycle regulation (SENO, YAMAO, KANEDA, KISHI and SEINO)

Division of Nucleic Acid Chemistry

Structural genetics: X-ray crystallography of proteins (MORIKAWA)
Isolation and characterization of yeast mRNA capping enzyme mutants (YAMAGISHI)

2. DEPARTMENT OF CELL GENETICS

Division of Cytogenetics

Mechanisms of genetic recombination (OGAWA)
Theoretical and experimental bases for chromosome evolution (IMAI)
Chromatin structure involved in recombination (TANAKA and OGAWA)

Division of Microbial Genetics

DNA replication in *E. coli* (YASUDA, HIGASHITANI and HORIUCHI)
Cell cycle regulation in *E. coli* (HARA, HIGASHITANI and HORIUCHI)

Division of Cytoplasmic Genetics

Studies on bacterial plasmids and transposons (OHTSUBO)
Molecular mechanisms of mammalian development and diseases (YAMAMURA)

3. DEPARTMENT OF ONTOGENETICS

Division of Developmental Genetics

Genetic and molecular analysis of developmental mechanisms in hydra (SUGIYAMA, FUJISAWA, SHIMIZU and HATTA)

Division of Phenogenetics

Gene expression in eukaryotes (HIROSE and UEDA)
Genetic studies on the life history characters in *Bombyx* (MURAKAMI)
Genetic studies on the nerve system characters in *Bombyx* (MURAKAMI)
Genetic studies on development and growth of insect (YAMADA and MINATO)

Division of Physiological Genetics

Studies on DNA replication timing (OKUMURA)
Systematic studies on neuropeptides (MUNEOKA)

4. DEPARTMENT OF POPULATION GENETICS

Division of Population Genetics

Theoretical studies of population genetics (OHTA and INA)
Theoretical studies on the evolution of multigene family (OHTA)
Theory of gene genealogy (INA)
Statistics for DNA polymorphisms (INA)
Experimental population genetics on DNA polymorphism and evolution in *Drosophila* (TAKANO)
Genetic studies on interspecific variation in *Drosophila* (TAKANO)

Division of Evolutionary Genetics

Studies on codon usage (IKEMURA)
Studies on chromosome band structures at the DNA sequence level (IKEMURA and TENZEN)
Studies on genes in the MHC region (IKEMURA and MATSUMOTO)
Studies on functions of extracellular matrix proteins (MATSUMOTO and IKEMURA)
Studies on DNA replication of human genome (TENZEN and IKEMURA)
Molecular evolutionary analysis of nucleotide sequence data (SAITOU)
Studies on the genetic affinity of human populations (SAITOU)

Division of Theoretical Genetics

Population immunogenetics (TAKAHATA)
Molecular anthropology (TAKAHATA)
Theoretical studies of population genetics (TACHIDA)

5. DEPARTMENT OF INTEGRATED GENETICS

Division of Human Genetics

Genetic and physical mapping of human genome (IMAMURA and IZUHARA)
Molecular genetics of human metabolic disorders (IMAMURA and IZUHARA)

Molecular biology of oncogenes (FUJIYAMA)
Studies on DNA polymorphisms in human populations (HORAI)

Division of Agricultural Genetics

Evolutionary and ecological genetics in wild and cultivated rice species (MORISHIMA)
Studies on plant gene expression (HIRANO and SANO)

Division of Applied Genetics

Molecular genetics of human genome imprinting mechanisms (SASAKI)
Preservation mechanisms of genetic diversity in plant populations (SHIMAMOTO)

6. GENETIC STOCK RESEARCH CENTER

Mammalian Genetics Laboratory

Recombinational hotspots in the mouse MHC (SHIROISHI and MORIWAKI)
Genetic Mechanisms for regulating tumor development in the laboratory and wild mice (MIYASHITA and MORIWAKI)

Invertebrate Genetics Laboratory

Molecular genetics of *Drosophila* melanogaster (HAYASHI and GOTO)

Plant Genetics Laboratory

Molecular genetics of rice (ITO)

Microbial Genetics Laboratory

Timing of cell division in *E. coli* (NISHIMURA)
Division apparatus of *E. coli* (NISHIMURA)
Environmental adaptation in *E. coli* (KANAMARU)

Genetic Resources Laboratory

Compilation of genetic resources information (SAITO and YAMAZAKI)

Mammalian Development Laboratory

Developmental mechanisms and manipulation of germ cells in mouse embryos (NAKATSUJI and SHIRAYOSHI)
Molecular analysis of cell differentiation and morphogenesis in postimplantation mouse embryos (SHIRAYOSHI and NAKATSUJI)
Cell differentiation and morphogenesis of the mouse central nervous system (NAKATSUJI and SHIRAYOSHI)

7. DNA RESEARCH CENTER

DNA-Protein Inateraction Laboratory

Nanobiology of DNA-protein interaction (SHIMAMOTO and NAGAI)

Recombinant DNA Laboratory

Genetics and molecular genetics of development and behavior of *Caenorhabditis elegans* (KATSURA and ISHIHARA)

DNA Synthesis Laboratory

Crystallographic study of supramolecules and transcription factors (SHIRAKIHARA)

Gene Library Laboratory

Molecular genetics of *Caenorhabditics elegans* development (KOHARA and ANDACHI)
Genome analysis of *Caenorhabditis elegans* (KOHARA)

8. CENTER FOR INFORMATION BIOLOGY

DNA Data Analysis Laboratory

Molecular evolution of pathogenic viruses (GOJOBORI and IKEO)
Molecular evolution of mosaic proteins (IKEO and GOJOBORI)
Human evolution based on polymorphisms in MHC genes (IMANISHI and GOJOBORI)
Genetic information analysis by using DNA databases (GOJOBORI, IKEO and IMANISHI)

Laboratory of Gene Function Research

Molecular phylogenetic analysis of DNA and protein sequence data (TATENO)

Laboratory for Gene-Product Informatics

Computational analysis and prediction of protein three-dimensional structure (NISHIKAWA)

9. RADIOISOTOPE CENTER

Molecular mechanisms of sporulation in *Bacillus subtilis* (SADAIE and FUJITA)

10. EXPERIMENTAL FARM

RESEARCH ACTIVITIES IN 1995

A. DEPARTMENT OF MOLECULAR GENETICS

A-a. Division of Molecular Genetics

(1) Functional Map of RNA Polymerase Alpha Subunit from *Escherichia coli*: Subunit-Subunit Contact Sites

Makoto KIMURA[1] and Akira ISHIHAMA ([1]Present address: Rockefeller University, New York)

The RNA polymerase core enzyme of *Escherichia coli* with a subunit composition of $\alpha_2\beta\beta'$ is assembled in the sequence: $\alpha \rightarrow \alpha_2 \rightarrow \alpha_2\beta \rightarrow \alpha_2\beta\beta'$. Dimerization of the α subunit is the first step in this assembly pathway and thus the α subunit plays a key role in RNA polymerase formation. The α subunit also plays an important role in molecular interactions with both a group of positive factors, which we defined as class I factors, and DNA UP (or upstream activation) elements.

Using the *in vitro* reconstitution system of RNA polymerase from deletion mutant α, we identified that the minimum fragment of α with the enzyme assembly activity is α(21-235) consisting of a region from residues 21 to 235 [wild-type α subunit contains 329 residues], while a carboxy-terminal region of α up to amino acid 235 is involved in the transcription activation. For fine mapping of the subunit-subunit contact sites, we made eleven species of α-insertion mutants, each containing an extra alanine-serine (AS) dipeptide at 20 amino acid intervals within this domain. From the results of subunit assembly *in vitro* for these α mutants (Kimura and Ishihama, 1995a), we proposed the functional map of subunit assembly domain on α as follows: α–α contact involves multiple sites; α–β contact site is located near residue 80; and β' assembly involves the site(s) near residues 80 and 180–200.

For detailed mapping, Ala was substituted for 26 highly conserved amino acids around residues 40, 80 and 170 to 210. The α-point mutants were also analyzed *in vitro* for their abilities to form dimers and to assemble $\beta\beta'$

subunits (Kimura and Ishihama, 1995b). New types of assembly-defective mutants were identified: α-R45A dimerized but did not assemble β (and β') subunits; and α-L48A showed a decreased level of $\alpha_2\beta$ subassembly formation, indicating that this region (residues 45 to 48) is responsible for β-binding. Isolation of two mutants, α-K86A and α-V173A, both forming $\alpha_2\beta$ but not $\alpha_2\beta\beta$ complex, confirmed our previous conclusion that two separated regions participate in β'-binding.

This year, we started the analysis of the subunit assembly *in vivo.* At first, we analyzed eleven species of AS (alanine-serine) dipeptide-insertion and four species of amino-terminal deletion mutants of α. In order to detect the subunit assemblies *in vivo*, we added hexa-histidine (H_6) tag to wild-type and mutant α, expressed H_6-tagged α, and isolated subunit complexes by Ni^{2+}-affinity chromatography. The assembly properties of α mutants agreed well with those observed *in vitro*, except that some mutants which showed decreased assembly *in vitro* were assembled into core and holoenzymes *in vivo*, suggesting that a specific condition(s) or factor(s) supports the efficient assembly *in vivo.*

These mutant α were also tested for their ability to complement two temperature-sensitive (*ts*) alleles, *i.e.*, assembly-defective *rpoA*112 and function-defective *rpoA*101. The *in vivo* results generally supported the functional map of α amino-terminal assembly domain previously established based on the *in vitro* studies. Most assembly-competent α mutants complemented two temperature-sensitive mutant alleles of *rpoA*, but the mutants carrying mutations at the extreme amino-terminal region and the mutant with a mutation at residue 60 failed to complement these *ts* mutants, suggesting that these assembly-competent but complementation-negative α mutants lack as yet unidentified function(s). With subunit complexes containing some assembly-competent/complementation-negative mutants and all the assembly-defective mutants was associated DnaK (hsp70) protein, suggesting that molecular chaperones are involved in RNA polymerase assembly.

(2) Functional Map of RNA Polymerase Alpha Subunit from *Escherichia coli*: Transcription Factor- and DNA UP Element-Contact Sites

Katsuhiko MURAKAMI, Tomofumi NEGISHI, Nobuyuki FUJITA and Akira ISHIHAMA

Escherichia coli RNA polymerase holoenzyme is composed of four different subunit ($\alpha_2\beta\beta'\sigma$). The α subunit, consisting of 329 amino acid residues, plays a key role in RNA polymerase assembly, of which as many as 94 carboxy (C)-terminal amino acid residues can be removed without preventing the formation of RNA polymerase complexes. On the other hand, the C-terminal proximal region is needed for transcription regulation by interaction with not only a group of transcription activators such as cyclic AMP receptor protein (CRP) (Murakami *et al.*, 1996), OxyR (Tao *et al.*, 1995), SoxS (Jair *et al.*, 1995), MarR (Jair *et al.*, 1996a), Rob (Jair *et al.*, 1996b) and OmpR, but also transcription repressor GalR (Choy *et al.*, 1995) [transcription factors which require the C-terminal domain of RNA polymerase α subunit, directly or indirectly, for action are classified into class-I factors]. The C-terminal proximal region also plays a role in the recognition of promoter upstream (UP) elements, which consist of (A+T)-rich sequences, with enhancing activity of transcription. Mutational mapping revealed that the major contact site for CRP on α subunit (CRP contact site-I) is located within a narrow region between amino acid residues 258 to 270. This region is also required for contact with OxyR (Tao *et al.*, 1995).

In order to investigate the role of each amino acid around this region (residues 258 to 275) and the secondary OxyR-contact region (residues 297 to 298), we carried out systematic mutagenesis to make α derivatives, each carrying a single amino acid (either Trp or Ala) substitution from 258 to 275, and from 297 to 298. The mutant α subunit proteins were overexpressed, purified and reconstituted into mutant RNA polymerases. The abilities of the mutant RNA polymerases to respond to CRP and the UP element were examined in transcription reactions *in vitro* initiated at the cAMP/CRP-dependent *lac*P1 and UP element-dependent *rrnB*P1 promoters. The mutations affecting CRP response were located on the surface of contact helix and moreover most of these mutatios also influenced the response to the *rrnB* UP element. Since Arg-265 was found to play a major role in response to both

CRP and UP, we also made another set of mutant RNA polymerases by replacing Arg-265 with amino acids other than Trp and Ala, and examined their responses to CRP and UP. From the location of mutations affecting CRP and UP responses on the three dimensional structure of α subunit C-terminal domain (Jeon *et al.*, 1995), we propose that the CRP contact surface is also involved in contact with the DNA UP element, although some amino acid residues within this region play different roles in molecular communication with CRP and the UP element.

The contact between CRP and αCTD was competitively inhibited by adding peptides with the sequence present on the CRP contact surface of α (Negishi *et al.*, in preparation). The competitive inhibition assay with synthetic peptides would be a useful method for identification of the location of transcription factor contact sites on RNA polymerase and the role of each amino acid in the molecular communication. Judged by DNase I footprinting analysis, the α mutants defective in transcription from the CRP-dependent *lac*P1 promoter showed decreased activity in the cooperative binding of CRP. Likewise, the mutants defective in *rrnB*P1 transcription showed decreased binding to the UP element. The amino acid residues important for the protein and DNA contact are conserved in the α subunits of not only bacterial but also chloroplast RNA polymerases.

(3) Structural Map of RNA Polymerase Alpha Subunit from *Escherichia coli*:

Tomofumi NEGISHI, Young Ho JEON[1], Masahiro SHIRAKAWA[2], Toshio YAMAZAKI[1], Katsuhiko MURAKAMI, Nobuyuki FUJITA, Olga OZOLINE[3], Yoshimasa KYOGOKU[1] and Akria ISHIHAMA ([1] Oaka University, Institute for Protein Research, [2] Nara Institute of Science and Technology, Graduate School of Bioscience, [3] On leave of absence from Institute of Cell Biophysics, Pushchino, Russia)

RNA polymerase of *E. coli* alters its promoter selectivity after interaction with transcription protein factors. One of the regions responsible for transcription regulation by transcription factors has been localized to the C-terminal domain of α subunit. This group of proteins requiring the C-terminal domain of α is designated as class-I factors. Fine mapping of the contact sites for CRP, OxyR and other class-I factors is being carried out by

using mutant α with single amino acid substitutions (Murakami *et al.*, 1996; Tao *et al.*, 1995). Limited proteolysis suggested that both N-terminl and C-terminal regions form independent compact globular domains, each being connected by a protease-sensitive and presumably fragile linker (Negishi *et al.*, 1995).

The structure of the C-terminal domain of α (αCTD), as determined by nuclear magnetic resonance (NMR) spectroscopy, is compactly folded and contains four helices and two long arms enclosing its hydrophobic core (Jeon *et al.*, 1995). Most of the mutations affecting activation by CRP and UP element are located on the surface of helix 1, indicating that the hydrophilic surface of helix 1 is involved in direct contact with the protein and DNA factors. The folding topology is distinct from those of other DNA-binding proteins. The UP element-biding sites as determined by monitoring the selective signal loose by NMR agreed well with the site of mutations affecting UP element response. In order to directly observe the molecular interactions of α with class-I transcription factors and DNA UP elements on this surface, we succeded to set up a condition to modify α by binding a fluorescent dye only at Cys269 within helix 1. The spectroscopic studies are being carried out for detection of protein-protein or protein-DNA contacts using the modified α subunits.

(4) Regulation and Specificity of RNA Polymerase Sigma Subunits from *Escherichia coli*: Intracellular Levels of Various Sigma Subunits

Miki JISHAGE, Shuichi KUSANO, Akira IWATA[1], Susumu UEDA[1] and Akira ISHIHAMA ([1] Nippon Institute of Biological Science, Ohme, Tokyo)

In order to adapt to such various environments as the mammalian gut, fresh and sea water and soil, bacteria carry sophysticated systems sensing the changes in the nutrient availability, osmolarity, temperature, and other external factors, and responding by turning on and off the specific sets of stress response genes. For the global changes in gene expression pattern, the RNA polymerase is considered to play a key role by rapidly modulating its promoter selectivity. One major mechanism of the promoter selectivity control of RNA polymerase is the replacement of σ subunit [the promoter recognition factor] on core enzyme, which alone is unable to initiate trans-

cription from promoter even though it carries the catalytic function of RNA synthesis. Up to the present time, seven different molecular species of σ subunit have been identified in *Escherichia coli*. The model of σ replacement relies on the change in the intracellular concentration of individual σ subunits, because the level of core enzyme stays almost at a constant level. However, little is known about the intracellular level of each σ subunit under various conditions. We then measured the intracellular levels of various σ subunits in *E. coli* at various growth phases. For this purpose, we prepared mono-specific antibodies against each σ subunit purified from over-expressed *E. coli* cells and determined the σ subunits in two *E. coli* strains, MC4100 and W3110, by a quantitative Western blot method.

The intracellular levels of the major σ subunit, σ^{70} (σ^{D}, the *rpoD* gene product) in *E. coli* MC4100 is maintained at 50–80 fmol of per μg of total protein throughout the growth transition from the exponential growth to the stationary phase, while the level of σ^{38} protein (σ^{S}, the *rpoS* gene product) is below the detection level at the exponential growth phase but increases to 30% the level of σ^{70} when the cell growth stops to enter into the stationary phase. Besides the stationary phase, the increase in σ^{38} level was observed in two cases: exposure to heat shock at the exponential phase; and osmotic shock at the stationary phase.

(5) Regulation and Specificity of RNA Polymerase Sigma Subunits from *Escherichia coli*: Promoter Selectivity of Various Sigma Subunits

Shuichi KUSANO, Tapas Kumar KUNDU, Quingquam DING[1], Kan TANAKA[2], Annie KOLB[3], Nobuyuki FUJITA and Akira ISHIHAMA ([1] On leave of absence from Wuhan Institute of Virology, Wuhan, China, [2] University of Tokyo, Institute of Molecular and Cellular Biology, [3] Pasteur Institut, Paris)

In *E. coli*, the total number of RNA polymerase core enzyme is fixed at a level characteristic of the rate of cell growth, which ranges from 1,000 to 3,000 molecules per genome equivalent of DNA. The levels of individual σ subunits vary depending on the cell growth conditions, but the total number of σ subunits is less than that of core enzyme (Jishage and Ishihama, 1995). On the other hand, the total number of genes on the *E. coli* genome is estimated to be about 4,000. Thus, competition must take place between

promoters for binding a small number of RNA polymerase. Among about 4,000 genes on the *Escherichia coli* genome, about 1,000 genes are expressed at various levels in exponentially growing cells under laboratory culture conditions, *i.e.*, at 37°C and with aeration. The rest of genes is considered to be expressed under various stress conditions that *E. coli* meets in nature.

Transcription of at least some of these stationary-specific genes is catalyzed by RNA polymerase holoenzyme containing σ^{38} (σ^{S}; the *rpoS* gene product). Promoters from the stationary-specific genes, however, do not have a single consensus sequence (Kolb *et al.*, 1995; Tanaka *et al.*, 1995). However, we found that transcription of the σ^{S}-dependent genes by RNA polymerase containing σ^{S} requires a specific condition(s) such as high concentrations of potassium glutamate for transcription of the osmo-regulated genes. This finding indicates that each stationary-specific promoter carries a specific sequence which is recognized by $E\sigma^{S}$ under a specific reaction condition and that the promoter sequences recognized by $E\sigma^{S}$ differ between gene groups sharing the same requirements. Our effort has since been focussed to identify specific conditions or factors required for transcription of each stationary-specific gene by $E\sigma^{S}$ holoenzyme and, as an extension along this line, to understand the molecular mechanism(s) of σ replacement under various growth conditions.

Along this line, we anlyzed the effect of trehalose on $E\sigma^{S}$ and $E\sigma^{70}$ holoenzymes. Trehalose is known to accumulate in stationary-phase cells of various microoganisms. Results indicated that high concentrations of trehalose specifically stimulate transcription by $E\sigma^{S}$. Likewise, we analyzed the effect of DNA superhelicity on the promoter recognition by $E\sigma^{38}$ holoenzyme. DNA superhelicity is known to change depending on the cell growth conditions. For instance, nutrient downshift and stationary growth-phase cause a decrease in the DNA superhelical density while high osmolarity leads to an increase in the superhelicity. The selectivity for stationary phase-specific promoters by $E\sigma^{38}$ increases concomitantly with the decrease in DNA superhelicity, and that the effects of decreased DNA superhelicity and high potassium glutamate concentrations are additive in enhancing the selectivity for $E\sigma^{38}$. The transcription by $E\sigma^{38}$ was, however, enhanced with the use of templates with low superhelical density, in good agreement with the decrease in DNA superhelicity in the stationary growth phase (Kusano and Ishihama, 1996). We thus propose that the selective transcription of stationary-specific genes by $E\sigma^{38}$ holoenzyme requires either a specific reaction condition(s) or

a specific factor(s). The same line of studies is being carried out for other holoenzymes containing other σ subunits.

(6) Subunit Composition of RNA Polymerase II from the Fission Yeast *Schizosaccharomyces pombe*: Subunit Composition

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Knowledge of the structure and function of RNA polymerase II is essential for understanding the molecular mechanism of transcription regulation in eukaryotes. At present, however, little is known about the molecular architecture of RNA polymerase II. Purified RNA polymerase II from yeasts, animals and plants all contain more than 10 polypeptides, but it has not been determined yet whether or not all these polypeptides are essential for RNA polymerase functions. One way to address the question whether all these subunits are necessary for the RNA polymerase functions is to develop an *in vitro* reconstitution system of RNA polymerase from isolated individual subunits and to analyze the functions of complexes reconstituted from different combinations of isolated subunits. For this ultimate goal, we started the cloning and sequencing of the *Schizosaccharomyces pombe* genes coding for the proteins associated with purified RNA polymerase II. Judging from the co-elution profiles in column chromatographies along the RNA polymerase activity and the two large subunits [subunit 1 (prokaryotic β' homologue) and subunit 2 (β homologue)], the minimum number of *S. pombe* RNA polymerase II-associated components was estimated to be 10, less than the subunit number (12) of *Saccharomyces cerevisiae* enzyme. Among these 10 putative subunits, the *S. cerevisiae* counterparts have been identified for nine. Up to the present time, we cloned and sequenced the genes for subunits 1, 2, 3 and 5.

(7) Subunit Function of RNA Polymerase II from the Fission Yeast *Schizosaccharomyces pombe*: Subunit 2, 3 and 5

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The genes coding for the two large subunits of RNA polymerase II have been cloned from a number of organisms. Sequence analysis indicated that subunits 1 and 2 exhibit notable homology with the β' and β subunits, respectively, of prokaryotic RNA polymerases. The functional roles of these two large subunits of eukaryotic RNA polymerases can now be predicted from the knowledge of the subunit functions of the well-studied *Escherichia coli* RNA polymerase: the catalytic site is located on β, while β' exhibits non-specific DNA binding activity. Within the subunit 2 sequence, there is a conserved sequence for barnase, an extracelluar ribonuclease from gram-positive bacteria. In order to identify possible function(s) associated with this domain, we expressed a short polypeptide including this barnase domain and examined activities for degrading RNA. So far, however, we failed to detect activities of RNA degradation, suggesting that this domain does not carry the RNase activity (Kawagishi-Kobayashi *et al.*, 1995).

On the other hand, the gene for subunit 3 has been cloned from *Saccharomyces cerevisiae*, *Tetrahymena thermophila*, and human. We also cloned the subunit 3 gene from *Schizosaccharomyces pombe*. Sequence analysis indicated that subunit 3 is a eukaryotic homologue of the prokaryotic RNA polymerase α subunit, of which the amino-terminal proximal domain plays a key role in RNA polymerase assembly by providing the protein-protein contact surfaces for α dimerization and for interaction with the two large subunits, β and β'. On other hand, the carboxy-terminal region of the prokaryotic α subunit provides the contact surfaces for class-I transcription factors, and DNA UP elements or prokaryotic enhancers. As judged from the sequence comparison, the subunit 3 of eukaryotic RNA polymerase II appears to lack this transcription activation domain. For the identification of subunit 3 function (s), we started to isolate temperature-sensitive mutants with mutations in the *rpb3* gene encoding subunit 3. We analyzed in details thermolabile RNA polymerase II from temperature-sensitive mutants of *S. pombe* with muta-

tions in the *rpb3* gene. Subunit α of prokaryotic RNA polymerases plays key roles in protein-protein contacts for both subunit assembly and transcription activation. To gain an insight into the roles of subunit 3, the eukaryotic homologue of α, temperature-sensitive mutants of *S. pombe* have been isolated after transformation of the mutagenized *rpb3* gene (Yasui *et al.*, 1995). A total of 68 *ts* mutants were classified into two groups: mutants comprising one group ceased growing immediately after a temperature up-shift, while mutants comprising the other group exhibited delayed growth arrest at high temperatures. RNA polymerase II partially purified from Ts54, one of the group 2 mutants, was thermolabile *in vitro*, as measured by a non-specific transcription assay. This mutant carries double mutations in domain A of subunit 3, and thus can be used as a reference mutant of RNA polymerase II.

(8) Subunit Function of Influenza Virus RNA Polymerase: Cross-linking Studies of Nucleotides

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Influenza virus RNA-dependent RNA polymerase plays an essential role in both transcription and replication of the viral genome. In transcription, the RNA polymerase cleaves cap-1(m^7GpppNm)-containing RNA of host cells at 10–13 nucleotides from 5′ cap structure, transcribes viral RNA (vRNA) by elongating the resulting capped fragments until nucleotides 17 to 22 from 5′ end of vRNA, and there adds multiple A residues to nascent RNA chains, altogether leading to formation of viral mRNA with both 5′-cap structure and 3′-poly(A) tail. The RNA polymerase, in conjunction with an yet unidentified host factor(s), is also involved in two step reactions of RNA replication, *i.e.*, the synthesis of full-sized complementary RNA (cRNA) without these modifications and the cRNA-directed synthesis of vRNA.

The influenza virus RNA polymerase consists of three virus-encoded proteins, PB1, PB2 and PA. Knowledge of the function of each subunit is still limited. Ultraviolet (UV) light-induced crosslinking studies indicated that PB2 is the cap 1-recognition protein, while PB1 can be UV-crosslinked to 3′ terminal nucleotides of elongating mRNA chains, indicating that PB1 is

the subunit involved in the catalytic activity of nucleotide polymerization. Both PB1 and PB2 can be crosslinked to synthetic RNA with the 3′ terminal sequence of vRNA. Thus, these two basic subunits may also be involved in recognition of transcription promoter and/or replication origin on template vRNA. In contrast, no specific function has been identified for PA protein. In order to get insight into the function of each subunit of influenza virus RNA polymerase and to make the functional map of each subunit polypeptide, we are trying to set up an efficient reconstitution system of RNA polymerase from isolated individual P proteins and to analyze functions of reconstituted mutant RNA polymerases.

In parallel, we carried out photoaffinity labeling of nucleotide-binding sites on influenza virus RNA polymerase with azido-substituted nucleotides. 8-Azidopurines are widely used for monitoring nucleotide-binding sites on proteins at the level of primary sequence. Upon exposure to a low dose of UV light, the azide moiety of these nucleotide analogues is converted to a highly reactive nitrene. If the analogue is associated at or near the active site of an enzyme at the time of UV exposure, the nitrene forms a covalent bond with most amino acids within the crosslinking distance. Once crosslinked these reagents can be used for isolation of active site peptides. Thus the site specificity imparted by the nucleotide, coupled with indiscriminate bond formation of the nitrene, permits tagging of the enzyme active site with regard to which amino acids are involved in forming the nucleotide-binding domain. This year, we used 8-azido guanosine 5′-triphosphate (8-N_3 GTP), an azido analogue of GTP, because GTP is the first substrate that is added to capped RNA primers in transcription initiation by the influenza virus RNA polymerase. Results demonstrated that: i) 8-N_3 GTP serves as a substrate for RNA synthesis by the influenza virus RNA polymerase; ii) the K_m value for the azido analogue of GTP in primer-dependent RNA synthesis was 94 μM whereas K_m for the natural substrate, GTP, was 6.7 μM; iii) 8-azido GTP (8-N_3 GTP) can be polymerized into RNA at about one tenth the rate of GTP incorporation; iv) 8-azido GTP is specifically photo-crosslinked to the PB1 subunit. These biochemical data provide a conclusive evidence supporting the prediction that the nucleotide binding site is located on the PB1 protein. In the presence of ApG primer, the 8-N_3 GTP binding was reduced to about 40% level, suggesting that the GTP analogue can bind not only to the substrate site (S site) but also to the primer- and product-binding site (P site).

(9) Subunit Function of Influenza Virus RNA Polymerase: RNA Synthesis by Subunits and Subassemblies

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Influenza virus contains eight single-stranded RNA segments of negative polarity as the genome and an RNA-dependent RNA polymerase as a virion component. After virus infection, the RNA polymerase participates in both transcription [the synthesis of plus-strand mRNA containing host cell-derived cap structure at 5′-terminus and poly(A) tail at 3′-terminus] and replication [the synthesis of full-length plus-strand complementary RNA (cRNA) and the cRNA-dependent synthesis of minus-strand viral RNA (vRNA)]. The viral RNA polymerase is composed of one molecule each of the three viral coded P proteins, PB1, PB2 and PA. Genetic and biochemical studies suggested that: PB1 is involved in polymerization of RNA chains; and PB2 is involved in recognition of host cell capped RNA. The role of PA is not clear yet, but temperature-sensitive mutations in the PA gene affect only vRNA synthesis, but not mRNA synthesis. In order to identify the minimal and essential viral component(s) required for transcription and replication, we analyzed *in vitro* activities of model template-directed RNA synthesis associated with nuclear extracts from cells infected with each of the recombinant baculoviruses or various combinations of these viruses (Kobayashi *et al.*, 1995). The nuclear extract of cells expressing all three P proteins catalyzed model template-directed RNA synthesis in the absence of primers (an indication of RNA replication), supporting the notion that the complete set of three P proteins is required for RNA replication. All the nuclear extracts containing the PB1 subunit, including the extract containing PB1 alone, were able to catalyze model template-directed dinucleotide-primed RNA synthesis (an indication of transcription).

In parallel, we also examined the transcription and replication activities of nuclear extracts of HeLa cells infected with one of the recombinant vaccinia viruses, each carrying cDNA for one of the three P proteins, or in various combinations of these viruses (Toyoda *et al.*, 1996). Results demonstrate that the complete replication, *i.e.*, vRNA→cRNA→vRNA, takes place in the extract containing the complete set of RNA polymerase subunits. Again, the

nuclear extract containing PB1 alone was found to catalyze primer-dependent RNA synthesis. Taken together, we conclude that PB1 is the catalytic subunit of RNA polymerase and propose that under certain conditions, PB1 alone is able to express the catalytic activity *in vitro.*

(10) Subunit Function of Influenza Virus RNA Polymerase: Mapping of Subunit-subunit Contact Sites

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Influenza virus RNA polyfmerase is composed of three subunits, PB1, PB2 and PA, all encoded by the viral genome. The P protein complex is able to catalyze transcription, as defined by the requirement of primers and vRNA templates (but not cRNA templates). For replication, an additional factor(s) present in infected cells is needed. Transfection experiments by recombinant viruses carrying one of the three P subunits indicated that the nuclear extracts containing PB1 alone is able to catalyze transcription (Kobayashi *et al.*, 1995; Toyoda *et al.*, 1996). However, reconstitution experiments indicate that none of the P proteins alone is able to catalyze transcription *in vitro* but that active enzymes are formed only by mixing all three P proteins. Moreover, the efficiency of reconstitution from isolated individual subunits is still at low level. In order to clarify the subunit assembly mechanism of influenza virus RNA polymerase, we made a series of deletion mutants for each P protein. Three combinations of P proteins, *i.e.*, PB1–PB2, PB1–PA and PB2–PA, were expressed simultaneouly in the same cells. Binary P protein complexes were immunoprecipitated with use of mono-specific antibodies again each P protein. Results indicated that the binary complexes are formed between PB1–PB2 and PB1–PA but not PB2–PA. Thus, PB1 is the core subunit for RNA polymerase assembly.

Using N-terminal and C-terminal deletion mutants of each P protein, we further identified the subunit-subunit contact sites on each P protein. Results indicate that the C-terminal 158 amino acids of PB1 associates with the N-terminal 249 stretch of PB2, while the N-terminal 140 amino acids of PB1 binds to the C-terminal region of PA.

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A–b. Division of Mutagenesis

(1) Abnormal Integrity of Nucleolus Associated with Cell Cycle Arrest Owing to the Temperature-Sensitive Ubiquitin-Activating Enzyme E1

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A mouse cell mutant, ts85 with the *temperature-sensitive* ubiquitin-activating enzyme E1, was arrested mainly at the G2 phase at a non-permissive temperature. In the arrested cells, Azure C, a nucleolar specific stain, revealed a U-shaped or ring-shaped arrangement of nucleolar lobes with an unstained region in the center. Silver staining of the nucleolar organizer region (NOR) and fluorescence in situ hybridization (FISH) with rDNA, both gave signals in azure C-positive regions. The extent of nucleolar abnormality was more severe when the mutant cells were synchronized in the G2 phase before the temperature shift-up. The Azure C-negative region of abnormal nucleoli coincided with a cloud of unidentified electron-dense particles (diameter, 70 nm) which grew in number following the temperature shift-up. The region also coincided with strong fluorescence signals detected by immuno-histochemical staining for polyubiquitin, proteasomes and inducible HSP70, but not HSP90, DNA topoisomerase I and so on. Using immuno-electron microscopy with anti-polyubiquitin, the gold particles were coincided with the 70 nm particles. However, it was not clear whether the signals represented a free form of polyubiquitin or ubiquitinated protein. Under the canonical heat-shock conditions, such a coincidence of signals for proteasomes and HSP70 in nucleoli was also detected for wild-type cells. When the arrested ts85 cells were released into the M phase, we observed a

frequent occurrence of persistent nucleolar materials in prophase and prometaphase chromosomes that resulted in a peculiar bouquet-like figure of metaphase-chromosomes in which regions of the nucleolar organizer seemed to have failed in condensation and separation as supported by FISH with rDNA and NOR-silver staining. Stable transformation of ts85 by a cloned mouse E1 cDNA recovered the mutant phenotypes. It appears the ubiquitin system plays a role in the dissolution processes of the nucleolus during G2–M transition, possibly involving a specific protein(s) that is degraded. For details, see ref. 1.

(2) Decrease in Intracellular Deoxyribonucleoside Triphosphate Pools Associated with Arrest in DNA Replication and Defective DNA Repair in a Mouse Cell Mutant tsFS20 with Thermolabile Ubiquitin-Activating Enzyme E1

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A mouse cell mutant, tsFS20, with the thermolabile ubiquitin-activating enzyme E1 is arrested in the S-phase of the cell-cycle with concomitant loss of survival when cultured at non-permissive temperature. The arrest was accompanied by inhibition of DNA replication, but not in total RNA and protein synthesis. When the arrested cells were permeabilized and incubated with an extracellular supply of dNTPs, DNA synthesis occurred normally. We found that the intracellular dTTP pool was specifically decreased in the arrested mutant cells. They showed increased sensitivity to hydroxyurea, a specific inhibitor of ribonucleotide reductase. tsFS20 cells also showed an increased sensitivity to cell kill by N-methyl-N′-nitro-N-nitrosoguanidine. However, this defect in DNA repair was not accompanied by increase in an induced mutation, the phenotype being reminiscent of *Saccharomyces cerevisiae rad6*, a mutant of the ubiquitin-conjugating enzyme UBC2. Hence, in addition to the regulation of DNA replication at the level of ribonucleotide reduction, DNA repair and induced mutation fixation may also be controlled by the ubiquitin system in this mammalian cell mutant. All of the phenotypes were corrected concomitantly by stable introduction of mouse E1 cDNA to the mutant. (Paper in preparation).

(3) Cell Cycle-Dependent Phosphorylation and Nuclear Targeting of Ubiquitin-Activating Enzyme, E1, in Mammalian Cells

Hiroaki SEINO, Yukio NAGAI, Takeshi SENO and Fumiaki YAMAO

The ubiquitin-activating enzyme E1 was found to be phosphorylated in cells of a mouse mammary carcinoma cell line, FM3A(2). Peptide mapping of trypsin digests of labeled E1 indicated that two oligopeptides were mainly phosphorylated *in vivo.* The same oligopeptides were also labeled *in vitro* on Cdc2 kinase-mediated phosphorylation of E1 which was affinity-purified from the same cell line. The phosphorylation of one of the two oligopeptides was prominent at the G2/M phase of the cell cycle, and dependent upon Cdc2 kinase activity *in vivo* since it was significantly reduced in tsFT210, a mutant cell line deficient in Cdc2 kinase. Mutation analysis indicated that the serine residue at the fourth position of the E1 enzyme was a phosphorylation site of Cdc2 kinase. A tentative nuclear targeting signal, KKRR, was found to link with the Cdc2 kinase target site, which implied the coupled function of Cdc2-mediated phosphorylation and the KKRR signal to localize E1 in the nucleus in a cell cycle-dependent fashion. In fact, the truncated E1 protein which was tagged with a FLAG sequence and transiently expressed in Cos7 cells, was found, by indirect immunofluorescent method with anti-FLAG, to be localized in nucleus, while the Ala4 mutant derivative, an unphosphorylatable form, was not. These are now being confirmed using synchronized cells that was stably transformed with E1 derivatives.

(4) Screening for cDNAs Encoding the Ubiquitin-Conjugating Enzyme in Fission Yeast, *Schizosaccharomyces pombe*

Fumio OSAKA, Hiroaki SEINO, Takeshi SENO and Fumiaki YAMAO

We isolated cDNAs for ubiquitin-conjugating enzymes (Ubc) of *S. pombe.* Among more than 20000 clones, twenty were found to encode proteins with Ubc-like activity, and subsequent sequence analysis identified four types of genes. The bacterially expressed proteins formed thiolester bonds with activated ubiquitin *in vitro* in the presence of E1 enzyme. An Ubc domain structure was found in their sequences, including a cysteine residue forming a thiolester bond with ubiquitin. Thus, we concluded that these four were *ubc* genes and designated them, *ubcP1*, *ubcP2*, *ubcP3* and *ubcP4.* *ubcP1* and

ubcP2, predominantly isolated by the screening, were found to encode the homologue of *Ubc4* and *Ubc2/Rad6*, respectively, in budding yeast, *Saccharomyces cerevisiae.* The other two, *ubcP3* and *ubcP4*, were novel ubc genes. *ubcP4* was found to be essential for growth, and arrested at two points, G2 and metaphase, of the cell cycle when its expression was abolished. The metaphase arrest resembled to that by *cut9* mutant cells. *cut9* has been shown to encode a subunit of ubiquitin-ligase complex (designated as the anaphase promoting complex, APC, or cyclosome) which is required for cyclin B degradation during the exit from mitosis. This strongly suggested that the UbcP4 mediated ubiquitin pathway specifically required at the onset of anaphase for ubiquitin-mediated degradation of both cyclin B and an as yet unknown protein necessary for sister chromatid pairing during metaphase.

(5) Regulation of G1/S transition by the Ubiquitin System in Yeast *Saccharamyces cerevisiae*

Tsutomu KISHI, Motoyasu UI and Takeshi SENO

The phenotypes of various cell division cycle mutants of *Saccharomyces cerevisiae* suggest a complex array of interactions among the corresponding gene products leading to DNA replication and other early functions of the cell cycle. Cells bearing cdc4, cdc34 and cdc53 mutations execute the Start function, which initiates the processes that lead ultimately to cell division, but fail to enter the S phase. Various specific functions including bud emergence and spindle pole body duplication take place in these mutants, but fail to proceed to bud maturation, formation of bipolar spindle or DNA replication. Molecular characterization of CDC34 has revealed that it codes for a ubiquitin conjugating enzyme, which catalyzes the addition of ubiquitin to the substrates and promotes degradation via a ubiquitin dependent proteolysis pathway. One of the substrates was suggested as the B-type cyclin dependent kinase inhibitor Sic1, since deletion of SIC1 allowed cdc34 cells to perform DNA replication at restriction temperatures. However, it did not suppress the lethality of the cdc34 mutation, suggesting that Cdc34 may have an other essential function in addition to promoting Sic1 degradation. We wished to identify mutations that rescue the temperature sensitivity of the cdc 34Δsic1 strain, in the hope of identifying genes acting downstream of the CDC34 (containing genes of proteins to be ubiquitinated by Cdc34). Twen-

ty-one mutants that were viable at restriction temperatures in the cdc34Δsic1 background were isolated. They were composed of three complementation groups. One group showed cold-sensitivity and the suppressor gene was cloned by complementation. DNA sequence analysis revealed that the suppressor gene was identical to GRR1. GRR1 is implicated in the regulation of glucose uptake, glucose repression, divalent cation transport, and the turn-over of G1 cyclins. We are now comparing the stability of G1 cyclins between the cdc34Δsic1 strain and its suppressor strain.

(6) Human Ubiquitin Pathway Homologous to that Mediated by Ubc P4 in Fission Yeast

Hiroaki SEINO, Fumio OHSAKA, Takesi SENO, Fumiaki YAMAO

Ubc P4, an E2 family gene isolated from fission yeast is involved in the regulation of G2/M and metaphase anaphase transition in the cell cycle (Ohsaka *et al.*, in preparation). We isolated the cDNA homologous to this gene in human and designated it *hubcP4*. The predicted amino acid sequence of *hubcP4* was highly homologous to fission yeast UbcP4 (identity 50%, similarity 80%). This suggested that the biological function of the ubiquitin pathway mediated by UbcP4 is conserved between yeast and human. Now, we are examining whether hUbcP4 cDNA is functional in fission yeast cells. Furthermore, we will investigate the biochemical function of the hUbcP4 in a mammalian culture cell line or Xenopus egg extract which are more suitable for biochemical experiments.

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A–c. Division of Nucleic Acid Chemistry

(1) Crystal Structure Determination of the *Escherichia coli* Replication Terminator Protein (Tus) Complexed with *Ter* DNA

Kosuke MORIKAWA[1] ([1] Biomolecular Engineering Research Institute)

Replication is terminated at defined sequences in the DNA of prokaryotic chromosomes. In *Escherichia coli*, this termination is mediated by a site-specific DNA-binding protein designated as Tus. The Tus protein (mw: 36 kDa) is a monomeric molecule that binds six specific sequences (*Ter*) within the replication terminus region, and it prevents the passage of DnaB helicase, which is a constituent of replication machinery. Knowledge of the three-dimensional (3D) structure at the atomic level is required to gain insight into the mechanism by which the Tus protein recognizes the *Ter* sites and blocks the replication fork in a polar manner.

The crystal structure has been determined at 2.7 A resolution by the multiple isomorphous replacement method and anomalous scattering (MIRAS), and has been refined to provide R-factor = 17.0%.

The Tus protein has a very unique structure as compared with other proteins. The protein embraces the *Ter* element from both sides of the DNA with two protruded α-helical domains. The most remarkable feature of the complex is an extensive protein-DNA interface which involves strikingly many direct and indirect polar interactions. Two β-sheets are responsible for the recognition of the DNA in the major groove. This structure motif is somewhat similar to the complexes of MetJ and Arc, although the β-sheets lies flat against the groove. Except for a slight bend and unwinding of the DNA in the vicinity of this β sheets region, the *Ter* site DNA in the complex deviates to a small but significant extent from the canonical B-form DNA.

A genetic approach allowed the isolation of the mutant Tus proteins. Most of these single mutants are mapped on the β-sheet region in the 3D-structure. These mutants that affect the efficiency of replication arrest probably impair the binding of the Tus protein with DNA. This suggests that replication arrest and Tus-DNA binding might be inseparable. This interpretation appears to be consistent with the collision action of Tus against DNA-

translocating and unwinding proteins.

(2) Yeast mRNA Capping Enzyme Mutants: Isolation of Temperature-Sensitive Mutations and Characterization of the Altered Enzymes

Masahiro YAMAGISHI, Kiyohisa MIZUMOTO[1] and Akira ISHIHAMA ([1] Kitasato University)

Eukaryotic mRNA is capped at the 5′ end by the addition of a guanylyl residue *via* a 5′–5′ triphosphate bridge followed by methylation to form an $m^7G(5')ppp(5')N$ structure. The cap structure is required for several important steps of gene expression; *i.e.*, mRNA stabilization, splicing, mRNA export from the nucleus, and initiation of translation. The mechanisms by which the cap structure functions in these steps have not yet been well characterized. One of the important features of capping is that the $m^7G(5')ppp(5')N$ end formation in the nucleus is specific to RNA transcribed by RNA polymerase II, although isolated capping enzyme is capable of adding caps to other RNA species *in vitro*. The mechanism that underlies the highly specific and efficient capping of polymerase II-transcribed RNA is also unknown.

Genetic and biochemical analyses of yeast capping enzyme will provide insights not only into the catalytic and structural properties of the enzyme but also into the regulation of mRNA capping and molecular mechanisms involved in the functions of the cap structure. The yeast capping enzyme is composed of α and β subunits possessing guanylyltransferase and triphoshatase activities, respectively. In this study, we isolated 10 recessive temperature-sensitive mutations of the *CEG1* gene encoding the α subunit and determined the mutation sites. Nine of the mutations (*ceg1-1* to *ceg1-9*) were isolated on a single-copy plasmid and the remaining one (*ceg1-10*) on a multi-copy plasmid. The presence of *ceg1-10* in multiple copies is essential for the viability of cells carrying the mutation, and a shift to a restrictive temperature resulted in quick growth arrest of *ceg1-10* cells while other mutants decreased growth rates gradually upon the temperature up-shift. Intragenic complementation was not observed for pairwise combinations of the mutations. Guanylyltransferase activity was examined for each mutant protein by assaying a covalent Ceg1p–GMP complex formation. While most

of the mutant proteins showed clear heat-lability, Cegl–8p maintained substantial activity during incubation at a high temperature. Detailed characterization of the *cegl* mutant cells, including investigation of synthesis, accumulation and subcellular localization of mRNA, remains to be done. In addition, isolation and analysis of extragenic suppressors will help us understand the regulation of mRNA capping and the functions of the cap structure in gene expression pathway, from transcription in the nucleus to translation in the cytoplasm.

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B. DEPARTMENT OF CELL GENETICS

B–a. Division of Cytogenetics

(1) A Species-specific Interaction of Rad51 and Rad52 proteins in Eukaryotes

Tomoko OGAWA, Akira SHINOHARA and Tomoatsu IKEYA

The properties of the *rad51* and *rad52* mutants in *Saccharomyces* cerevisiae are very similar to the *recA* mutant in *Escherichia coli.* The wide distribution of the recA-like *RAD51* and *RAD52* homologs in a variety of organisms suggests the presence of similar fundamental mechanisms of recombination in all living cells. Although the protein structure of Rad51 homologs are highly conserved (about 80%), the human *RAD51* gene did not complement the *rad51* deletion mutant of *S. cerevisiae.* In the case of Rad52 homologs, only one third of the N-terminal region was conserved. We found that the N-terminal region of the Rad51 protein interacts with the C-terminal region of the Rad52 protein and the interaction is species-specific. This species-specificity probably plays an important role in the functioning of these proteins in meiotic recombination and repair of damaged DNA. Ref. 1.

(2) Homologous Recombination and The Roles of Double-strand Breaks

Akira SHINOHARA and Tomoko OGAWA

Double-strand breaks (DSBs) and single-strand gaps in damaged DNA are efficiently repaired by mechanisms associated with recombination. Recombination is a series of complex biological reactions, requiring at least 20 gene products, even in *Escherichia coli.* Genes homologous to bacterial and yeast recombination genes have been cloned in higher eukaryotes, suggesting there might be a common fundamental mechanism of recombination among a wide variety of species. In eukaryotes, protein-protein interactions play important roles in recombination: by interacting with a specific protein(s), the complex involved in repair of DSBs is modified to carry out specialized cellular

functions, such as meiotic recombination and switching of mating types in yeast. Ref. 2.

(3) Localization of RecA-like Recombination Protein on Chromosomes of The Lily at Various Meiotic Stages

Masahiro TERASAWA, Akira SHINOHARA, Tomoko OGAWA

The Rad51 and Lim15 proteins of lily, which are homologs of the bacterial RecA protein, were found on chromosomes in various stages of meiotic prophase 1. The presence of both Rad51 and Lim15 proteins as discrete foci on leptotene and zygotene chromosomes and their colocalization suggest that meiotic recombination begins at the leptotene stage with the cooperation of these proteins and continues in the zygotene stage. Localization of the foci on or adjacent to the chromosomes suggests that these proteins bind to the chromatin loops that extend from the axial cores. The protein in these foci may participate in the searching and pairing of homologous DNA sequences, as the RecA protein does. The different pattern of localization of the Rad51 protein between the leptotene and pachytene stages and the absence of the Lim15 protein in the pachytene stage suggest that the Rad51 protein plays different roles in these stages. Ref. 3.

(4) Localization of Mouse Rad51 and Lim15 on Meiotic Chromosomes in Late Stages of Prophase 1

Tomoatsu IKEYA, Akira SHINOHARA, and Tomoko OGAWA

Studies on the localization of Rad51 and Lim15 proteins on meiotic chromosomes of lily showed that these proteins participate in searching for homologous chromosomes, which is initiated in the leptotene stage and lead to pairing in the zygotene stage. However, the roles of these proteins in chiasma formation and chromosomal exchanges are not known. Taking advantage of the much shorter length of mouse chromosomes which facilitates the examination of an entire chromosome with a chiasma region through optical methods, we studied the localization of Rad51 and Lim15 homologues on mouse chromosomes.

In the chromosomes of mouse testis, during the late stages of meiotic

prophase 1, the Rad51 and Lim15 proteins were present in the core regions of the chromosomes which remained after removal of the chromatin by DNase II. Rad51 located along the core of the synaptonemal complexes (SC) in the pachytene stage and in the chiasma regions in the diplotene stage. The protein was not present on separated homologous cores in the diplotene stage. On the other hand, the Lim15 protein was present almost exclusively at both ends of the core of chromosomes from the pachytene to diplotene stages and even in diakinesis. Differences in the roles of these proteins in the late stage of recombination were evident from these findings. Ref. 4.

(5) Roles of the Rad52 Protein of *Saccharomyces cerevisiae* in Homologous Recombination

Akira SHINOHARA, Miki SHINOHARA and Tomoko OGAWA

It has been reported that the *RAD52* gene of *Saccharomyces cerevisiae* plays crucial roles in several pathways of homologous recombination. The Rad52 protein is a single-stranded (ss) DNA binding protein which catalyzes annealing of complementary ssDNAs. The protein interacts with a yeast ssDNA binding protein, RPA. The RPA protein stimulates Rad52 activities by recruiting Rad52 and forming a Rad52-RPA complex on ssDNA. The Rad52 protein interacts directly with the Rad51 protein and the incorporation of the Rad52 protein stabilizes the complex formed by Rad51 on ssDNA. The Rad52 protein appears to act by promoting the assembly of proteins involved in recombination machinery for pairing of homologous sequences in Rad51-dependent pathways, and also by directly promoting homology searches and homologous pairing in Rad51-independent recombination by virtue of its annealing activity.

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B–b. Division of Microbial Genetics

(1) The Initiator Protein of Filamentous Phage Forms a Covalent Complex with the Origin DNA

Satoshi ASANO, Atsushi HIGASHITANI and Kensuke HORIUCHI

The plus-strand replication of filamentous coliphage is initiated by gpII, which is a phage-encoded initiator protein. gpII binds to the plus-strand origin and introduces a specific nick in the plus-strand. The 3′-hydroxyl end of the nick serves as the primer for plus-strand rolling-circle-type replication.

We showed that DNA bending and duplex melting around the nicking site are induced by gpII binding. The melting required negative superhelicity of DNA, and the nicking reaction required the Mg^{++} ion. gpII showed single-stranded DNA-specific and sequence-specific endnuclease activity. A nicking substrate was prepared through hybridization of 39mer and 25mer deoxyoligonucleotides. It contained a 25-nucleotide dsDNA domain for gpII binding and a 9-nucleotide ssDNA domain around the nicking site, and was completely cleaved by gpII in less than 5 min. When this substrate was used for nicking reactions, a small fraction of the nicked DNA product was found covalently attached at the 5′ end to gpII. This covalent bond was extremely unstable in the presence of active gpII.

G73A, a mutant gpII, formed the covalent complex more efficiently than wild type gpII. To identify the amino acid residue that forms the covalent bond, we used this mutant gpII. A substrate DNA carrying ^{32}P internally at the nicking site was nicked with G73A gpII to yield a ^{32}P-labelled gpII-DNA complex. The result of hydrolysis of the complex in 6N HCl followed by high voltage paper electrophoresis indicated that it is a phospho-tyrosine that bridges the 5′ end of the DNA and gpII.

(2) Mutational Analysis of the Minus Strand Replication Origin of Filamentous Bacteriophage

Nahoko HIGASHITANI, Atsushi HIGASHITANI and Kensuke HORIUCHI

The single-stranded viral DNA of F-specific filamentous bactriophages (f1, M13, and fd) is converted upon infection to the double-stranded replicative form through the synthesis of the minus strand, which is initiated by a RNA primer. The RNA primer is synthesized by the host RNA polymerase on the viral DNA template in the presence of the *E. coli* SSB protein, and is extended by the DNA polymerase III holoenzyme (Geider, K. *et al.* (1974) J. Biol. Chem. **249**, 3999–4005). The minus-strand origin contains two inverted repeats that can form a structure containing two hairpins named [B] and [C] (Gray, C. P. *et al.* (1978) Proc. Natl. Acad. Sci. USA. **75**, 50–53). The mechanism of recognition of the minus-strand origin by RNA polymerase is not well understood. The specificity of the priming reaction depends on the σ^{70} subunit of RNA polymerase (Kaguni, J. M. *et al.* (1982) J. Biol. Chem. **257**, 5437–5443). We previously determined the nucleotide sequence of the primer RNA, and this prompted us to search for structures that are recognized by RNA polymerase in the priming reaction by taking into account the analogy to the promoter recognition mechanism. By depicting the two hairpins [B] and [C] horizontally, and by assuming that they correspond to a stretch of a double-stranded molecule, it was possible to draw the origin schematically as if it was comparable to a transcriptional promoter (Higashitani, N. *et al.* (1993) J. Virol. **67**, 2175–2181).

We carried out footprinting experiments with *E. coli* RNA polymerase on the minus strand origin. The results indicated that the bottom half of the stem in hairpins [B] and [C] was protected by RNA polymerase and that the footprint was dependent on the presence of the σ^{70} factor. The protected portions of DNA corresponded to the -35 region and a region ranging from -10 to $+5$, respectively, in the schematic presentation of the origin. We constructed and analyzed a number of base substitution mutants on the hairpin [B]. The results showed that a specific sequence which is required for the origin function exists within the stem of hairpin [B], located about 35 nucleotides upstream of the primer RNA start site in the scheme of the origin described above.

We then constructed mutations in the region about 10 nucleotides up-

stream of the primer RNA start site in the scheme. The dA5 sequence on the upper strand (non-template strand) was replaced by dC5 or dT5. These mutant origins were inactive. When the dT5 sequence on the lower strand (template strand) was replaced by dG5, the origin was active. Furthermore, we replaced six nucleotides on the non-template strand of this region by [TATAAT], the consensus sequence for the promoter −10 region. This mutant was active as the origin. These results indicated that in this region the sequence of the non-template strand was important for the origin activity while that of the template strand was not.

We further constructed a set of mutants in which the region ranging from −12 to −1 was replaced by the sequence of the lacUV5 promoter. When the sequences of both strands were replaced by those of lacUV5, the origin was inactive. Starting from this mutant, replacement of the template strand with another sequence to destroy the base pairing in this region produced a fully active origin. On the other hand, replacement of the non-template strand by another sequence to destroy the base pairing produced a totally inactive origin. The results indicated that in the region ranging from −12 to −1 in the origin, there are specific bases in the non-template strand that are required for the origin recognition, but not in the template strand, and that the DNA in this region must be in a single-stranded form for the origin to be active.

We also constructed mutants by changing the sequence of the region ranging from +2 to +10 of the origin. The result clearly indicated that double-strandedness of this region was vital for the origin activity and that the nucleotide sequence of either strand was irrelevant.

Altogether, the results obtained suggest that the interaction of RNA polymerase with the minus-strand origin shares some common features with the interaction with transcriptional promoters, in spite of the fact that a significant portion of the minus-strand origin is single-stranded.

In addition, we examined the role of SSB in the recognition of the origin. When the viral single-stranded DNA of 6.4 kb was used as the template, RNA polymerase produced a large amount of non-specific RNA in the absence of SSB. In the presence of more than one molecule of SSB per 5 nucleotides of the template DNA, synthesis of non-specific RNA was abolished, and synthesis of primer RNA was observed. Primer RNA was not synthesized in the absence of SSB. To test if this was due to sequestration of RNA polymerase by the non-specific single-stranded region of the DNA, a 140-nucleotide fragment of the viral DNA containing the origin was pre-

pared, and used as the template. An equal amount of primer RNA of normal size (20 nucleotides) was produced no matter whether SSB was present or not. This suggests that the function of SSB in primer synthesis is to block non-specific RNA synthesis rather than to be directly involved in a specific interaction between RNA polymerase and origin DNA.

(3) A Promoter at the Beginning of the Cell Envelope Biosynthetic/ Cell Division Gene Cluster in the *Escherichia coli* Chromosome 2-Minute Region is Required for Expression of Nine Genes up to *ftsW*

Hiroshi HARA and Kensuke HORIUCHI

A promoter required for expression of the *ftsI* gene encoding penicillin-binding protein 3, a membrane-bound enzyme essential for cell division, of *Escherichia coli* is located upstream of the gene by 3 open reading frames. It was identified by analyzing chromosomal fragments necessary to complement a chromosomal null allele of the gene (*ftsI*Δ::*cat*) at a single copy. In this null allele an internal fragment of *ftsI* was replaced with the chloramphenicol resistance gene (*cat*) of the same orientation. An attempt to construct a similar chromosomal null allele but with *cat* of the opposite orientation was unsuccessful in the presence of a plasmid carrying a chromosomal fragment from the promoter to *murF*, the second downstream gene of *ftsI*, which complemented *ftsI*Δ::*cat*. The promoter (P*mra*) is at the 5′ end of the *mra* (murein biosynthetic gene cluster *A*) region at the map position of about 2 min, in which cell envelope biosynthetic and cell division genes are tightly clustered in the same orientation with the only known terminator at the 3′ end. We suspected that P*mra* might be responsible for expression of the genes further downstream of *murF*. To examine the function of this promoter we disrupted it on the chromosome by inserting the *lac* promoter of the same orientation between the −35 and −10 sequences of P*mra*. The resultant strain did not grow at all in the absence of a *lac* inducer IPTG. Removal of IPTG from the culture medium caused cell lysis, indicating defective expression of a cell wall synthetic gene(s). Complementation of such a defect required a chromosomal fragment from P*mra* to *ftsW* that is the fifth downstream gene of *ftsI* and is essential for cell division. With a plasmid carrying genes up *to murD*, the fourth downstream gene, cell division was

blocked upon the removal of IPTG, and this defect was corrected with the additional introduction of a plasmid carrying *ftsW*. Thus we concluded that the P*mra* promoter is required for expression of the first nine genes, up to *ftsW*, of the *mra* cluster.

(4) Functional Penicillin-Binding Protein 7 is a Multicopy Suppressor of Thermosensitive Growth Defect at Low Osmolarity Due to an *spr* Mutation of *Escherichia coli*

Hiroshi HARA and Kensuke HORIUCHI

Escherichia coli Δprc mutants lacking Prc, a periplasmic proteolytic enzyme involved in the C-terminal processing of penicillin-binding protein (PBP) 3, show thermosensitive growth at low osmolarity. An extragenic suppressor mutation (*spr*) that reversed the thermosensitivity was isolated and found to cause thermosensitivity at low osmolarity in the prc^+ background. Thus *prc* and *spr* are mutual suppressors. In the course of the study on *spr*, the gene for PBP7, a DD-endopeptidase that hydrolyzes peptide crossbridges of macromolecular murein sacculi, was identified as a multicopy suppressor of *spr*. PBP7 seemed to be degraded by Prc, and we suspected that the multicopy suppression might be due to overproduction of the protease substrate mimicking a Prc-deficient state. We converted the serine residue at the 42nd position from the N terminus of the mature form of PBP7, which was predicted to be the active site by its occurrence in the SXXK motif conserved among PBPs and β-lactamases, to alanine by oligonucleotide-directed mutagenesis. This resulted in the loss of penicillin-binding ability, indicating the serine residue is actually the active site. Overproduction of this active-site mutant protein did not suppress the *spr* growth defect. The presence of the gene product was demonstrated by a maxicell experiment. The multicopy suppression of *spr* did not seem simply due to an excess amount of the Prc substrate, but the DD-endopeptidase activity of PBP7 was necessary. Spr protein might also function in murein metabolism. For details, see Ref. 1.

(5) C-terminal Region of an *Escherichia coli* Cell-Division Inhibitor SulA is Involved in Negative Regulation of the SOS Response

Atsushi HIGASHITANI, Yasuyuki ISHII[1], Yasuhiko KATO[1] and Kensuke HORIUCHI ([1]Department of Applied Chemistry, Faculty of Engineering, Kyushu Institute of Technology)

The *sulA* gene of *Escherichia coli* codes for a cell-division inhibitor that is expressed in the bacterial SOS response to prevent premature segregation of the damaged DNA into daughter cells during the DNA repair process. The intracellular concentration of SulA is well regulated so that the division inhibition in SOS response is transient. *sulA* mutants were first isolated as suppressors of *lon*, which codes for an ATP-dependent protease. The SulA protein is rapidly degraded by Lon protease. The average half-life of SulA molecules is 1.2 min in wild-type cells and 19 min in *lon*$^-$ cells. In this study, we constructed a series of deletion mutants of *sulA* gene and identified the negative regulatory region of *sulA* for the degradation by Lon protease.

From deletion analyses, the N-terminal amino acid residues from the 3rd position to the 27th position were found to be dispensable for the activity of the cell-division inhibitor. Likewise, the 21 C-terminal residues were dispensable. We found that the inhibitory activity of the C-terminal deletions was greater than that of the wild-type SulA in *lon*$^+$ cells, but not in *lon*$^-$ cells. *In vitro* experiments using SulA fused to the maltose binding protein (MBP-SulA) as a substrate for Lon protease indicated that wild-type SulA was specifically cleaved by Lon at two sites located within hydrophobic domains at about the 50th and 110th codons. On the other hand, SulA lacking 21 C-terminal residues which was similarly fused to MBP, (MBP-SulAC148) was not cleaved by Lon. Thus, several C-terminal residues of SulA are likely to have an important role in regulating cell division via the stability of the protein, in the presence of ATP-dependent protease Lon, during the SOS response. The C-terminal region of SulA contains a sequence similar to that found in the λ phage N protein which is also cleaved by Lon protease (4 amino acids residues identity in 9 amino acids). This amino acid sequence may be recognized by Lon. However, it is not enough by itself to make the protein sensitive to Lon. The LacZ protein that was fused at its C-terminus to the 8 or 20 amino acid residues of the SulA C-terminus was stable in the *lon*$^+$ cells. Lon is a heat shock protein whose function appears to be the

degradation of heat-denatured proteins. *In vitro* analysis has indicated that heat-denatured proteins are good substrates for Lon, while their native forms are not. The SulA and N protein, natural substrates of Lon protease, may harbor a unique structure in common for recognition and cleavage by Lon.

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B–c. Division of Cytoplasmic Genetics

(1) Identification and Characterization of the Linear IS3 Molecules Generated by Staggered Breaks

Eiichi OHTSUBO

Transposable elements are characterized by their ability to transpose. IS3 (1258 bp in length) is an insertion element present in the *Escherichia coli* chromosome and in plasmid F. IS3 is supposed to transpose in a non-replicative manner. This element encodes two, out-of-phase, overlapping open reading frames, orfA and orfB. The OrfAB transframe protein, that is IS3 transposase, is produced by −1 translational frameshifting between orfA and orfB. Efficient production of the IS3 transposase in cells harboring the IS3-carrying plasmid has been shown to generate miniplasmids as well as characteristic minicircles, called IS3 circles, consisting of the entire IS3 sequence and one of the 3-bp sequences flanking IS3 in the parental plasmid. We showed that IS3 transposase also generates the linear molecules of IS3 with 3-nt overhangs at the 5′ ends. The nucleotide sequences of the overhangs are the same as for those flanking IS3 in the parental plasmid,

suggesting that the linear IS3 molecules are generated from the parental plasmid DNA through staggered double-strand breaks at the end regions of IS3. We pointed out that the transposition reaction in IS3 is similar to the transposition reaction in other transposons, such as Tn10 and Tn7, and even more similar to the integration reaction in retroviruses, which generate linear DNA molecules as the early intermediates in insertion.

(2) Characterization of TraI Helicase-Endonuclease Which is Involved in the Initiation and Termination Steps of DNA Transfer

Eiichi OHTSUBO

In conjugal DNA transfer promoted by bacterial sex factor plasmids, such as R100 and F, one of the initial events is supposed to be strand- and site-specific nicking at the origin of transfer, oriT, by the plasmid-specified endonuclease. Unwinding from the nick subsequently occurs to provide single-stranded DNA, which is known to be transferred to a recipient cell. The TraI protein of R100 specifies both DNA helicase activity and site- and strand-specific nickase activity. We have recently shown that the TraI protein specifies a new activity in addition to the helicase-nickase activity, that is the single-strand DNA specific endonuclease-rejoining activity at oriT, where nicking occurs. The TraI protein consequently generates one complete unit of the single-stranded plasmid DNA, which is supposed to be transferred into a recipient cell at the final stage of conjugation. We have also obtained results indicating that in the nicking reaction, the TraI protein recognizes and binds to a specific single-stranded DNA sequence around oriT, which appears due to local melting of the double-stranded DNA molecule, and it cleaves the sequence at oriT.

(3) A 6-kb Upstream Region of the Human Transthyretin Gene Can Direct Developmental, Tissue-Specific and Quantitatively Normal Expression in Transgenic Mouse

Yasushi NAGATA, Fumi TASHIRO, Shigehiro YI, Tatsuhumi MURAKAMI, Shuichiro MAEDA, Kiyoshi, TAKAHASHI, Kazunori SHIMADA, Hitoshi, OKAMURA and Ken-ichi YAMAMURA

To ascertain whether a 6-kb upstream region of the human transthyretin (TTR) gene contains the cis-element(s) required for proper specificity and level of expression, transgenic mice carrying the human mutant TTR gene containing either 6-kb (6.0-hMet30) or 0.6-kb (0.6-hMet30) of the upstream region were produced and studied. The 6.0-hMet30 gene was expressed in the yolk sac, liver and choroid plexus, where the mouse endogenous TTR gene is also expressed. In contrast, expression of the 0.6-hMet30 gene was restricted to the yolk sac and liver. The expression levels of the 6.0-hMet30 gene in the liver and serum were similar to those of the mouse TTR gene, and about 10-fold those of the 0.6-hMet30 gene. Before birth, the developmental profiles of the expression of both transgenes in each tissue were similar to those of the mouse TTR gene. However, the expression levels of the 6.0-hMet30 gene in the liver and serum increased after birth to reach adult levels at an age of 4 weeks, while expression of the 0.6-hMet30 gene remained at a low level after birth. These results suggest that the 6-kb upstream sequence contains the cis-elements required for developmental, tissue-specific and quantitatively normal expression. For the details, see Ref. 7.

(4) Cell-Cycle-Dependent Expression of the STK-1 Gene Encoding a Novel Murine Putative Protein Kinase

Hitoshi NIWA, Kuniya, ABE, Takahiro KUNISADA and Ken-ichi YAMAMURA

We have cloned a novel putative serine/threonine kinase-encoding gene, designated STK-1, from murine embryonic stem (ES) cell and testis cDNA libraries. The kinase most closely related to STK-1 is Xenopus laevis XLP46 protein kinase which shows 71% amino acid identity to STK-1 between their kinase domains. Nevertheless, STK-1 is conserved throughout phylogeny with hybridizing sequences being detected in DNA from mammals, amphi-

bians, insects and yeast. STK-1 mRNA is detected in testis, intestine and spleen, tissues that contain a large number of proliferating cells, but not in other tissues. All cell lines tested expressed STK-1 mRNA with levels being dependent upon proliferation rates. In NIH 3T3 cells, STK-1 is expressed in a cell-cycle-dependent fashion. These findings suggest a role for STK-1 in cell growth. For the details, see Ref. 8.

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C. DEPARTMENT OF ONTOGENETICS

C–a. Division of Developmental Genetics

(1) Nerve Cell Differentiation in Epithelial Hydra: Epithelial Tissue Provides Cues for Region-Specific Differentiation

Sumiko MINOBE[1], Osamu KOIZUMI[1], and Tsutomu SUGIYAMA ([1] Fukuoka Women's University)

Nerve precursor cells were introduced into nerve-free epithelial hydra by grafting of normal hydra tissue, and their differentiation into nerve cells was examined and compared in tentacles and hypostome of epithelial hydra host.

In tentacles, ganglion cells first appeared in tentacle base at 1.5 days after grafting. A boundary was present between tentacle base tissue containing nerve cells and more distal tissue without them. The boundary moved gradually, reaching tentacle tip in 7–8 days. Sensory cells also appeared in tentacle base, but about 1 day later than ganglion cells. The boundary for the sensory cells also moved distally. The rates of boundary movement for both nerve cell types were similar to the rate of displacement of carbon-marked epithelial tissue from base to tip of tentacles.

In hypostome, a small number of ganglion cells first appeared in the apex at 1.5 days. More nerve cells appeared during following days, eventually forming a cluster of sensory cells at the apex surrounded by numerous ganglion cells in adjacent tissue as in the hypostome of normal hydra.

These results show that nerve cell differentiation in the epithelial hydra host does not occur randomly. Instead, it occurs in a strongly region-specific manner as in normal hydra. This observation suggests that epithelial cells in different regions provide different cues or signals for region-specific nerve cell differentiation.

(2) Hydra Peptide Project II. Biological Function Assay

Toshitaka FUJISAWA, Hiroshi SHIMIZU, Masayuki HATTA, Osamu KOIZUMI[1], Hans BODE[2], Thomas BOSCH[3], Maria deHARO[3], Jan LOHMANN[3], Charles DAVID[3], Toshio TAKAHASHI[4] and Yojiro MUNEOKA[4] ([1] Fukuoka Women's University, [2] University of California at Irvine, [3] University of Munich, [4] Hiroshima University)

Using the approach described in Hydra Peptide Project I, we have isolated 286 hydra peptides, determined the structure of 112 peptides and synthesized 19 peptides. We selected 2 families of peptides (GLWamide family and PW family) to examine their biological functions.

1) *GLWamide family.* Six peptides belong to this family. They consist of 7 or 8 amino acids and all have a consensus sequence of GLWamide at the C-terminus (*e.g.* Hym-54 GPMTGLWamide). Similar peptide, Metamorphosin-A (pEQPGLWamide), is isolated from sea-anemone, which can induce metamorphosis of planula larvae of a marine hydrozoan, *Hydractinia* (Leitz *et al.*, 1994). All 6 hydra GLWamide peptides can also induce metamorphosis of *Hydractinia* planulae. In hydra they induce the contraction of ectodermal sphincter muscles which in turn promotes detachment of buds from the parental body. They also induce contraction of parietal muscles of the sea anemone body wall.

2) *PW family.* Four peptides belong to this family. They are 5–8 amino acids long and have PW in their C-termini (*e.g.* Hym-33H AALPW). All of the PW peptides specifically inhibits neuron differentiation in hydra.

All of the members of each family affect the expression of the same transcripts when analyzed in DD–PCR. The results suggest that they have redundant functions being good agreement with the results of biological function analysis. Further characterization of these peptide families as well as other synthetic peptides are currently in progress.

(3) Disappearance and Reappearance of Mesoglea in Hydra Tissue During Head Regeneration

Hiroshi SHIMIZU and Michael P. SARRAS, Jr[1] ([1] University of Kansas Medical Center)

Mesoglea is a thin (2–3 μm) layer of extracellular matrix (ECM) that

intervenes the ectodermal and endodermal epithelial layers in hydra. As an attempt to elucidate the involvement of ECM in hydra morphogenesis, changes that occur in the mesoglea during head regeneration were examined. Immunoflourescence staining using a monoclonal antibody m52 which specifically recognizes laminin B1 chain in hydra mesoglea was employed to visualize the mesoglea as a marker.

Immediately after head amputation, the apical edge of mesoglea was located basal to that of epithelial tissue with a distance of about 50 μm. The tissue at the site of amputation was thus free of mesoglea. The edge of mesoglea was a ragged line with very high staining intensity. It was found by sectioning tissue that this bright staining represents a notable thickening of mesoglea there up to 20–30 μm compared to 2–3 μm of intact mesoglea.

At the site of regeneration, no staining was seen until 18 hr after amputation, followed by a gradual and overall increase of staining intensity. Formation of tentacles began morphologically at 30–48 hr. There tentacles emerged in the area of tissue which was free of mesoglea.

The notable thickening of mesoglea at the amputation site strongly suggests that mesoglea retracted away the site thereby making the tissue there free of mesoglea. The gradual and overall increase of staining intensity after 18 hr suggests that new synthesis of mesoglea occured at the amputation site to fill the gap of mesoglea. The close correlation between the site of retraction of mesoglea and the site of tentacle formation suggests that the retraction of mesoglea is involved somehow in determining the site of tentacle formation. Two ways of involvement is possible. First, the direct contact of ectodermal and endodermal tissue layers enabled by the retraction of mesoglea may give rise to massive interaction of the two layers resulting in commitment of tentacle formation at the spot. Second, the intact mesoglea might work as a mechanical barrier for tentacle formation because of its possible elasticity or rigidity. The long duration of time when there is much less mesoglea than normal might make tentacle formation mechanically easier at the spot.

(4) Analysis of Hydra Cell Adhesion Molecules

Masayuki HATTA, Engelbert HOBMAYER[1] and Tsutomu SUGIYAMA
([1] Frankfurt University)

Hydra can regenerate even if it is dissociated to single cells and allowed to form reaggregated cell mass. This process gives a good model system to analyze principles of morphogenesis of multicellular animals. During regeneration from reaggregates, cell to cell recognition/adhesion and cell movement are thought to play key roles for morphogenesis giving rise to cell sorting and epithelium formation.

We investigated cell adhesion properties of dissociated hydra cells in rotation culture and detected two kinds of activities. In the early stage of rotation culture, ectodermal and endodermal cells sorted out making ectodermal or endodermal homotypic small cell clumps. Once homotypic cells adhered, they altered cell adhesion specificities quickly to accept the other type of cells. This alteration resulted to make large mixed cell masses as the later phase of reaggregation in rotation culture. When antiserum against hydra cell membrane was added to the culture, the both type of the cells mixed making small cell clumps and never grew to lager cell mass. These results suggest that the quick alteration of cell adhesion specificities is one of major driving force for epithelium formation and that this adhesion specificities are performed by molecules recognized by the antiserum.

Another approach on cell adhesion is identification of homologues of known cell adhesion molecules. We isolated cDNAs for β-catenin (ref.) and integrin β-chain, and identified ABP-280 as a component of septate junction that was first identified to crosslink actin fibers and membrane glycoproteins in mammalian blood cells. Both description of cell adhesion activities and identification of cell adhesion molecules will complement each other to figure out molecular bases of epithelium formation.

(5) Evolutionary Study of Reef-Building Corals, Genus *Acropora*

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([1] Tokyo University of Fishery, [2] Akajima Marine Science Laboratory)

Genus *Acropora* shares dominant species in reef-building corals. Dozens of *Acropora* species spawn at the same time in the small areas. This mass-spawning gives a problem how they could speciate without geological isolation. We tried to find out the evidences for the coral evolution by molecular phylogenetic analysis and experimental cross.

First we isolated the gene for mini-collagen encoding a major component of nematocyst capsule from *A. donei* (ref.) and *A. nasuta*, and designed primers for amplification of a fragment of the gene by PCR. DNA was extracted from sperms used for cross experiments and subjected to PCR. DNA sequences obtained were analyzed by molecular phylogenetic methods to estimate genetic distances and variation, and compared with the results of cross experiments. Five conclusions were obtained; (1) two species which do not join mass-spawning are genetically distant from the others, (2) DNA polymorphism are higher in two similar corymbose shape species than the others, (3) intraspecific polymorphisms and interspecific divergence were indistinguishable in the two corymbose species and interspecific cross were observed between the two, (4) one major staghorn shape species cross-fertilized with one of the corymbose shape species at high frequency, (5) the major table shape species revealed low frequency of intraspecific fertilization suggesting it is a sibling species.

It is suggested that *Acropora* is evolving through repeating speciation by rapid establishment of reproductive isolation and gene introgression or species fusion by interspecific fertilization, giving rise to variety of morphology.

(6) Cloning of Female Germ Stem Cells from a Male Strain of *Hydra magnipapillata*

Chiemi NISHIMIYA-FUJISAWA and Tsutomu SUGIYAMA

Three types of interstitial stem cell subpopulations were isolated from *Hydra magnipapillata*, and their roles in sex determination were examined.

A subpopulation of interstitial stem cells restricted to the sperm differentiation pathway was isolated previously from strain nem-1 (male). Another subpopulation restricted to the egg differentiation pathway was also isolated from the same strain. Hydroxyurea treatment was used for isolation in both cases.

"Pseudo-epithelial hydra" containing only sperm- or egg-restricted stem cells but no other interstitial stem cell types were maintained by force-feeding for 2 years. Sex reversal from egg-restricted to sperm-restricted stem cells occurred 3 times during this period. Both of these two stem cell types are numerous in the central gastric region of the pseudo-epithelial hydra, but absent in the foot region below the budding zone. Foot tissue was cut out from normal nem-1 polyps (male), and allowed to regenerate. The regenerates produced eggs but no sperm upon sex induction. These and other results suggest that the foot tissue contains multi-potent stem cells capable of differentiating into eggs during sexual differentiation.

These observations suggest that strain nem-1 (male) contains 3 types of interstitial stem cell subpopulations: (1) sperm-restricted stem cells, (2) egg-restricted stem cells, and (3) multi-potent stem cells capable of differentiating into nerve cells, nematocytes and eggs. Upon sex induction, however, differentiation of eggs by the latter two types is suppressed, and only sperm are produced by the sperm-restricted stem cells. Evidence is presented which suggests that similar "phenotypic males", which normally only produce sperm but contain the stem cell types capable of differentiating into eggs, occur widely in *Hydra magnipapillata.* A possible relationship between phenotypic male hydra and hermaphroditic hydra is discussed.

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C–b. Division of Phenogenetics

(1) Cloning of a cDNA for DNA Supercoiling Factor Reveals a Distinctive Ca^{2+}-Binding Protein

Tsutomu OHTA, Masatomo KOBAYASHI and Susumu HIROSE

The DNA supercoiling factor is a protein capable of generating negative supercoils into a relaxed DNA in conjunction with eukaryotic DNA topoisomerase II. A cDNA clone encoding the *Bombyx mori* supercoiling factor has been isolated and characterized. The cDNA sequence contains an open reading frame of 322 amino acids rich in acidic residues. A local region of the predicted polypeptide shares a weak homology with the central portion of the bacterial DNA gyrase A subunit. The cDNA sequence also predicts two direct repeats within the factor. Each repeat unit consists of a potential EF-hand domain. The factor contains three other potential EF-hand domains and actually binds Ca^{2+}. Moreover, the supercoiling reaction has been shown to be activated by Ca^{2+}. These results implicate Ca^{2+} in the control of the superhelical state of DNA. For details, see Ref. 1.

(2) Molecular Cloning of a cDNA for Transcriptional Positive Cofactor MBF2

Feng-Qian LI, Ken-ichi TAKEMARU, Hitoshi UEDA and Susumu HIROSE

Transcriptional activation of the *Drosophila melanogaster fushi tarazu* gene by FTZ–F1 or its silkworm counterpart BmFTZ–F1, requires two additional factors MBF1 and MBF2. Neither of these factors directly binds to DNA.

MBF1 serves as a bridging molecule that connects BmFTZ–F1 (or FTZ–F1), MBF2 and the TATA binding protein TBP (F.-Q. Li, H. Ueda, and S. Hirose, Mol. Cell. Biol. **14**, 3013–3021, 1994). To clarify the role of MBF2 in transcriptional activation, we isolated a cDNA coding for the factor from the silkworm *Bombyx mori.* The deduced amino acid sequence of the factor showed no homology with proteins in the databases. Farwestern analyses demonstrated that MBF2 makes direct contact with the β-subunit of TFIIA. In a HeLa cell nuclear extract, bacterially expressed recombinant MBF2 activated transcription from various promoters as natural MBF2 did. When recombinant MBF2 was added to the HeLa cell nuclear extract in the presence of MBF1 and BmFTZ–F1 (or FTZ–F1), it selectively activated transcription of the *fushi tarazu* gene. We propose that MBF2 activates transcription through its interaction with TFIIA to allow selective activation in a FTZ–F1 site dependent manner.

(3) Molecular Cloning of a cDNA Encoding Transcriptional Mediator MBF1

Kin-ichi TAKEMARU, Feng-Qian LI, Hitoshi UEDA and Susumu HIROSE

MBF1 is a transcriptional mediator that connects the TATA element-binding protein TBP and the *Drosophila melanogaster* nuclear hormone receptor FTZ–F1 or its silkworm counterpart BmFTZ–F1 (F.-Q. Li, H. Ueda, and S. Hirose, Mol. Cell. Biol. **14**, 3013–3021, 1994). A cDNA clone coding for MBF1 has been isolated from the silkworm *Bombyx mori.* The cDNA sequence encodes a basic protein consisting of 180 amino acids. Homology search in the databases revealed that the deduced amino acid seguence of the factor is conserved across species from yeast to human. Bacterially expressed recombinant MBF1 is functional in interactions with TBP and a positive cofactor MBF2. Recombinant MBF1 also makes direct contact with FTZ–F1 and stimulates the FTZ–F1 binding to its recognition site. When recombinant MBF1 was added to a HeLa cell nuclear extract in the presence of MBF2 and BmFTZ–F1, it supported selective transcriptional activation of the *fushi tarazu* gene as natural MBF1 did.

(4) Consideration of the Hormonal Function of Aging Cells and Organisms in Insects

Kikuo IWABUCHI[1] and Akio MURAKAMI ([1]Fac of Agric., Tokyo Univ. of Agriculture and Technology, Fuchu, Tokyo)

The scenario for the developmental process of organisms might already be described as a grand design in the genome. The life history of insects is comprised of processes taking the organisms from through embryonic, larval, pupal and adult forms: senescence begins after the reproductive phase in the last form and is followed by the death event. As is well-known, the prothoracic gland hormone (ecdysone) and corpus allatum (juvenile) hormone (JH) take part in various processes in holometabola insects, among other things, the larval growth period. Ecdysone plays a role in each larval molt—the periodic shedding of the larval cuticle in insects—and in each step of metamorphosis from embryo to larva, larva to pupa, and pupa to the imaginal form in *Bombyx*, for example. As has widely been accepted, JH has a juvenilizing function in larval stages (Wigglesworth, 1934). The relative concentrations of ecdysone and JH determine whether juvenile or mature forms will follow a given molt. Thus, the function of ecdysone is to set the stage for a more mature form. In other words, the hormone pushes organisms to the approach of death by opening several gates in developmental steps.

The central nervous system is essential to the life of organisms. Accordingly, the neurons which comprise the neural networks in the system affect the life span of individual organisms. Of note is another function of ecdysone in organisms, as an activator in the cell nucleus inducing cell transformation and/or differentiation. At a cellular level, ecdysone changes aspects of cell proliferation and differentiation leading to cell death. The fate of each differentiated cell in tissue at a different developmental step is genetically determined. The cellular mechanisms at each developmental step has a special function to change cells from dividing to non-dividing types. It has been observed in the polyembryonic wasp that JH induces a vegetable cell propagation in the polymorula formation without differentiation (Iwabuchi, 1995). From such an observation, it can be imagined that a similar event may occur in cultured cells of the insect: when *Drosophila* Kc cells were treated with ecdysone, it revealed a cell transformation from the dividing to non-

dividing nerve cell type (Courgeon, 1972). Again, ecdysone is closely related to the longevity in both cells. Thus, it is clear that in insects, the longevity of cultured cells is analogous to that of individual organisms: the nerve cells derived either *in vivo* (organism) or *in vitro* (transformed cell types) possess the key function to control life span for both cell types. Cultured cell populations possess an inherently neuron type nature. When such a transformation once begins, cell division ceases and finally results in death. For reference, it is worthwhile to note that cell lines derived from imaginal discs of insects maintain a more undifferentiated state than those of vertebrates, and in consequence insect cells are apt to exhibit a nature as the non-dividing nerve cells among cultured cell populations.

It can be inferred that in insects, ecdysone exhibits cell transforming activity from undifferentiated to differentiated forms in cultured cells. It is also possible to infer that the hormone pushes both insect cells and organisms toward death.

(5) Neuro-Endocrinological Studies on a Mutant *sdi* (Short Duration of Imaginal Lifespan) in the *Bombyx* Silkworm

Jun SHIMADA[1], Masako KANBARA[2] and Akio MURAKAMI ([1] Fac. of Agric., Tokyo Univ. of Agric. and Technology, Fuchu, Tokyo, [2] Fac. of Agric., Univ. of Tokyo, Hongo, Tokyo)

The continuation of each biological species through transmission of genetic information to progeny, is ensured by sophisticated reproductive mechanisms. However, some hold the view that when the mechanisms of life maintenance in the post-reproductive phase fail it results in a death events through the process of senescence. But the senescence process, which starts at the time of completion of the reproductive phase is also a life phenomenon that is part of the genome design.

As is well-known, the larvae of the *Bombyx* silkworm feed on mulberry leaves, and both the pupa and moth use nutrients stored-up in the larval stage. Accordingly, moths are required to make efficient use of the stores to maintain the post-reproductive period as possible. To do this, the *Bombyx* moth should have well-organized physiological functions to manage the consumption of stored nutrients under control of the brain-nerve system, to maintain homeostasis during the post-reproductive period of the insect.

A spontaneous autosomal recessive mutant, *sdi*, was detected and fixed by Murakami and its genetic characteristics analyzed by him and his co-workers since 1986. In the present study, the effect of brain removal and/or implantation of the moths of the *sdi* strain, those of the J106 strain having a wild type gene *sdi* (or $+^{sdi}$), and F_1 moths obtained from a cross between *sdi* and J106 strains were used to investigate the brain function of moths in regard to the imaginal lifespan. The experiment was performed at a temperature of 25°C and a humidity of 75% using moths from the above groups. Brain removal and implantation were performed according to conventional methods, and moths wounded by a sham operation used as the control.

Results indicated that certain endoneurosecretory substances from the implanted brain did not change the imaginal lifespan in each line observed in this experiment. Accordingly, it is likely that the short adult lifespan of the *sdi* mutant line is caused by an abnormal mechanism concerning intercellular transmission of neural information among brain neurons rather than by a substance secreted from the brain.

The typical death in the moth of the *sdi* strain, suddenly occurred after completion of the reproductive phase (or two to three days) after emergence without a marked reduction of their body-weight (Murakami *et al.*, 1986, 1989). Similarly, if pupal brains were removed in certain wild-type stocks (or lines) having an average adult lifespan a drastic shortening of the imaginal lifespan was observed (Murakami and Shimada, 1988).

As is well-known, ecdysone is able to activate genes of *Diptera*, as observed in the polytene chromosome (Clever and Karlson, 1960) and this induces adult transformation from pupal forms in various insect species. Furthermore, the hormone seems to have the ability to transform cells from undifferentiated type cells to differentiated ones as described in another report. Accordingly, it is possible to infer that ecdysone makes renews and/or transforms undifferentiated neurons into differentiated ones in the central nervous system. In addition, the hormone acts as an inducer of transformation from larval to adult forms.

From these observations, it appears that the short adult lifespan, as a typical feature of the *sdi* mutant line, may be caused by a marked disorder of the life sustaining mechanism or homeostasis at an early stage of the post-reproductive phase. In this regard, the possibility cannot be excluded that some functional disorder in the ecdysone receptor exists and that a genetically programmed cell replacement of used neurons with new ones

occurs in the preceding developmental stage.

(6) Linkage Analysis of the *sdi* (short duration of imaginal lifespan) Gene

Hirosi FUJII[1], Hirosi DOIRA[1] and Akio MURAKAMI ([1]Fac. of Agric., Kyushu Univ., Fukuoka)

In the *Bombyx* silkworm the growth period from embryo via larva to pupa in the mutant line *sdi*, is no different than that in the normal line. However, the adult lifespan in the *sdi* line is shorter than that in common silkworm lines. The adult lifespan of this mutant lasts at most two to three days after emergence from the pupal form. Murakami and his colleagues have analyzed the genetic as well as biological characteristics of the mutant *sdi* since 1986 and their findings indicate that the mutant characteristic is controlled by a single recessive gene located on some autosome. It is not yet known which linkage group the gene *sdi* belongs to.

For linkage analysis, the marker genes used were 2(*Y*), 3(*Ze*), 4(*L*), 5(−), 6(E^kp), 7(*q*), 8(*st*). 9(*Ia*), 10(−), 11(*K*), 13(*ch*), 14(*U*), 15(*bl*), 16(cts), 18 (*m*/*n*), 19(*nb*), 20(−), 21(−), 22(*mw*), 23(−), 24(*Sel*), 25(*Nd*), 26(−), 27(*so*) and 28(*Etr*). The numbers indicate linkage chromosome groups. When the dominant marker was used for the analysis, the F_1 hybrid females were mated with the test male (*sdi* and $+^{Nd}$, a normal cocooning wild type), and then BF_1 progenies were examined for longevity. If the recessive marker was used, F_2 progenys were examined for longevity. The results showed that the gene *sdi* was an independent relation to the following linkage groups, 2, 3, 4, 6, 7, 8, 9, 11, 13, 14, 15, 16, 18, 19, 22, 24, 27 and 28. In analyses conducted so far, when silkworms marked with genes, *sdi* and $+^{Nd}$, were crossed with moths having the gene *Nd* and $+^{sdi}$ and then F_1 female moths showing the *Nd* characteristics were again mated with moths having the genes *sdi* and $+^{Nd}$. The BF_1 progenys obtained were examined for longevity. The results showed that all 216 $+^{Nd}$ type moths, of both sexes, had short adult lifespans, while thirty-three moths of the *Nd* type also had a short adult lifespan and died within 4 days after eclosion. But a hundred and two (102) *Nd* type moths had a long lifespan, over 5 days after emergence. The thirty-three moths seem have a normal lifespan ($+^{sdi}$) (as a death symptom of the moths showed natural) and the observed segregation ratio was far from expected in an independent relationship. If the genes *sdi* and *Nd* had an

independent relationship when *sdi* and normal ($+^{Nd}$) moths were crossed with *Nd* $+^{sdi}$ moths and F_1 females showing the *Nd* characteristics were crossed with $+^{Nd}$ *sdi*, moths the segregation of the BF_1 progeny would have the ratio of 1 *sdi* $+^{Nd}$, 1 $+^{sdi}$ *Nd*, 1 *sdi Nd*, 1 $+^{sdi}+^{Nd}$, but in the cross, only 33 *sdi* and *Nd* moths appeared, and moreover, no $+^{sdi}+^{Nd}$ moths were observed. The *sdi* and *Nd* moths, which died soon after emergence, were different from those having natural (senescence) death: they were not *sdi* mutants. Hence, the segregation of the BF_1 progeny should be considered to have a ratio of 1 $+^{Nd}$ *sdi* to 1 *Nd* $+^{sdi}$. Accordingly, we concluded that the gene *sdi* is linked with *Nd* on the 25th linkage group of *B. mori* (L.).

Then, we carried out the following cross to determine the recombination value between the gene *sdi* and *Nd*. The BF_1 male moths, *sdi* $+/+^{Nd}$, were mated to females homozygous for *sdi* $+^{Nd}$, the progenys were segregated into *Nd* and $+^{Nd}$ type cocoons. Each type progeny was examined for longevity. The resultant progenys were segregated in the ratio of 151 *Nd* $+^{sdi}$: 52 *Nd sdi*: 252 $+^{Nd}$ *sdi*; 20 $+^{Nd}$ $+^{sdi}$. Thus, the recombination value between *sdi* and *Nd* was calculated to be 12.3%. But as already noted above, *Nd* moths die in consequence of physiological disorders throughout the entire developmental period from embryo to pupa, and among other things, at the larval-pupal ecdysis event. Therefore, we can calculate the recombination value using $+^{Nd}$ *sdi* individuals exclusive of *Nd* ones. The value estimated was 7.3% and the gene *sdi* was located ±7.3% units from the gene *Nd* on the 25th linkage group in the *Bombyx* silkworm.

(7) Genetic Studies on Chromosomal Translocation in *Bombyx Mori* (L.)
(a) Construction of a New Reciprocal Translocation

Akio MURAKAMI

In *Bombyx* silkworms, several numbers of the *Y*-chromosome marked with various kinds of visible gene characters have been constructed for breeding strategies, to discard female insects before they enter the actively growing instar phase, since male silkworms more efficiently produce silk-proteins than females. To construct a certain type of translocated chromosome, it is convenient to make use a chromosome marked with visible gene characteristics. In this insect, there are known to be various kinds of mutant genes in terms of egg and cocoon color, larval marking patterns, and others. The Y

chromosome is known only as a female determining factor, but other genes having a biological function have not been detected so far. At present, several marked *Y*-chromosomes have been preserved in many biological institutions. However, neither their origins, attachment or translocation, have been sufficiently analyzed.

Such being the case, we have purposely produced a certain reciprocal translocation between the Y and 5th chromosomes. Wild type stocks, Aojuku

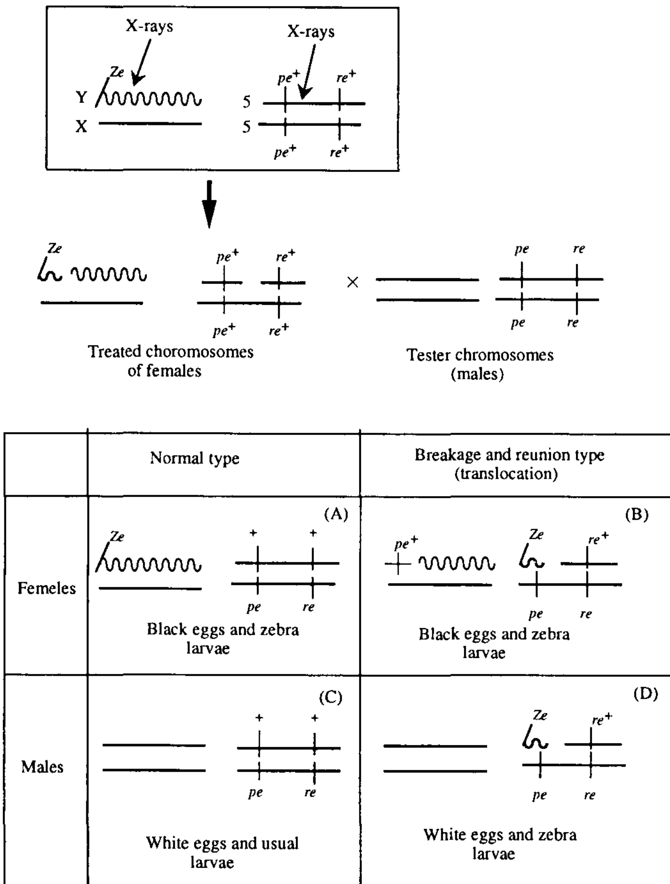

Fig. 1. A schematic construction procedure of a reciprocal translocation between the Y and 5th chromosomes in *B. mori* (L.).

and C108, in the latter of which the Y chromosome is marked with the Zebra gene, were adopted for use in this study. A dozen F_1 hybrid female pupae obtained from crosses between stocks were treated with X-rays (*ca.* 1000 R) to break the chromosomes. These females were mated with the marker males, homozygous *pe* (5–0.0) and *re* (5–31.5), in expectation of breakage events on the two chromosomes.

In consequence, we detected a small number of whole-body type mutant eggs colored with either yellowish-white or red serosa cells and mosaic type mutants with black and either yellowish-white or red cells among many normal black eggs. Theoretically, these mosaic mutants were considered to include chromosomal aberrations on the 5th chromosome concerned with egg-color gene loci. The female moths that hatched from mosaic mutants, were again crossed with *pe : re* tester males for progeny tests.

A female moth, which originated from the mosaic egg with yellowish-white and black cells, laid black ($+^{pe}$) and yellowish-white (*pe*) eggs in the ratio of approximately 1 : 1. Egg-color mutant were separately cultured. The sex of larvae derived from the yellowish-white eggs was male, but zebra and normal (*p* or p^+) type larvae were also found in the ratio of 1 : 1 without exception, while the sex of black eggs was female in the ratio 1 : 1 for normal and zebra types. Viability of females with black colored eggs and zebra type larva was 100%, but that of males with yellowish-white eggs and zebra markers was poor to some extent, indicating that the female did not suffer from a loss of chromosomes (or genetic information) from a shift of the genes located on the chromosomes. But the male was completely lacking in some genes on the proximal region of the 5th chromosome. From these analyses, the chromosomal aberration type mutant can be regarded as a reciprocaltranslocation between the Y and 5th chromosomes, rcpT[t_1(Y:5)pe^+:t_2(5:Y)*Ze*]. The reciprocal translocation line together with the egg-color *pe* and *re* genes, has been preserved in our laboratory.

In this line, the sex-ratio of females to males is normal, suggesting that a translocated part of the Y-chromosome marked with the *Ze* gene on the 5th chromosome in the male showed maleness. Males having new constitution in the 5th chromosome received a certain portion of the Y-chromosome marked with the zebra gene did not express femaleness and females, which had a Y-chromosome suffering from some structural changes, an exchange between the Y and 5th chromosomes, did not change because of the original gender. Those observations suggest that a female determinant factor is not dispersed

along the Y-chromosome, but is localized at a specific site on the chromosome. Furthermore, it suggests that the factor did not exist or a translocated part of the Y to the 5th chromosome did not exist, and female silkworms can't exist (survive) without the factor on the Y chromosome.

(8) Genetic Studies on Chromosomal Translocation in *Bombyx Mori* (L.) (b) Genetic Function of the Y-Chromosome

Akio MURAKAMI

In the reciprocal translocation reported above, the sex-ratio of females to males was normal, indicating that silkworms, with an exchange between the 5th chromosome and the Y chromosome marked with the zebra gene, were all males, while silkworms, which have a Y chromosome with a proximal part of the 5th chromosome, are females. As is well-known, in *Bombyx* silkworms, only a female determinant factor has been recognized on the Y chromosome, but no other genes relating to life activities of this insect. This shows the factor is localized on a specific site of the Y chromosome rather than being dispersed along the chromosome. It is also possible to say that the Y chromosome fragment translocated onto the 5th chromosome does not only retain the factor, but the remainder of the Y chromosome still maintains the factor. In any case, the Y chromosome of several Y chromosome translocation lines established showed a perfect function in expressing the sex of females. Moreover, it is likely that a would-be female silkworm does not exist without the female determinant factor. Furthermore, it is possible to deduce that the Y chromosome fragment shifted onto the autosome does not have a killing effect on males in the translocation line.

The Y-chromosome marked with the zebra gene was constructed by Hashimoto (1956). This chromosome has been considered to be a product of a reciprocal translocation between the Y and 3rd chromosomes, T[t_1(Y:3) *Ze*:t_2(3:Y)(−)]. There is a low viability of males in the line, but not a severe lethality in either embryonic or young larval male. Hashimoto interpreted this phenomenon as being caused by a structural disorder of the 3rd chromosome, which received a fragment of the Y chromosome autosome on the occasion of chromosome breakage. However, it may be possible that as an alternative explanation, the poor viability of males in Hashimato's translocation line depended on a large deficiency of the 3rd chromosome from the *lem*

locus (3–0.0) to the *Ze* locus (3–20.8). As discussed above, there was also low male viability in the rcpT(Y:5) line, probably, becouse of a deletion of a small portion of the 5th chromosome. In the sex chromosome constitution of *Bombyx* silkworms, males are XX and females XY. Accordingly, there is no change of genome constituents in spite of a drastic shift of chromosomal structure owing to chromosomal exchanges in the females. While, in the males, there is a deficiency of genome composition in the nature of the sex chromosome composition of males. Consequently, it can be said that the deficiency of a certain region in the autosome seems to be reflected directly in lethality in the male. The differential male viability observed between Hashimoto's and the present translocation lines is thought to be due to the size of deletions on the autosomes concerned in each line.

(9) Origin of Holometabolous Metamorphosis in Insects

Kiyoshi MINATO

Among the organisms, those in the insecta classes are most successful in not only the total number but also the number of species. One of the reasons is thought to be in the very skillful life styles through the holometabolous metamorphosis or the complete metamorphosis seen in groups of the animals. That is, the holometabolous metamorphosis is specific in their larval forms relatively simple and dissimilar to adult ones, and the intermediate phase of pupa lying between larva and adult. Since the origin of the holometabolous metamorphosis, and hence the origin of the pupal stage in evolution of insects have never been thoroughly classified, we focused on the matter by investigating the life styles in various insect and examining the theories seen in available literature's.

The Berlese theory (1913) that the larval forms in Holometabola derived from a precocious hatching of embryos owing to less yolk reserves is now received by few authors from some negative evidences. And many think that endopterous conditions in Holometabola larva derived from a similar process seen in the earlier nymphal stages of some Heterometabola, such as Thysanopter and Homoptera in which wing anlages development internally.

Furthermore, the larval forms in Holometabola are though to develop to be simplified and specialized more and more through the physiological and morphological independency between larva and imago made by the metamor-

phic phase of pupa.

However, the origin of other features seen in the larvae of Holometabola, such as defects in compound eyes and less-developed thoracic legs, have not explained satisfactorily by the preceding authors. The precocious hatch of embryo in the Berlese theory may not be acceptable, it is likely that Holometabola utilized a part of embryonic forms in making their larval forms by suppressing the morphogenesis of some tissues, such as compound eyes and thoracic legs appeared later in embryonic development.

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C-c. Division of Physiological Genetics

(1) Hydra Peptide Project I. Isolation, Structure Determination and Chemical Synthesis

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Peptide signaling molecules are widely found among animal tissues and bear important biological functions, as neurotransmitters, hormones, growth factors *etc.* However, very little is known about their involvement in development. In order to systematically isolate peptide signaling molecules

involved in hydra development, we initiated a new project based on a novel approach (see Annual Report, 1994). The project consists of the following 5 parts: (1) Peptide isolation from hydra tissue, (2) selection of peptides which are capable of altering gene expression in hydra, by using Differential Display PCR (DD–PCR) (Liang and Pardee, 1992), (3) structure determination of peptides active in DD–PCR, (4) chemical synthesis of active peptides, and (5) examination of biological functions of synthetic peptides. Part (1), (3) and (4) are reported here and the rest are reported in the abstract "Hydra Peptide Project II".

(1) Peptide isolation. As we have reported previously (see Annual Report, 1994), two batches of crude peptide preparations were made from *Hydra magnipapillata* tissue. Batch 1 (150 g) using hot acetic acid extraction method and batch 2 (500 g), the cold acetone method. Each extract was separated by reverse-phase HPLC into 15 factions. Fraction 8 from batch 1 and fractions 8 and 9 were first selected and subjected to 8–9 alternating steps of reverse-phase and ion-exchange HPLC to isolate peptides. No screening was involved in these steps. So far, 286 peptides were isolated. The results suggest that hydra tissue contains close to 1,000 peptides.

(2) Amino acid sequence. Out of 286 peptides 193 peptides were subjected to amino acid sequence analysis by using an automatic amino acid sequencer (and a mass spectrometer in a few cases) and 112 peptides were successfully analyzed. However, it was not successful for 81 samples (42%) because of N-terminal blocking (or non-peptides).

(3) Chemical synthesis. 19 peptides were chemically synthesized and confirmed to be identical to the native peptide by HPLC. They include a family of 6 peptides which have a common sequence of GLWamide at the C-terminus (as in Metamorphosin-A, Leitz *et al.*, 1994) and a family of 4 peptides which have PW at the C-terminus. PW peptides are not reported so far. Isolation, structure determination and chemical synthesis of peptides from the remaining fractions are currently underway.

(2) Analyses of Mammalian Genome by Fluorescence *in situ* Hybridization

Katsuzumi OKUMURA

We analyzed the human and mouse genomes by fluorescence *in situ* hybridization (FISH), including the mapping of several genes, judgment of

YAC clone chimerism, detection of a transgene and its amplified unit, ordering of genomic clones. For details, see Ref. 1, 2, 6–8, 10.

On the other hand, FISH was used to analyze how DNA replication timing is related to the genome structure and how the specific genome domains are arranged in the nucleus.

First, we determined the replication timing pattern of DNA segments over 2 Mb within the human MHC on HL60 cells. This region is composed of long-range G+C% (GC) mosaic structures related to chromosome bands and a boundary of the GC mosaic domains exists between MHC classes II and III. FISH with elongated chromatin fibers showed that the flanking genome structures of the boundary are strictly consistent with the reported mapping data. This confirmed that HL60 cells may not have extensive structural abnormality in this region. Multi-color FISH with interphase nuclei was useful in comparing the temporal replication timing of two DNA fragments at adjacent genomic positions and which have similar replication timing in the cell cycle. Using this procedure, it was possible to determine the precise replication timing of each DNA fragment which was randomly selected from the MHC region. The fragments at the boundary region were found to relatively replicate latest among all examined. It was also suggested that this region consists of at least two replicon clusters. Interestingly, there is a close correlation between the temporal order of replication and the GC gradient of genome sequences in human MHC on HL60 cells. These results suggested the possibility that replication of DNA in the mammalian cell genome reflects GC mosaic domains at the level of genome sequences as well as chromosome bands.

Second, we used FISH to delineate the intranuclear arrangement of specific genome domains by using 16 cosmids scattered all over the entire chromosome 12. Each set of two probes was hybridized to the HL60 nuclei fixed with paraformaldehyde to keep the 3D structure and detected by different fluorochromes. The analysis of the distribution of the nuclear periphery or the interior on each probe suggested that chromosome 12 exhibits a distinctly non-random arrangement in G1 nuclei; centromeric and q21 flanking regions are localized on the nuclear periphery, while the other domains are largely localized in the interior. The relationship between DNA replication and the intranuclear arrangement of specific genome domains are also discussed. For details, see Ref. 12.

We also studied biosynthesis and the metabolism of NAD and its related

compounds. For details, see Ref. 3–5, 9, 11.

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D. DEPARTMENT OF POPULATION GENETICS

D–a. Division of Population Genetics

(1) Synonymous and Nonsynonymous Substitutions in Mammalian Genes and the Nearly Neutral Theory

Tomoko OHTA

The nearly neutral theory of molecular evolution predicts larger generation-time effects for synonymous than for nonsynonymous substitutions. This prediction is tested using the sequences of 49 single-copy genes by calculating the average and variance of synonymous and nonsynonymous substitutions in mammalian star phylogenies (rodentia, artiodactyla, and primates). The average pattern of the 49 genes supports the prediction of the nearly neutral theory, with some notable exceptions.

The nearly neutral theory also predicts that the variance of the evolutionary rate is larger than the value predicted by the completely neutral theory. This prediction is tested by examining the dispersion index (ratio of the variance to the mean), which is positively correlated with the average substitution number. After weighting by the lineage effects, this correlation almost disappears for nonsynonymous substitutions, but not quite so for synonymous substitutions. After weighting, the dispersion indices of both synonymous and nonsynonymous substitutions still exceed values expected under the simple Poisson process. The results indicate that both the systematic bias in evolutionary rate among the lineages and the episodic type of rate variation are contributing to the large variance. The former is more significant to synonymous substitutions than to nonsynonymous substiutions. Isochore evolution may be similar to synonymous substitutions. The rate and pattern found here are consistent with the nearly neutral theory, such that the relative contributions of drift and selection differ between the two types of substitutions. The results are also consistent with Gillespie's episodic selection theory. For details, see Ref. 1.

(2) Gene Conversion vs Point Mutation in Generating Variability at the Antigen Recognition Site of Major Histocompatibility Complex Loci

Tomoko OHTA

In order to assess the roles of gene conversion followed by natural selection and balancing selection for point mutations in polymorphisms at major histocompatibility complex (MHC) loci, DNA sequences of several mammalian taxa were analyzed. Synonymous and nonsynonymous diversities were estimated separately for the antigen recognition site (ARS) and the remaining region of class I and class II genes. In some sequence pairs, the number of nonsynonymous substitutions exceeds that of synonymous substitutions at the ARS. This result indicates that some kind of balancing selection for point mutation is operating. In other sequence pairs (particularly of bovine and of rabbit), the number of synonymous substitutions at the ARS exceeds the same number at the remaining region. This result indicates that gene conversion involving a short region followed by natural selection is important. In general, a combination of gene conversion, point mutation, natural selection and random drift is thought to have contributed to polymorphisms. For details, see Ref. 3.

(3) Genetic Study of Species Differences as Observed as Loss of Bristles in Interspecific Hybrids of *Drosophila*

Toshiyuki TAKANO

Species differences can be revealed through inviability, sterility and/or morphological anomalies of interspecific hybrids even if there is remarkable morphological similarity between species. An analysis of bristle number in interspecific hybrids has been used to search for genes responsible for genetic differences among *D. melanogaster* and three closely related species, *D. simulans*, *D. mauritiana*, and *D. sechellia.* There are clear-cut genetic differences between *D. simulans*, on the one hand, and *D. mauritiana* and *D. sechellia*, on the other hand, in reduction of the bristle number of hybrids with *D. melanogaster.* Interspecific hybrids between a line of *D. melanogaster* and *D. simulans* iso-female lines exhibited a wide range of the number of missing bristles on the thorax. On the contrary, *D. mauritiana* and *D. sechellia* lines showed almost no reduction of the bristle number in hybrids

with *D. melanogaster.* Furthermore, the application of deficiency screening identified two potential regions on the X chromosome.

(4) New Methods for Estimating the Numbers of Synonymous and Nonsynonymous Substitutions

Yasuo INA

I developed two new methods for estimating the numbers, d_S and d_N, of synonymous and nonsynonymous substitutions. Using computer simulations, I evaluated the accuracies of the two new methods and of four previously proposed methods: Nei and Gojobori's method (NG), Miyata and Yasunaga's method (MY), Li, Wu, and Luo's method (LWL), and Pamilo, Bianchi, and Li's method (PBL). The following results were obtained: (1) When there is a strong transition/transversion bias at the mutation level, the NG, MY, and LWL methods overestimate d_S and underestimate d_N. (2) Unless there is a strong nucleotide-frequency bias, the new methods and the PBL method give good estimates of d_S and d_N. For details, see Ref. 2.

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D-b. Division of Evolutionary Genetics

(1) Studies of Human Pseudoautosomal Boundary-Like Sequences (PABLs)

Tatsuo FUKAGAWA, Mizuki OHONO, Yasukazu NAKAMURA, Katsuzumi OKUMURA, Asako ANDO[1], Hidetoshi INOKO[1], Naruya SAITOU and Toshimichi IKEMURA ([1] School of Medicine, Tokai University, Isehara)

The human genome is composed of long-range mosaic structures of G+

C% (GC%), which are thought to relate with chromosome bands. We previously found a boundary of Mbp-level mosaic domains in the human major histocompatibility complex (MHC) region disclosing a sharp GC% transition. Near this boundary to differentiate the global GC% distribution along the genome, a sequence highly homologous (*ca.* 80% nucleotide identity) with the pseudoautosomal boundaries of short arms of human sex chromosomes was found and designated "PABL1": the pseudoautosomal boundaries, PABX1 and PABY1, are unique in the human genome as the strict physical site at which the unusually high rate of recombination in the pseudoautosomal region PAR1 (known to be 20-fold greater than the genome average) terminates abruptly, differentiating human sex chromosomes into functionally distinct regions, *i.e.*, 2.6 Mbp of PAR1 and sex-specific regions.

To investigate structures and functions of the highly homologous sequences both of which are found in the boundaries differentiating global genome characteristics, other pseudoautosomal boundary-like sequences (PABLs) were cloned and characterized. Comparing ten genomic PABLs situated on different chromosomes, a *ca.* 650 nt consensus sequence of the PABL core could be defined. From six independent cDNA libraries, we isolated cDNA clones having PABL and found fairly strict conservation of the 3′ or 5′ edge of their PABL cores. Northern blot experiment showed sizes of PABL transcripts to range 5–10 kb length, and FISH analysis using the cDNAs as probes showed characteristic localization of these transcripts in interphase nuclei.

Divergence time of PABLs was estimated to be 60–120 million years ago and their evolutionary rates showed evolutionary (and thus most likely functional) constrains on PABL sequences. Based on structures of pseudoautosomal boundaries of short and long arms of human sex chromosomes, evolutionary process to form the present day pseudoautosomal boundary of Y chromosome, PABY1, is proposed postulating an illegitimate recombination between two PABLs of the ancestral Y chromosome. For details, see Ref. 1.

(2) Studies of Notch-Related MHC-Class III Gene INT3: Structure of the Human Counterpart of Mouse Mammary Tumor Gene int-3 and (CTG)n Polymorphism in the 5′-Upstream Region

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The cDNA clones for the human counterpart of mouse mammary tumor gene int-3, which is a human MHC class III gene and designated INT3, were isolated and sequenced. Mouse int-3 has been thought a Notch-homologous gene, but the extracellular portion of this predicted transmembrane gene has not been clarified. The human INT3 sequence determined in this work corresponds not only to the intracellular portion but also to the extracellular portion, being composed of Notch/lin-12 and EGF-like repeats, which are essential domains in Notch-homologues but uncharacterized for mouse int-3. A phylogenetic comparison of INT3 with other Notch homologues of a wide range of species revealed four subfamilies for mammalian Notch. In the 5′-upstream region of human INT3, we found (CTG)n repeats showing VNTR polymorphism for different HLA haplotypes.

Eight genes mapped on 6p21.3, including INT3, were found to have counterparts structurally and functionally similar to those mostly mapped on 9q33–q34, indicating the segmental chromosome duplication during the course of evolution (submitted).

(3) Precise Replication Timing around a Boundary of Long-Range G+C% Mosaic Domains in the Human MHC Region

Toyoaki TENZEN, Asao FUJIYAMA, Katsuzumi OKUMURA, Asako ANDO[1], Hidetoshi INOKO[1], Takashi GOJOBORI and Toshimichi IKEMURA ([1] School of Medicine, Tokai University, Isehara)

The human genome is composed of long-range G+C% (GC%) mosaic structures related to chromosome bands. The human MHC region has been found to have a boundary of megabase-level GC% mosaic domains in the junction area between MHC classes II and III. We determined the precise DNA replication timing for several loci in the classes II and III, especially focusing the GC% boundary region. The replication timings could be

determined at the kb-level resolution by quantifying pulsely labeled nascent DNAs which were isolated from synchronized HL60 cells. The class III region including TNX, AGER, and INT3 replicates in the second hour of S phase whereas the class II region including HLA-DRA, PABL1, and dense LINE-1 cluster replicates in the fourth hour of S-phase, *i.e.*, it makes the lag of two hours between the classes II and III. The replication timing changes around the 20-kb dense Alu cluster where a sharp GC% transition point exists, showing a good correlation between replication timing and GC% distribution.

(4) Detection of Genes in *Escherichia coli* Sequences Determined by Genome Projects and Prediction of Protein-Production Levels, Based on Multivariate Diversity in Codon Usage

Shigehiko KANAYA[1], Yoshihiro KUDO[1], Yasukazu NAKAMURA and Toshimichi IKEMURA ([1] Department of Electric and Information Engineering, Faculty of Engineering, Yamagata University, Yonezawa)

We developed measures (called Z-parameters in this study) which reflect the diversity of codon usage in *Escherichia coli* genes using principal component analysis. Protein-production levels for 1500 CDSs (protein-coding sequences) proposed by genome projects in Japan and USA were estimated by a correlation equation between Z1 and cellular protein content, which was obtained through advance analysis of the genes which were experimentally characterized. Through a profile analysis of Z1 for *E. coli* sequences determined in the Japan genome project, we predicted additional 36 CDSs that had not yet been annotated in the International DNA Databases. Thirty-two out of the thirty-six CDSs could be assigned to presumed protein genes also through a BLASTX search of recent protein databases in the Genome Net in Japan. Detailed examination of the Z1-parameter profile also led us to assess sequencing errors which cause frame-shift. For details, see CABIOS, in press.

(5) Tenascin-X Expression in Tumor Cells and Fibroblast in Culture

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Tenascin-X, a novel member of tenascin family, has recently been shown to be distinct and its distribution is often reciprocal to that of tenascin-C in the developing mouse embryo. We have investigated the expression of tenascin-X in fibroblasts and carcinoma cells. Tenascin-X protein was secreted *in vitro* in the conditioned media at an apparent molecular weight of 450 kDa, and fibroblasts contained a major tenascin-X isoform of 220 kDa. On Northern blots, a single major transcript with a size of 13 kb was detected. Overexpression of tenascin-X protein was not found in primary fibroblasts of knockout mice for tenascin-C gene. Steroid hormone glucocorticoids, were found to downregulate tenascin-X mRNA levels and protein synthesis in fibroblasts at the physiological concentrations. On the other hand, neither growth factors nor cytokines examined affected the expression level of tenascin-X.

As *in vivo* study, carcinoma cells were transplanted into nude mice. In contrast to the distinct tissue distribution of tenascin-X in adult skin, expression of tenascin-X protein during tumorigenesis was found to be downregulated considerably not only in tumor cells themselves but also in tumor stroma. These findings provide evidence that the expression of tenascin-X can be influenced by stromal-epithelial interactions. We have identified glucocorticoids as physiological inhibitors for tenascin-X and suggest that glucocorticoids may in part participate in the downregulation of tenascin-X in fibroblasts *in vivo*. For details, see Ref. 3.

(6) Codon Usage Tabulated from the International DNA Sequence Databases

Yasukazu NAKAMURA, Takashi GOJOBORI and Toshimichi IKEMURA

Codon usage in 87,602 genes was calculated using the nucleotide sequence data obtained from the GenBank Genetic Sequence Data Bank (Release 90.0; Sep., 1995). The database is called the CUTG Database. To reveal the characteristics of codon use of a wide range of organisms, as well as viruses and organella, the frequency (per one thousand) of codon use in 461

organisms for each of which more than 20 genes were available was calculated by summing up numbers of codons used. WWW clients, such as NCSA Mosaic and Netscape, can be used to query this file. A user can display a codon usage table by clicking an anchor for selecting species or searching with species' name. Complete form of the database is available by anonymous ftp from DDBJ: ftp://ftp.nig.ac.jp/pub/db/codon/GB90. The frequencies of codon use in the 461 organisms can be accessed on the following WWW server: http://tisun4a.lab.nig.ac.jp/codon/CUTG.html. For details, see Ref. 2.

(7) A Genetic Affinity Analysis of Human Populations

Naruya SAITOU

Genetic affinity of human populations based on allele frequency data was studied from two viewpoints. (1) The effect of the number of polymorphic loci on the reconstruction of a phylogenetic tree of human populations was empirically investigated. Affinity networks (unrooted trees) were constructed based on data for 1–12 polymorphic loci, by using the neighbor-joining method. Geographical clustering of populations gradually appeared while the number of loci was increased. A new classification and terminology of higher order human population clusters is proposed based on those and other studies. (2) A new method of estimating absolute divergence time of two populations is proposed, which is based on a diffusion equation that describes random genetic drift. For details, see Ref. 5.

(8) Methods for Building Phylogenetic Trees of Genes and Species

Naruya SAITOU

This chapter deals with phylogenetic trees of genes and species, that are fundamental not only for evolutionary studies but for molecular biology in general. The mathematical properties of phylogenetic trees such as the difference between rooted and unrooted trees and the number of possible tree topologies are first explained. Then the biological properties of phylogenetic trees in general is discussed with special reference to the difference between gene trees and species trees. The next section gives description of various

tree-building methods such as the unweighted pair group method with arithmetic mean (UPGMA), the neighbor-joining, the maximum parsimony, and the maximum likelihood methods with worked-out examples. Results from computer simulation studies and statistical tests of estimated phylogenetic trees follow. Introduction of various computer packages for tree-building analyses and future trends are given at the end. For details, see Ref. 6.

(9) Application of a Parallel Logic Programming for Reconstruction of Molecular Phylogenetic Trees Using the Maximum Likelihood Method

Satoshi OOTA[1], Naruya SAITOU and Susumu KUNIFUJI[1] ([1] Japan Advanced Institute of Science and Technology)

With rapid increase of DNA sequence data, it is required to develop reliable application programs to infer molecular phylogenetic trees in parallel environment. To implement a practical maximum likelihood method in parallel environment, we have developed programs *Contour/1* and *Traverse/3* to calculate likelihood values of arbitrary molecular phylogenetic trees. KLIC/KL1, a parallel logic programming language, was used for those programs. *Contour/1* generates log likelihood surfaces and we can investigate the surface to reach the maximum likelihood point, while *Traverse/3* searches the maximum likelihood values by traversing branches. We propose ideas how to implement the maximum likelihood method in a parallel environment. For details, see Ref. 7.

(10) Mitochondrial DNA Sequences of Various Species of the Genus Equus with a Special Reference to the Phylogenetic Relationship between Przewalskii's Wild Horse and Domestic Horse

Nobushige ISHIDA[1], Tsendsuren OYUNSUREN[2], Suguru MASHIMA[1], Harutaka MUKOYAMA[1] and Naruya SAITOU ([1] Equine Research Institute, Japan Racing Association, Japan, [2] Department of Molecular Biology, Institute of Biotechnology, Mongolia)

The non-coding region between $tRNA^{Pro}$ and the large conserved sequence block is the most variable region in mammalian mitochondrial DNA D-loop region. This variable region (*ca.* 270 bp) of four species of *Equus*, including

Mongolian and Japanese native domestic horses as well as Przewalskii's (or Mongolian) wild horse, were sequenced. These data were compared with our recently published Thoroughbred horse mitochondrial DNA sequences. The evolutionary rate of this region among the four species of *Equus* was estimated to be $2\text{–}4 \times 10^{-8}$ per site per year. Phylogenetic trees of *Equus* species demonstrate that Przewalskii's wild horse is within the genetic variation among the domestic horse. This suggests that the chromosome number change (probably increase) of the Przewalskii's wild horse occurred rather recently. For details, see Ref. 8.

(11) A Genetic Study of 2,000-Year-Old Human Remains from Japan Using Mitochondrial DNA Sequences

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We present nucleotide sequence data for mitochondrial DNA extracted from ancient human skeletons of the Yayoi era (*ca.* 2,000 BP) excavated from the Takuta-Nishibun site in northern Kyushu of Japan. Nucleotide sequence diversity showed that the Yayoi people of the Tak excavated from the Takuta-Nishibun site in northern Kyushu of Japan. Nucleotide sequence diversity showed that the Yayoi people of the Takuta-Nishibun site were not a genetically homogeneous population. This site shows a diversity in the burial style. Phylogenetic analysis indicated a statistically significant correlation between burial style and the genetic background of the Takuta-Nishibun individuales, and revealed no discrete clustering patterns for the Yayoi individuales, for early modern Ainu, or for the Jomon people. For details, see Ref. 9.

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D–c. Division of Theoretical Genetics

(1) A Genetic Perspective on the Origin and History of Humans

Naoyuki TAKAHATA

Recent topics in molecular anthropology are reviewed with special reference to hominoid DNA sequences and population genetics theory. To cover a wide range of demographic situations in the human lineage since the Miocene, a model is introduced that allows temporal changes in population structure and size. The coalescence process of neutral genes is formulated and used to make quantitative inferences on the origin and history of humans. Nuclear DNA sequence data support the theory that humans and chimpanzees diverged from each other 4.6 million years (*my*) ago and the gorilla lineage branched off as early as 7.0–7.4 my ago. The same data estimate the effective size of the Pliocene hominoid population as 10^5, a figure similar to that obtained independently from alleles that have persisted in the human population for more than 5 *my*. Hypotheses about the origin of *Homo sapiens*, genetic differentiation among human populations, and changes in population size are quantified. None of the hypotheses seems compatible with the observed DNA variation. The effective size decreased to 10^4 in the Pleistocene, suggesting an important role of extinction/restoration in *H. sapiens* populations. Natural selection against protein variation might be relaxed in the Pleistocene. The history of *H. sapiens* appears to have been more dynamical than is postulated by the hypotheses. For the details, see Ref. 1.

(2) MHC Diversity and Selection

Naoyuki TAKAHATA

The major histocompatibility complex (*Mhc*) contains a family of genes whose products play crucial roles in self and nonself discrimination. Self is one's own peptides that bind to Mhc molecules and restrict the T-cell repertoire in the thymus, whereas nonself is processed peptides of foreign antigens that can be presented in the context of Mhc molecules and trigger

the immune response. Since the immune system thus controlled by Mhc molecules affects the survival of individuals, the dual function of *Mhc* itself must be subjected to natural selection. The operation of natural selection calls for the necessity of studying *Mhc* genes from a viewpoint of evolutionary population genetics. This paper is concerned with convergence in sequence motifs in the peptide-binding region (PBR) and T-cell mediated evolutionary forces for Mhc molecules. For the details, see Ref. 2.

(3) Divergence Time and Population Size in the Lineage Leading to Modern Humans

Naoyuki TAKAHATA, Yoko SATTA and Jan KLEIN

We have developed maximum likelihood (ML) methods for comparisons of nucleotide sequences from unlinked genomic regions. In the case of a single species, the ML method primarily estimates the effective population size (N_e) under both constant size and abrupt expansion conditions. In the case of two or three species, the ML method simultaneously estimates the species divergence time and the effective size of ancestral populations. This allows us to trace the evolutionary history of the human population over the past several million years (my). Available sequences at human autosomal loci indicate $N_e = 10{,}000$ in the Late Pleistocene, a figure concordant with the results obtained from mitochondrial DNA sequence and allele-frequency data analysis, and there is no indication of population expansion. The ML analysis of two species shows that humans diverged from chimpanzees 4.6 my ago and that the human and chimpanzee clade diverged from the gorilla 7.2 my ago. Furthermore, the effective population size of humans more than 4.6 my ago is nearly ten times larger than N_e of modern humans. The effective population size in the human lineage does not seem to have remained constant over the past several million years. The ML model for three species predicts slightly results different from, but consistent with those obtained by the two-species analysis. For the details, see Ref. 3.

(4) A Population Genetic Study on the Evolution of SINEs. II. Sequence Evolution under the Master Copy Model

Hidenori TACHIDA

A transient population genetic model of SINE evolution which assumes the master copy model was theoretically investigated. The means and variances of consensus frequency, nucleotide homozygosity, and the number of shared differences considered to have been caused by mutations occurring in master copy lineages were computed. All quantities investigated were shown to be monotone functions of the duration of the expansion period. Thus, they can be used to estimate the expansion period although their sampling variances were generally large. Using the theoretical results, the Sb subfamily of human *Alu* sequences was analyzed. First, the expansion period was estimated from the observed mean and variance of homozygosity. The expansion period was shown to be short compared to the time since the end of the expansion of the subfamily. However, the observed number of shared differences was less than one half of that expected under the master copy model with the estimated expansion period. Alternative models including one with multiple master copy loci as explanation of this observation were discussed.

(5) Molecular Evolution of the 5′-flanking Regions of Duplicated *Amy* Genes in the *Drosophila melanogaster* Species Subgroup

Eisaku OKUYAMA, Hiroki SHIBATA, Hidenori TACHIDA and Tsuneyuki YAMAZAKI

The nucleotide sequences of the 5′-flanking regions of duplicated *Amy* genes in eight sibling species belonging to the *melanogaster* species subgroup were analyzed. In *Drosophila melanogaster*, about 450 bp immediately upstream of the translation initiation site of the two paralogous genes (the proximal and distal genes) have sequence similarities. However, we could not detect any significant sequence similarity in the upstream region beyond −450. This result indicates that the coding regions of the ancestral *Amy* gene were duplicated together with 450 bp of the 5′-flanking region as one unit. Multiple alignment of these 450 bp sequences in the proximal and distal genes of all eight species revealed a mosaic pattern of highly conserved and

divergent regions. The conserved regions included almost all the putative regulatory elements identified in previous analyses of the sequences. A phylogenetic analysis of the aligned sequences shows that these 450 bp sequences are clustered into proximal and distal groups. As a whole, the divergence between groups in this region is very large in contrast to that in the coding regions. Based on the divergence between groups, the 450 bp region is divided into two subregions. We found that ratios of the divergence between groups to that within groups differ in the two subregions. From these observations, we discuss the possibility of positive selection acting on the subregion immediately upstream of the *Amy* coding region to diverge regulatory elements of the paralogous genes.

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E. DEPARTMENT OF INTEGRATED GENETICS

E–a. Division of Human Genetics

(1) Isolation of Transcribed Sequences in Cloned Genomic DNA with Known Location on the Human Chromosome 18

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Chromosome 18 is involved in tetrasomy 18p and in trisomy 18 syndromes. The latter is the second most common abnormality after Down syndrome which is caused by an excessive number of chromosome. To identify genes involved in these disorders, construction of high-resolution expression map of this chromosome is a rational strategy. Identification and recovery of transcribed sequences from cloned mammalian genomic DNA remains an important problem in isolating genes on the basis of their chromosomal location. We have developed a strategy that facilitates the recovery of transcribed sequences from random pieces of cloned genomic DNA. Thus, we isolated 600 cosmid clones of which 60 new cosmids are mapped on the short and long arms of chromosome 18 either by R or by DAPI banding and simultaneous fluorescence *in situ* hybridization. In the further extension of this approach, cDNA library has been probed with pools of microclones from a cosmid clone. Our approach to generate region-specific cDNA libraries used hybridization of cDNA library inserts to genomic DNA from cosmids, and subsequent cloning of annealed cDNAs. In one application of the scheme, human fetal brain cDNA library was hybridized to linker-adaptor ligated fragments of a digested cosmid, and selected cDNAs are amplified by PCR before cloning.

While considerable progress has been made in genetic and physical mapping of the human genome, approaches are now needed to identify the protein coding sequences contained therein. The ultimate goal would be an integrated genetic, physical and transcriptional map of the genome, combining information on the position of a gene, its sequences and its pattern of expression. Since most genes are composed of multiple exons and the

identification of a gene requires the recovery of only a single exon, this consideration should not be a limiting factor. These candidate exons are ideally suited for establishing the presence of a gene in a cosmid insert and facilitating the subsequent isolation of this gene. Our current experience suggests that as many as 20 cosmids can be screened concurrently in a 4 week period, and that every one or two cosmids contain at least one transcribed sequence. Screening of uncharacterized cosmids will determine the utility of transcribed sequence fragments of known chromosomal location, as identifiers of candidate coding sequences. For the details, see reference 1.

(2) Isolation of Transcribed Sequences by Kinetic Enrichment of cDNA Fragments Derived from Chromosome 15 and Molecular Definition of the Prader-Willi Syndrome

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The prader-Willi syndrome (PWS) is characterized by generalized muscular hypotonia and severe feeding problems in early infancy, followed by hyperphagia and subsequent central obesity in childhood. In addition to short statue and characteristic dysfunction causing hypogonadism, dysmorphic signs such as almond-shaped palpebral fissures, narrow bifrontal diameter, down-turned mouth and acromicria become evident. In time, patients may manifest intellectual deficiency, behavioral abnormalities, and poor articulations. Originally defined as a clinical entity, PWS was later found to be associated with abnormalities of chromosome 15, in particular a small interstitial deletion of bands q11–q13 that involve the paternally derived chromosome 15. Non-deletion PWS patients exihits maternal disomy for chromosome 15 further demonstrating that loss of the expressed paternal alleles of maternally imprinted genes is responsible for the PWS phenotype. Conversely, maternally derived deletions of this region or paternal disomy are associated with the Angelman syndrome (AS). AS can also be caused by paternal disomy or maternally inherited as yet unidentified submicroscopic mutations. Thus, PWS and AS are distinct neurogenic disorders, caused by the loss of function of closely linked genes on chromosome 15. Paternal deletion and maternal disomy in PWS suggest that the PWS genes are transcribed from the paternal chromosome only. Likewise, maternal dele-

tions and paternal disomy in AS suggest that the AS genes are transcribed from the maternal chromosome only.

Lymphoblastoid cell lines from 5 PWS patients and one AS patient and those from healthy individuals were made in this laboratory. Messenger RNAs were prepared from the cells, from which the cDNA libraries were constructed. A system was developed in which subtraction and kinetic enrichment was used to purify restriction endonuclease fragments present in a population of cDNA fragments from the normal individual but not in the other from the patient. Application of this method to cDNA library of reduced complexity as compared to the genomic DNAs resulted in the isolation of 13 probes present as single copies in the normal library. These probes were 120–500 base pairs in length, which could detect difference between transcribed sequences of patient's cells and those of normal individuals. In principle, this system was used for isolating probes linked to maternally imprinted genes on the chromosome 15 PWS region. Results of initial analysis of mRNAs from lymphoblastoid cell lines implied that these sequences are transcribed in the normal individuals but not in PWS patients. A mechanism of imprinting may have evolved in mammals because of conflicting interests of maternal and paternal genes in relation to the transfer of nutrients from the mother to her offspring. This hypothesis predicts most imprinted genes will affect how much nourishment an offspring receives from its mother at the expense of its siblings. Imprinted genes will include loci that influence placental growth, suckling, neonatal behavior, appetite control, nutrient metabolism, and postnatal growth. Perhaps imprinted gene in PWS region, and in Angelman region as well, is just such a gene. The mapping of genes identified here to within the PWS critical region, together with the indications that they function in the brain and may play a role in regulating feeding behavior, should prompt us further to predict these genes as a candidate imprinting loci responsible for syndrome.

(3) A Signal Transduction Pathway of Interleukin-4 and Interleukin-13 in Human B Cells Derived from X-linked Severe Combined Immunodeficiency Patients

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Interleukin-4 (IL-4) activates members of a few tyrosine kinase family, JAK1 and JAK3, and a cytoplasmic protein termed STAT (signal transducers and activators of transcription) by tyrosine phosphorylatioion. The IL-4 receptor has been demonstrated to share the common gamma chain (gc) with IL-2, IL-7 and IL-9 receptor systems. Mutations of gc are known to result in human X-linked severe combined immunodeficiency (XSCID). Since JAK3 has been shown to associate with gc, we analysed activation of JAK–STAT pathway in EBV-transformed B cells established from two XSCID patients (XSCID-B) upon IL-4 stimulation in order to investigate the involvement of gc in IL-4 mediated signal transduction. We detected no tyrosine phosphorylation of JAK3 in two XSCID-B cell lines, whereas tyrosine phophorylation of JAK3 in EBV-transformed normal B cell lines was clearly detected. Using electrophoretic mobility shift assay, we found that STAT was activated by IL-4 in normal B cells, but not in XSCID-B cell lines. These results indicate that JAK–STAT pathway in IL-4 signal transduction is impaired in XSCID, further suggesting that gc plays a pivotal role in activation of JAK–STAT pathway by IL-4. For the details, see Ref. 1.

(4) A Synthetic Peptide Corresponding to a Critical Intracellular Signalling Region of the Human IL-4 Receptor Inhibits IL-4-Dependent Proliferation

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We have identified the critical region for growth signal transduction in the cytoplasmic domain of the human IL-4 receptor (hIL-4R). IL-4R is likely to associate with other signal transducing molecules through this critical cytoplasmic region. We tested how a synthetic peptide (SP-1), corresponding to

this critical cytoplasmic region interfered with IL-4-dependent proliferation by competition with the IL-4R in binding to intracellular signal transducing molecules. Our results indicated that 100 mg/ml of SP-1 peptide completely inhibited hIL-4-induced proliferation of Ba/F3 cell transfectants expressing the full-length hIL-4R. In contrast, an alternated synthetic peptide corresponding to the C-terminal region of the hIL-4R (SP-2) did not affect hIL-4-induced proliferation of hIL-4R–Ba/F3 transfectants. The specificity of SP-1 mediated inhibition of IL-4-induced proliferation was further supported by the fact that SP-1 peptide had no effect on IL-3-induced proliferation of the same hIL-4–Ba/F3 transfectants. In addition, SP-1 peptide did not affect either IL-2-induced proliferation of Ba/F3 transfectants expressing the human IL-2 receptor b chain (hIL-2Rb), or hIL-4-induced proliferation of Ba/F3 transfectants expressing a chimeric receptor consisting of a hIL-4R extracellular domain and a hIL-2Rb cytoplasmic domain. For the details, see Ref. 2.

(5) Recent African Origin of Modern Humans Revealed by Complete Sequences of Hominoid Mitochondrial DNAs

Satoshi HORAI, Kenji HAYASAKA, Rumi KONDO[1], Kazuo TSUGANE and Naoyuki TAKAHATA[1] ([1]The Graduate University for Advanced Studies)

Extensive analyses of cleavage sites and sequences of mitochondrial DNA (mtDNA) have shown that African is most variable among various ethnic groups, and have supported an earlier suggestion that the last common ancestor (LCA) of contemporary human mtDNAs existed some 200,000 years ago. These results have been taken as evidence for the notion that modern humans originated in Africa and migrated to Eurasia, replacing *Homo erectus* and the Neanderthals with no or little gene exchanges. However, this notion was strongly disputed by proponents of the multiregional hypothesis, who claim that modern humans originated simultaneously in various geographic regions and that regional continuity in human characters has evolved over the past 1 million years (Myr). Under this hypothesis, the age of the LCA is unlikely to be as low as 200,000 years. The main controversy, therefore, has centered around the estimated age of the LCA and the reliability of the mitochondrial molecular clock (constancy of nucleotide substitution rates) on which it is based. To resolve close relation-

ships among humans, recent studies have focused on sequence analysis in the rapidly evolving D (displacement)-loop region of mtDNA. Sequence data from the D-loop region alone are insufficient for the accurate estimation of substitution rates and divergence times of hominoid mtDNAs, and sequences from coding regions are required. To this end, we have analyzed the complete mtDNA sequences of three humans (African, European and Japanese), three African apes (common and pygmy chimpanzees, and gorilla), and one orangutan in an attempt to estimate most accurately the substitution rates and divergence times of hominoid mtDNAs. Nonsynonymous substitutions and substitutions in RNA genes have accumulated with an approximately clock-like regularity. From these substitutions and under the assumption that the orangutan and African apes diverged 13 million years ago, we obtained a divergence time for humans and chimpanzees of 4.9 million years. This divergence time permitted calibration of the synonymous substitution rate (3.89×10^{-8}/site/year). To obtain the substitution rate in the D-loop region, we compared the three human mtDNAs and measured the relative abundance of substitutions in the D-loop region and at synonymous sites. The estimated substitution rate in the D-loop region was 7.00×10^{-8}/site/year. Using both synonymous and D-loop substitutions, we inferred the age of the last common ancestor of the human mtDNAs as $143{,}000 \pm 18{,}000$ years. The shallow ancestry of human mtDNAs, together with the observation that the African sequence is most diverged among humans, strongly supports the recent African origin of modern humans, *Homo sapiens sapiens.* For the details, see Refs. 15, 16 and 17.

(6) Different Waves of Migration to the New World: Implications of Mitochondrial DNA Polymorphism in Native Americans

Satoshi HORAI

Mitochondrial DNA sequences of the major noncoding region (482 base pairs) were determined for 72 Native Americans from 16 different local populations in the South, Central and North Americas. On the basis of a sequence comparison, 43 different types were observed. Phylogenetic analysis revealed that most Native American lineages are classified into four major distinct clusters. Individuals belonging to each cluster share at least two specific polymorphic sites that are nearly absent in other human populations,

indicating a unique phylogenetic position of Native Americans. Phylogenetic analysis was extended with 193 individuals including Africans, Europeans, Asians and Native Americans, indicating that the four Native American clusters are distinct and dispersed in the tree. Although a majority of members are Native Americans, there are some Asian individuals in each cluster. For example, in cluster II, one Japanese appeared together with two Native Americans in a small cluster which diverged at a final branching point. This coalescence occurs at only 7% in length of the total way from the tips to the root of the tree. As the Japanese and the Native Americans reside on two different continents, ancestors of these Native Americans must have migrated after the time of initial coalescence of the two lineages. Assuming that the human mitochondrial lineage divergence took place 143,000–179,000 yr. B.P. (Horai *et al.* 1995), the dates of this initial coalescence were estimated as 10,000–12,500 yr. B.P. The same magnitude of coalescence between the Asians and Native Americans are also observed in other three clusters. The phylogenetic tree also suggests that the four distinct clusters containing Native Americans may represent different waves of migration to the New World. The coalescence time within each cluster is less than one-fourth of the coalescence time for the entire tree. However, the coalescence time of the four clusters is a little longer than half the latter coalescence time. It appears more likely that individuals in the four clusters are descendants of four different ancient populations, which had been well isolated from each other for a relatively long period of time. I therefore postulate that the four clusters represent different waves of migration to the New World. Although it is difficult to determine the times of occurrence of the four waves of migration, they seem to have taken place 10,000–12,500 yr. B.P. For the details, see Ref. 18.

(7) Y Chromosomal DNA Variation and the Peopling of Japan

Michael F. HAMMER[1] and Satoshi HORAI ([1] Arizona University)

Four loci mapping to the nonrecombining portion of the Y chromosome were genotyped in three Japanese populations (Okinawa, Shizuoka and Aomori) and Taiwanese. The Y Alu polymorphic (YAP) element is present in 42% of the Japanese and absent in the Taiwanese, confirming the irregular distribution of this polymorphism in Asia. Data from the four loci were used

to determine genetic distances among populations, construct Y chromosome haplotypes, and estimate the degree of genetic diversity in each population. Evolutionary analysis of Y haplotypes suggests that polymorphisms at the YAP (DYS287) and DXYS5Y loci originated a single time, whereas restriction patterns at the DYS1 locus and microsatellite alleles at the DYS19 locus arose more than once. Genetic distance analysis indicated that the Okinawans are differentiated from Japanese living on Honshu. The data support the hypotheses that modern Japanese populations have resulted from distinctive genetic contributions involving the ancient Jomon people and Yayoi immigrants from Korea or mainland China, with Okinawans experiencing the least amount of admixture with the Yayoi. It is suggested that YAP(+) chromosomes migrated to Japan with the Jomon people >10,000 years ago and that a large infusion of YAP(−) chromosomes entered Japan with the Yayoi migration starting 2,300 years ago. Different degrees of genetic diversity carried by these two ancient lineages may be explained by the different lifestyles of the migrant groups, the size of the founding populations, and the antiquities of the founding events. For the details, see Ref. 19.

(8) Molecular Phylogeny of Gibbons Inferred from Mitochondrial DNA Sequences: Preliminary Report

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A large amount of data have been accumulated to elucidate phylogenetic relationships within Hominoidea (humans and apes). These studies revealed that the closest relative to human are African apes, ultimately chimpanzees. The phylogenetic position of gibbons (family Hylobatidae) as a sister group to human and great apes was also supported by these studies. Gibbons, genus *Hylobates*, inhabit in a wide range of Southeast Asia, from eastern India to southern China on the mainland, as well as Borneo, Java, Sumatra, and the Mentawai islands. Nine nominal species are generally accepted. They are the concolor gibbon (*H. concolor*, lives in Vietnam, Laos, Yunnan, and Hainan Island), the siamang (*H. syndactylus*, in Sumatra and Malaya), the hoolock gibbon (*H. hoolock*, in Assam, northern Burma, and Bangladesh), the Kloss's gibbon (*H. klossii*, in the Mentawai Islands), the white-handed gibbon (*H.*

lar, in Thailand, southeastern Burma, Malaya, and northern Sumatra), the agile gibbon (*H. agilis*, in Malaya, central and southern Sumatra, and southwestern Borneo), the pileated gibbon (*H. pileatus*, in southwestern Thailand and Kampuchea), the silvery gibbon (*H. moloch*, in Java), and Muller's gibbon (*H. muelleri*, in Borneo). The former three species are placed in monotypic subgenera *Nomascus*, *Symphalangus* and *Bunopithecus*, respectively. The latter six species are classified into the subgenus *Hylobates*. In the subgenus *Hylobates*, the latter five species, *lar*, *agilis*, *pileatus*, *moloch*, and *muelleri*, are called the *lar*-group or *lar* species complex, because they share derived morphological characters, such as very dense fur and large ears. *klossii* is excluded from lar-group due to lack of the synapomorphic characteristic of the lar-group. We analyzed the 896 base-pair (bp) mitochondrial DNA fragment comprising partial sequences of ND4 and ND5 (NADH dehydrogenase complex) genes and adjacent three complete tRNA genes ($tRNA^{His}$, $tRNA^{Leu(CUN)}$, and $tRNA^{Ser(AGY)}$) from seven gibbons, representative of three out of four subgenera. A result from our molecular analysis is consistent with the previous studies as to the monophyly of subgenus *Hylobates* species, yet the relationship among subgenera remains slightly ambiguous. A striking result of the analysis is phylogenetic location of the Kloss's gibbon (*H. klossii*). The Kloss's gibbon has been considered to be an initial off-shoot of the subgenus *Hylobates* because of its morphological primitiveness. However our molecular data strongly support the Kloss's gibbon speciated most recently within the subgenus *Hylobates*. For the details, see Ref. 20.

(9) Orosomucoid Phenotyping with Monoclonal Antibodies: Polymorphic Occurrence of *ORM1*Q0* in Aboriginal Taiwanese Populations

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Human orosomucoid (ORM) or α_1-acid glycoprotein is a major acute-phase plasma protein with a molecular mass of approximately 49 kD, of

which 45% is carbohydrate. ORM is predominantly produced in the liver and suppresses blastogenesis of lymphocytes and neutrophil activation. ORM shows extensive genetic variation, and about 30 alleles have been described at the two loci ORM1 and ORM2. The ORM1 and ORM2 alloproteins are encoded by two closely linked genes on the long arm of chromosome 9 and are distinguishable from each other in band intensity. In the course of ORM phenotyping, we have occasionally encountered some bands for which it was difficult to determine whether they belonged to the ORM1 or ORM2 product. To overcome such confusions, we attempted to prepare locus-specific monoclonal antibodies. Using polyclonal and monoclonal antibodies, we performed ORM phenotyping on native Taiwanese populations and carried out ORM1 Q0 typing.

Three monoclonal antibodies (OR35, OR40 and OR48) against orosomucoid (ORM) were prepared for the phenotyping of the human ORM system. The OR35 and OR48 antibodies recognized ORM1 and ORM2 products, respectively. OR40 reacted strongly to the products of ORM1 but poorly to those of ORM2. With the help of these monoclonal antibodies, ORM phenotyping was performed on 658 individuals from nine subpopulations of aboriginal Taiwanese, with close attention to two individuals with an ORM1 Q0 homozygous phenotype. The *ORM1*Q0* allele was found to be at a polymorphic frequency in eight of the nine subpopulations. For the details, see Ref. 21.

(10) Genetic Polymorphism of ADA in Taiwan Aboriginal Populations: New ADA Variants Detected by Isoelectric Focusing Method

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Human adenosine deaminase (ADA) catalyzes the conversion of adenosine to inosine with the release of ammonia. Genetic polymorphism of ADA has been recognized by Spencer *et al.* (1968) by means of starch-gel electrophoresis. In the following two decades, several new genetic variants of ADA were recognized by the same method. In the past, the investigation of the ADA polymorphism has been carried out in most human population studies

using starch gel electrophoresis. In the present study, we improved the method in typing of ADA by employing isoelectric focusing (IEF). Nine aboriginal populations (Gaoshan) of Taiwan including 654 individuals were investigated. The improved method led us to find two new variants, which were tentatively named *ADA*Taiwan1* and *ADA*Taiwan2*. It was also found that one of the common alleles *ADA*2*, had relatively high frequencies in some (the Paiwans and the Amis) of the Taiwan aboriginal populations. For the details, see Ref. 22.

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E-b. Division of Agricultural Genetics

(1) Isozyme Variation in Asian Common Wild Rice *Oryza rufipogon*

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A total of 106 strains of Asian wild rice, *Oryza rufipogon*, collected from 13 countries were examined for isozyme variation. Polymorphism at 21 loci was

detected using starch and poly-acrylamide gel systems.

The geographical distribution area was divided into four regions, China, Indochina, the Indian subcontinent and the Malayan Peninsula. Strains from China were found to carry some alleles at high frequencies which were rarely found in other regions. Of them, *Cat-1*^{2}, *Est-13*^{1} and *Est-12*^{0} are known to be specific to Japonica cultivars. Genetic distances between the four regions and average gene diversities within region were estimated. Genetic distances ranged from 0.085 (Indian subcontinent and Indochina) to 0.254 (China and the Malayan Peninsula).

To examine overall variation patterns, data for 21 loci was analyzed by factor analysis. Variation explained by the first three factors was only 24% of the total variation, indicating that differentiation within *O. rufipogon* is not so distinct at the isozyme level. The first factor extracted a variation characterized by the alleles at *Cat-1* and *Est-13*, which are differentially distributed in the wild rices of China and other regions and are also diagnostic in classifying Indica and Japonica cultivars. The second factor distinguished a few strains carrying a rare allele, *Est-5*^{3}. The third factor extracted a variation characterized by the alleles at *Acp-1* and *Pox-2*, which are also Indica-Japonica diagnostic loci. A scatter diagram of 106 strains plotted by the first and third factor scores showed that Chinese strains and the strains from other regions are separated. Strains from the Indian subcontinent tend to be located in between the Chinese and Indochina/Malayan Peninsula strains. Perennial and annual types, which are the two major ecotypes, differentiated within this species were not detected at the isozyme variation level.

In order to examine to what extent alleles at different loci are nonrandomly associated in Asian wild rice, corresponding to Indica-Japonica differentiation in cultivars, we calculated the R^2 (squared correlation coefficient of allelic frequencies) between 8 Japonica-Indica diagnostic loci. Several combinations showed significant nonrandom associations. But generally the R^2 values were much lower ($R^2=0.022$) than those found in *O. sativa* ($R^2=0.382$, Sano and Morishima 1992). This indicates that no clear Japonica-Indica differentiation occurs in *O. rufipogon*.

From the above results, it may be concluded that *O. rufipogon* has a continuum at the isozyme level, but tends to be differentiated geograpically. For the details, see Ref. 2.

(2) QTL Analysis of Annual/Perennial Life-History Variation in *Oryza rufipogon*

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The use of molecular markers to identify quantitative trait loci (QTL) has become a key approach in modern breeding programs. This approach, however, has been little used as a means to understand the genetic basis of ecologically important adaptation. In this study, we use molecular markers to map genomic regions controlling trait variation among annual and perennial ecotypes of wild rice, *Oryza rufipogon.* Annual types exhibit early heading, self-fertilizing flowers, high seed yield and low regenerating ability. Perennial types exhibit late heading, mixed-mating flowers, low seed yield and high vegetative reproduction.

One hundred and one F_2 offspring from a cross between annual and perennial strains were used. Each F_2 plant was split into two clones and grown as replications. Various quantitative traits related to life history were measured. The broad sense heritability of each trait and genetic correlations among traits were calculated. Each plant was scored for 40 molecular markers (one isozyme locus and 39 genomic RFLP probes provided by the Rice Genome Research Program, Japan and from Cornell University). Single and multiple regression were used to estimate the proportion of phenotypic and genotypic variation explained.

Genotype effects were significant for all traits and broad-sense heritabilities ranged from 0.81 to 0.37. Traits expressed early in the life cycle (*e.g.* heading date) or features of highly canalized floral structures (*e.g.* anther length) had higher heritabilities than did traits expressed late in the life cycle, or composite traits such as the ratio of panicle number to tiller number. As expected, many traits were correlated and negative genetic correlations were found between most measures of fertility and vegetative persistence.

Single QTL explained up to 27% of the phenotypic variation in the traits examined. Multiple regressions explained 7–38% of the phenotypic and 18–62% of the genotypic variation in each trait examined. Some QTL appear to explain significant proportions of variation in several different traits, suggesting the possibility of pleitropic effects. In particular, marker G1184Ca located on chromosome 1, explained significant variation in heading date,

height, panicle number/tiller number, panicle length, and post-harvest regeneration.

In many other studies, chromosomal regions controlling large fractions of phenotypic variation have been found. In contrast, we found only one case in which greater than 25% of the phenotypic variation was explained by a single marker. One reason was the moderate heritability of the traits under study. Lack of marker saturation might also have reduced the explanatory capacity of markers. Finally, ecological adjustments that contribute to intraspecific annual/perennial differentiation may be more polygenic than the species-level differences examined in previous studies. Annual and perennial ecotypic syndromes reflect differences in allocation to reproduction versus survival and vegetative spread. Little is known about the genetics of alternative allocation patterns and the results of this study should be of interest to evolutionary and developmental biologists as well as to those involved in plant breeding.

(3) Ecological Genetic Studies on American Wild Rice (*Oryza glumaepatula* Steud.) Distributed around the Amazon Basin

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Oryza glumaepatula distributed in the tropical America is a wild-rice species having an AA genome in common with cultivated rice and their wild relatives. Populations of this species growing in the flood plain of the Amazon, where the annual oscillation of water level is about 10 m, show unique life-history traits. Most probably they propagate mainly by seeds. To study the influence of life-history on the genetic population structure, we examined several phenotypic characteristics supposedly related to life history and allozyme variability of natural populations of *O. glumaepatula* collected in the Amazon basin.

a) Germination tests indicated that the kernel dormancy (physiological dormancy) is not so strong. However, the seeds showed grain dormancy (physical dormancy) to some extent which restricted germination under deep water.

b) They had high deep-water tolerance conferred with internode elonga-

tion and culm brittleness. On the other hand, they seemed to have weak deep-water resistance. Large amounts of resources are used to vegetative organs even after heading. This characteristicis is assumed to be necessary for surviving the flood conditions of the Amazon.

c) *O. glumaepatula* had well developed anthers and produced a large amount of pollen, as much as the outbreeding ecotype of Asian *O. rufipogon.* But the proportion of resource allocation to anther and stigma was not so high as expected from outbreeding plants.

d) Observed heterozygosities were much lower than expected and the Hardy-Weinberg equilibrium did not hold in the populations. This suggests that Amazonian *O. glumaepatula* developed self-pollination system.

e) Allozymes were not so variable and overall gene diversity was low (=0.113) as compared with those of Asian wild rice *O. rufipogon.* Presumably, a bottleneck caused by periodical extinction of populations, scarceness of inter-populational hybridization owing to a self-pollinating nature and the absence of related species growing sympatrically may hamper the development of allozyme variability.

f) It was found that gene diversities gradually increased going down from the upper to lower basin. This is probably because gene flow has proceeded in a one-way direction from the upper to lower basin. A low degree of inter-populational differentiation ($D_{ST}=0.050$ and $H_S=0.068$) was found, contrary to the expectation that they would be predominantly selfing. The plants appear to be able to migrate a long distance by seeds drifting on the river, resulting in frequent inter-regional gene flow in the basin. For the details, see Ref. 1.

(4) Gene Regulation of Two Alleles at the *wx* Locus of Rice

Hiro-Yuki HIRANO, Mitsugu EIGUCHI and Yoshio SANO

The rice *wx* locus controls amylose synthesis in the endosperm and has two functional alleles, Wx^a and Wx^b, which were initially defined on the basis of the amount of their gene product, the Wx protein. The Wx^a allele produces about 10-fold higher levels of mRNA and protein than Wx^b does. Wx^b is found in *Oryza sativa* Japonica, and Wx^a is found in *O. sativa* Indica and various wild rice species including *O. rufipogon.* Since both cultivated rices, Japonica and Indica, evolved from a wild rice, *O. rufipogon*, Wx^b is thought

to have been derived from *Wx*^{a}. What kind of mutation caused such a large change in the level of *Wx* gene expression during the short period of the evolution of Japonica? We have great interest in the molecular mechanism(s) underlying the differences in expression levels of *Wx*^{a} and *Wx*^{b} as related to the evolution of gene regulation.

The rice *Wx* gene has a long intron (>1 kb) upstream of the ATG codon. The DNA sequence (PI-DNA) that includes the promoter region (P) and the first intron (I) was fused to the GUS gene and GUS activity analyzed in a transient assay system using rice protoplasts. The PI-DNA from *Wx*^{a} showed about 100-fold higher activity for driving the GUS gene than that from *Wx*^{b}. To identify the sequence responsible for the large difference in expression level in the two alleles, various chimeric sequences of the PI-DNA consisting of *Wx*^{a} and *Wx*^{b} were constructed and used for a transient assay. The results indicated that the sequence responsible for the difference was not in the promoter region but in the downstream region (about 140 bp) of the initiation codon, where two sequence differences were detected between *Wx*^{a} and *Wx*^{b}. One difference was the number of CT repeats before the 1st intron. Another difference was the splice donor site of the 1st intron. The donor sequence of *Wx*^{b} was TT, while that of *Wx*^{b} was the normal sequence, GT. The Northern blot analysis for the transcript from the seeds of Japonica showed a larger band including the 1st intron, in addition to the mature transcript. It seems likely that poor efficiency in splicing causes the lower transcription of *Wx*^{b}, or that quick degradation of the abnormal transcript results in a reduced level of the mature transcript in Japonica. Alternatively, the number of CT repeats may affect the transcription. Further analysis involving site-directed mutagenesis at the splice donor site are in progress to ascertain which mutation affects the level of transient expression of GUS derived by PI-DNA.

(5) Genetic Analysis of Chromosome 7 in an Annual Type Wild Rice

Mitsugu EIGUCHI and Yoshio SANO

In order to look into the genetic divergence of wild and cultivated rice, a segment of chromosome 7 was examined after introducing it into a cultivated form from a common wild rice through successive backcrossings. An annual type wild rice from India (W107) was crossed to a near-isogenic line of

Taichung 65 (Japonica type from Taiwan) having *g* (long empty glume) and *lg* (liguleless) which was used as the reccurent parent (T65*glg*). W107, like most common wild rice shows red pericarps (*Rc*) and short empty glumes (g^+) and T65*glg* shows white pericarps and long empty glumes. The two dominant genes were used to transfer the segment with *Rc* and g^+. The genetic divergence on the segment linked to *Rc* needs to be studied since weedy forms frequently show red pericarps even after introgression, suggesting the adaptive significance of the segment under natural conditions.

Two dominant genes were detected on the segment. One was responsible for short stature only in the vegetative phase (tentatively called *Ssv*) while no difference in culm length was observed at the flowering stage. Dominant dwarfism expressed at the vegetative phase has not been reported so far. The other gene was responsible for late heading in Mishima (tentatively called *Lh*) while plants with *Lh* headed earlier than T65*glg* in a short-day field, which indicated that the dominant gene may be a photosensitivity gene. A ratio of 3 : 1 was repeatedly found from BC_3F_2 to BC_5F_2 showing that short stature and late heading both are controlled by a single dominant gene in the genetic background. Allelism tests between *Lh* and other known photosensitivity genes remain to be carried out.

Segregation in B_5F_2 showed that these genes were linked as *g-Ssv-Rc-Lh* on chromosome 7. Distorted segregations were detected for *Rc* and *Lh*, showing significant values in χ^2. Although the mechanism for the distortion is unknown, the frequency of phenotypes with *Rc* decreased more sharply in B_5F_2 than expected (60.8%, significant at 1%) , suggesting that the introduced segment is quickly eliminated when backcrossed with T65*glg*.

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E-c. Division of Applied Genetics

(1) Mitochondrial DNA Analysis Reveals Cytoplasmic Variation within a Single Cultivar of Perennial Ryegrass (*Lolium perenne* L.)

Yoshiya SHIMAMOTO

The extent of mitochondrial DNA diversity among individual plant samples of perennial ryegrass was determined by using RFLP. Polymorphisms can be observed among the cultivars, showing that the cultivars were classified into the three major groups by similarity of fragment length. Three cultivars revealed polymorphisms among individual plants, whereas most of the cultivars were monomorphic in fragment length. Two of the three cultivars showed several different haplotypes of the mitochondria genome, respectively, and had a common haplotype. These cultivars were the ecotypes based on a number of selected plants which were collected from the old sward. It seems most likely that mitochondrial genome diversity among individual plants within the cultivar has resulted from non-homogeneous ancestor cytoplasms in perennial ryegrass. For the details, see Ref. 1.

(2) Genetic Differentiation between Summer and Autumn Maturing Cultivars of Soybean (*Glycine max* L.) in the Kyushu district of Japan

Yoshiya SHIMAMOTO

Soybean landraces and pure line selections collected from the Kyushu district of Japan were assayed for isozyme and seed protein loci in order to determine the genetic structure of the groups of summer and autumn maturing cultivars. Four of the 16 loci tested, *Dia1*, *Enp*, *Est1*, and *Ti*, exhibited a marked difference in allelic frequency between the two groups. The summer and autumn cultivar groups had different predominant alleles at these four loci. The analysis of multi-locus genotypes revealed that both

groups mostly consisted of different allele-combinations at four loci. Two major genotypes, *Dia1-b*, *Enp-b*, *Est1-a*, *Ti-b* and *Dia1-a*, *Enp-a*, *Est1-b*, *Ti-a*, were unique to the summer and autumn groups, respectively. The former type has not been observed in China or Korea. These results indicated that both groups were appreciably differentiated from each other and the summer group was originally established in the Kyushu district of Japan. For the details, see Ref. 2.

(3) Molecular Genetic Studies of Genomic Imprinting

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In order to understand the molecular mechanisms involved in the phenomenon of genomic imprinting in mammals, we are characterizing an imprinted DNA region on mouse chromosome 7. This region contains three imprinted genes, *i.e.* *Ins2*, *Igf2* and *H19*, and has an approximate size of 100 kb. Both *Ins2* and *Igf2* are only expressed from the paternally derived copy and *H19* from the maternal copy. One mechanism that has been shown to be involved in imprinting is DNA methylation. Our previous studies showed that the inactive, paternally derived *H19* copy is highly methylated at its promoter. We have now shown that this methylation occurs around the time of implantation during early development. However, the inactivation of the paternal copy preceded this methylation. Thus the promoter methylation is probably a stabilizing mechanism rather than the primary inactivation mechanism. Because some sites further upstream were more methylated on the paternal copy than on the maternal copy even at these early stages of development, these sites could serve as targets for primary imprinting.

The recent study has shown that a 13 kb deletion, including the *H19* gene and its upstream region, causes loss of imprinting of both *Ins2* and *Igf2*, which are located more than 90 kb upstream of *H19*. This imprinted region proposes a excellent model to study gene regulation at the chromosomal domain level. To identify regulatory elements such as enhancers, silencers, insulators and imprinting elements, in this domain, we are now carrying out a systematic screening for DNaseI hypersensitive sites, which could serve as a marker for such regulatory elements. So far, three novel clusters of hypersensitive sites were identified. We found that some of them were

constitutively present and others were embryonic stage-specific. These findings were reported in meetings and symposiums. We are currently studying the rest of the domain and will set out to assay the functions of these hypersensitive sites. For the details, see Ref. 3.

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F. GENETIC STOCK RESEARCH CENTER

F-a. Mammalian Genetics Laboratory

(1) Establishment of System for Tetrad Analysis in Mouse Female Meiosis II

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Recombination occurs in two distinct ways, crossover and gene conversion. Single crossover displays reciprocal segregation of parental alleles at a locus. On the other hand, gene conversion exhibits non-reciprocal segregation. In the meiosis of lower eukaryotes such as yeast, tetrad analysis allows us to find out which type of recombination, crossover or gene conversion, occurred at allelic loci. This tetrad analysis and assignment of recombinational breakpoints have given clues to understand recombinational processes. In higher eukaryotes including mammals, tetrad analysis has never been established, because four gametes derived from a single oogonium eventually dispersed and it is by no means easy to trace all four gametes. Hence, the mechanism of meiotic recombination in mammals has been poorly understood.

The meiotic process in female mice is relatively synchronized. Female germ cells that migrate into prospective ovaries cease proliferation and enter meiosis at 13.5 d.p.c. In postnatal ovary, oocytes approach in diplotene, the late stage of meiotic prophase I, and then meiotic division is arrested until sexual maturation. In this stage, there are four chromatids in a single nucleus and they contain four times the haploid amount of DNA (4C). Under the influence of sex hormones, oocytes complete meiotic division I, release a small polar body and proceed to division II of meiosis. A egg and a polar body derived from a oocyte are enveloped in single zona pellucida. Finally, oocytes are arrested at meiotic metaphase II until fertilization occurs. Since there are two chromatids in a single nucleus in an oocyte of sexually matured female mouse, meiotic recombination should have finished. Therefore, we can analyze the segregation of the alleles of four chromatids from a single

matured mouse oocyte.

In the mouse major histocompatibility complex (MHC) class II region, the breakpoints of meiotic recombination are not distributed at random, but are clustered in specific sites, called hotspots. One of the hotspots is located downstream of the *Lmp2* gene in the MHC class II, when *wm7* and *cas3* MHC haplotypes derived from Asian wild mice are crossed with laboratory standard haplotypes. Within 2 kb of the DNA segment of this *Lmp2* hotspot, meiotic recombination occurs at a 2% frequency, the highest value observed among hotspots in the mouse MHC until now. Since this hotspot could be of great use in analyzing the segregation of the alleles flanking the recombinational breakpoints, we decided to establish the first experimental system for tetrad analysis in mammalian meiosis.

We used a polymerase chain reaction (PCR) to amplify the recombinational breakpoints and each allele of the flanking regions from a single oocyte. For this purpose, we established a method for preparing a single mature oocyte. Ovaries were removed from sexually mature female mice, and mechanically picked with a needle in medium containing a chelate agent. Mature oocytes could be distinguished by cell size, and easily isolated with a capillary. A single oocyte was purified by washing several times and transferred into newly prepared excessive medium with a capillary. Second we set primers across the *Lmp2* hotspot and tried to select conditions for DNA amplification from a small amount of purified genomic DNA corresponding to a single haploid (1C) and found a suitable one. Based on this, we tried to amplify the DNA segment of the *Lmp2* hotspot from a single oocyte. After lysis of the oocyte, we could amplify 1.3 kb of DNA from a single oocyte by PCR. To establish a system for tetrad analysis in mammals, we need to establish the conditions for comparative-quantitative PCR from single cells in order to detect two types of parental DNA molecules and other two recombinant molecules, if present. Once the whole system is established, we can analyze rate, fashion and timing of meiotic recombination. The same system might be applicable to analyzing the timing of DNA replication in single cells.

(2) Fine Structure of the *Pb* Hotspot in the Mouse MHC class II Region

Taku ISOBE, Masayasu YOSHINO, Ken'ichi MIZUNO, Tsuyoshi KOIDE, Kazuo MORIWAKI and Toshihiko SHIROISHI

In mammals, the murine major histocompatibility complex (MHC) is the only region where breakpoints of meiotic recombination are systematically studied at the molecular level. In this region, meiotic recombinations do not occur at random but are clustered in limited regions known as hotspots. Thus far, four hotspots have been identified in this region. The presence or absence of a hotspot on a certain DNA segment depends on the MHC haplotypes involved in genetic crosses. For example, the *Eb* hotspot is observed in crosses between standard laboratory haplotypes. In genetic crosses including *cas3* and *wm7* haplotypes which were derived from Asian wild mice, most of the recombinations occurred at the *Lmp2* hotspot. *Lmp2* and *Eb* hotspots have been well characterized at the molecular level. Sequences around these two hotspots were determined and the fine locations of the breakpoints were analyzed. Comparison of the sequences between *Lmp2* and *Eb* hotspots revealed several molecular motifs commonly shared by the two hotspots. In order to elucidate the roles of these motifs in recombinations at the hotspots and to understand the mechanism by which recombinations are restricted to hotspots, we need to characterize other hotspots at the molecular level. Meiotic recombination takes place at a high frequency at a hotspot in the vicinity of the *Pb* gene, when the wild mouse derived *cas4* haplotype is used in the genetic cross. Molecular characterization of this hotspot, designated *Pb* hotspot, has not yet been done. So far, we have obtained six independent recombinants at the *Pb* hotspot after screening six hundred mice generated from crosses between *cas4* and *wm7* haplotypes. The breakpoints of these recombinants were localized to a 15 kb of DNA fragment in the vicinity of the *Pb* gene. In this study, we attempted to make a more complete map of the breakpoints of the six recombinants. First, we constructed the restriction map of the 15 kb of DNA fragment including the breakpoints. Subsequently, we determined the parental origins of several short DNA segments consisting of the 15 kb of DNA fragment in the six recombinants, by examining polymorphisms between parental DNA segments through PCR-SSCP analysis. At a result, at least five recombinations were found to be confined to a 5 kb of DNA segment located proximal to the 3'end of the *Pb* gene.

(3) Genetic Analysis of Coat-Color Mutation, *rim2*. II

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An MHC haplotype *wm7*, which was derived from Japanese wild mouse, enhances recombination specifically during female meiosis at the *Lmp2* hotspot. The mouse mutation, *rim2*, is one of a series of spontaneous mutations which arose in intra-MHC recombinants between *wm7* and laboratory haplotypes. As described in the previous report, *rim2* is a single recessive coat-color mutation and is allelic to the old mutation *pearl*, *pe*, mapped on chromosome 13. It is known that many coat-color mutants in mouse have abnormalities similar to human hemorrhagic symptoms accompanied with albinism, platelet storage pool deficiency (SPD). Recently, it was shown that platelet aggregation and ATP release in platelets of the *rim2* mutant mouse using several agonists were clearly reduced in comparison to those of wild type mouse. These results supported the possibility that *rim2* may serve as a mouse model for heterogeneous human SPDs.

On the basis of linkage analysis with 1570 backcross progeny, we identified two tightly linked microsatellite markers on chromosome 13, which did not segregate from *rim2*. The screening of the YAC libraries gave two YAC clones positive for each microsatellite marker and FISH analysis confirmed that these clones were not chimaeric. Construction of YAC contig between these markers is now in progress. We are planning to make transgenic mice with the DNA prepared from the YAC clones positive for these tightly linked markers and rescue the *rim2* phenotype in order to identify the clone which includes *rim2* gene.

(4) Linkage Analysis of the Mouse Mutant, *Rim3*, Affecting Skin and Eye

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Rim3 is a dominant mutation derived from an intra-MHC recombinant between wild mouse-derived *wm7* and laboratory haplotypes. It is characterized by hairless and corneal opacity. Based on linkage analysis with backcrosses between C57BL/10J-*Rim3*/+ and an MSM inbred strain derived from Japanese wild mouse, the *Rim3* locus appeared to be tightly linked to three microsatellite markers, *D11Mit14*, *124* and *197*, on chromosome 11. A *Keratin type 1* (*Krt-1*) locus has been mapped to the same position. Keratins are a group of water-insoluble proteins that form 10-nm intermediate filaments in epithelial cells. These can be divided into two groups, type I and type II. Type I keratins are relatively acidic and type II basic-neutral. The two types differ in immunoreactivity and genes have higher sequence homology within each type. It is known that the *Krt-1* region consists of a cluster of more than 10 genes and each gene has, to some extent, tissue-specific expression patterns in normal epithelia. Since *keratin-10*, *12*, *14*, *16* and *17* genes are expressed in epidermis, hair follicle and cornea, they seemed to be candidates for genes affected in *Rim3*. At present, it appears that *keratin-10* is not a candidate because it was genetically segregated from *Rim3*. *Keratin 12* was also excluded from being a candidate, because we could not find any alteration of sequence in the *Rim3* mutant gene. Several mouse mutations affecting the skin and cornea have also been mapped to the region close to the *Rim3* locus. The mutation *Bare skin* (*Bsk*) causes baldness and corneal opacity. Another mutation *Rex denuded* (Rex^{den}) exhibits a similar phenotype. To characterize corneal opacity in more detail, we carried out a histological analysis of the cornea of *Rim3* and Rex^{den}. In both mutants, the corneal epithelium is hyperstratified at the center of the cornea and a cluster of unidentified cells are accumulated in that subepithelial region. In addition, keratin-like substances are observed on the surface of hyperstratified epithelium. Now we intend to carry out a immunohistochemistrical analysis for these two mutants and *Bsk*.

(5) Multigenic Control of Localization of the Zone of Polarizing Activity in Mice Limb Morphogenesis

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In vertebrate limb development, the pattern formation along the anteroposterior and proximodistal axes is controlled by two signaling centers. Polarizing activity along the anteroposterior axis of limb morphogenesis is provided from the Zone of Polarizing Activity (ZPA) located at the posterior margin of the limb bud mesoderm. Now it is accepted that *Sonic hedgehog* (*Shh*), a mouse homolog of the segment polarity gene, *Hedgehog*, in *Drosophila*, mediates the key activity of the ZPA. Expression of *Shh* in the posterior mesenchyme of limb bud, and *fibroblast growth factor-4* (*Fgf-4*) in the posterior portion of apical ectodermal ridge (AER), coordinately regulate pattern formation along the anteroposterior axis. We demonstrated the ectopic expression of *Shh* and *Fgf-4* at the anterior margin of limb buds in three preaxial polydactyly mutants, *Recombination induced mutant 4* (*Rim 4*), *Hemimelic extra toes* (*Hx*) and *Extra toes-J* (Xt^J), indicating that mirror-image duplications of the skeletal pattern of the digits could be caused by duplication of ZPA at the anterior margin of limb buds. For details, see Ref. 1.

One of the above mutations, Xt^J, is thought to be a loss of function type, because the affected gene, *Gli3*, which encodes a transcription factor has a long deletion including a Zinc-finger DNA binding domain in this mutation. Our finding of the duplication of ZPA in Xt^J mice suggests that there is a genetic control that suppresses ZPA activity at the anterior margin of the limb bud, and *Gli3* is involved in this cascade. To explore the gene function of *Gli3* in limb morphogenesis, we examined the localization of the *Gli3* transcript during limb development by whole-mount in situ hybridization. As a result, *Gli3* was expressed in the limb bud mesenchyme except the posterior margin which overlaps the location of ZPA. This expression pattern, which is complementary to that of *Shh*, shows that GLI3 functions in the anterior portion of the limb mesoderm to suppress *Shh* expression.

Three other mouse mutants, *luxate* (*lx*), *Strong's luxoid* (*lst*), and *X-linked*

polydactyly (*Xpl*), exhibit a mirror-image duplication similar to those of, *Hx* *Rim4* and Xt^J showing hyperphalangy and reduction of tibia, except that *lx* and *Xpl* show polydactyly only in the hindlimbs. To examine whether the preaxial polydactylies of *lx*, *lst*, and *Xpl*, are caused by ectopic ZPA activity at the anterior region of the limb buds, we carried out whole-mount in situ hybridization of 12.5 days embryos using riboprobes of the *Shh*, and *Fgf-4* genes. Ectopic expression of *Shh* and *Fgf-4* were observed in the limbs of all mutants. These results in conjunction with previous ones from our study on *Rim4*, *Hx* and Xt^J suggested that most preaxial polydactyly mutations with mirror-image duplication are caused by ectopic formation of ZPA at the anterior margin of limb buds. In the *lx* mutant, ectopic expression of *Fgf-4* was found in the AER in the forelimb buds, but no ectopic expression of *Shh* was detected in the mesoderm. Hence, the anterior margin of AER was posteriorized in the limb buds of this mutant, but not its mesodermal mesenchyme. Thus, *lx* seems to be a candidate gene which functions to suppress *Shh* via the signaling pathway in AER.

When mice heterozygous for the *Rim4* mutant were crossed with MSM, an inbred strain derived from Japanese wild mouse, *Mus musculus molossinus*, the appearance of the polydactylous phenotype was reduced to 0%, though half of the F1 hybrids carried the *Rim4* mutant gene (data not shown). When (C57BL/10J-*Rim4*/+ and MSM) F1 mice heterozygous for *Rim4* were backcrossed to C57BL/10J, 14.5% of the progeny exhibited polydactyly. These findings indicate that the polydactylous phenotype of *Rim4* was completely rescued by genes of the MSM strain in the F1 hybrids, and the genes responsible for the rescue of the polydactylous phenotype are not many in number. To investigate whether the same genes are able to rescue the mutant phenotypes of other polydactylous mutations, we crossed the MSM strain and heterozygotes of other polydactylous mutants. The polydactylous phenotype of *Rim4*, *Hx*, and *lst*, was ameliorated in crosses with the MSM strain, but the phenotype of *Xpl* did not change at all. These mating experiments indicate the possibility that *Rim4*, *Hx*, *Xt*, and *lst* genes play functions in upstream of the rescue gene in the MSM strain, and the *Xpl* gene plays function in downstream of the rescue genes. Normal functions of all these genes are likely to be involved in the down regulation of ZPA activity at the anterior margin of limb buds.

(6) Searching for Modifier Genes for the Phenotype of Mutation *Tail-short*

Junko ISHIJIMA, Kikue UCHIDA, Akihiko MITA, Tsuyoshi KOIDE and Toshihiko SHIROISHI

Mice homozygous for *Tail-short*, *Ts*, exhibit abnormalities in early development and embryonic lethality at about the time of implantation. On the other hand, heterozygotes have various kinds of skeletal abnormalities throughout the whole body and severe defects in head structural development. These phenotypes differ depending upon the genetic background in which the mutant gene is introduced. For example, when (TSJ/Le-*Ts*/+ × C57BL/6J) F1 mice were backcrossed to C57BL/6J, the viability of the resultant progeny carrying the *Ts* allele is almost 0%. In turn, when the MSM inbred strain derived from Japanese wild mouse, *Mus musculus molossinus*, was used as a backcross partner, mice with the *Ts* allele were less defective and viability increased to 40%. The difference in expressivity of the mutant phenotype indicates that there is a gene(s) which modifies the expressivity of *Ts* phenotypes and is polymorphic between the C57BL/6J and MSM strains. We intend to map these modifier gene(s) using backcross progeny from (TSJ/Le-*Ts*/+ × MSM)F1 × C57BL/6J. At present, no major gene has been detected as a candidate for the modifier, but it is becoming more apparent that strain differences in expressivity of the mutant phenotype is under the control of a number of minor genes.

(7) Morphological Characterization of the Mouse Mutant *Tail-short*: Skeletal Patterning Along the Anteroposterior Axis

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We explored the characterization of the morphological phenotype of the mouse mutant *Tail-short* in embryonic development and skeletal patterning. Detailed analysis of the skeleton of *Ts*/+ mice revealed that a number of homeotic transformations occur along the anteroposterior axis. In particular, transformation of the seventh cervical vertebrae to the first thoracic vertebrae was highly reproducible in *Ts* heterozygotes generated from the cross of (C57BL/6J-*Ts*/+ × MSM)F1 × C57BL/6J. It is of interest to note that this transformation was observed asymmetrically, namely, the left side of

vertebrae was predominantly transformed. Besides the above skeletal anomalies, a neural tube defect in the rhombomere region was frequently observed. Thus it is possible that the *Ts* gene plays some roles providing segmental identity in mouse embryogenesis. More detailed analysis of the mutant phenotype is underway to characterize the primary defect in the *Ts* mutation, using *in situ* hybridization with a number of probes for genes whose expression patterns seems to be related to the defect in the *Ts* mutation.

(8) Fine Gene Mapping of the Skeleton Mouse Mutant, *Tail short* (*Ts*), Toward Positional Cloning

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Tail short (*Ts*) was initially found as a mutant which exhibits shortened kinked tail in heterozygotes. They also have numerous skeletal abnormalities throughout the entire body. Homozygous embryos die at around the time of implantation. This is probably due to a retardation in forming morulae because pale-staining embryos have been found at this stage in homozygotes. These various phenotypes indicate that the gene for *Ts* plays a crucial role throughout development including the early embryonic stage and skeletal formation. To elucidate the function of the *Ts* gene, we are trying to clone the gene by the method of positional cloning. We have conducted backcross mating between *Ts*/+ and the MSM inbred strain derived from Japanese wild mouse. The *Ts* gene was mapped within a region of 0.2 cM that located between two microsatellite markers, *D11Mit128* and *D11Mit214*. Calculations estimate the distance between the two markers as approximately 400 kb. We screened the mouse YAC library and obtained one YAC clone which carries *D11Mit214*. Further screening of the mouse genomic library is underway to make a contig between the two markers.

(9) Establishment of Intersubspecific Consomic Mouse Strains

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Two strains of mouse are said to be consomic when they differ by one complete chromosome pair. The prefix "con" means that any two consomic strains, in spite of specific differences, have a similar genetic background and the suffix "somic" refers to a complete chromosomal element. Given that mouse has a karyotype with 21 different chromosomes (19 autosomes+X+Y), 21 consomic mouse strains are required for full coverage of mouse chromosomes by donor strains, from which one complete chromosome is introduced into the common genetic background referred to as recipient. Chromosome substitution is achieved by six to seven times backcrossing of individuals identified as heterozygous for the selected chromosome to the recipient strain.

Consomic strains can facilitate the genetic analysis of penetrance and expressivity. Variation in expressivity depends upon both genetic and non genetic determinism, and it is clear that a couple of backcrossings to three or four different consomic strains may help in detecting genes likely to be involved in the phenomenon of expressivity. Consomic strains also help in the genetic analysis of phenotypes with multigenic determinism. It has been well established that many pathological conditions and diseases like cancer and diabetes are under multigenic control. They are generally difficult to analyze when a great number of genes are involved and epistatic interactions are present among the genes. In these cases, if a phenotype of interest appears to deviate in a consomic strain from what is known in the genetic background strain, its genetic control must be related to chromosomal substitution. Further genetic analysis will then be considerably simplified. In addition, consomic strains can facilitate chromosomal assignment of cloned DNAs.

Considering all these benefits from using consomic strains, we have started to establish consomic strains. We chose the MSM strain, which is derived

from Japanese wild mouse, *Mus musculus molossinus*, as donor strain and, a C57BL/6J laboratory strain as recipient. These two strains are genetically very remote from each other so that they differ in a number of genetic traits. This genetic difference facilitates in chromosomal assignment of any phenotype to be examined and is of great use in linkage analysis based on polymorphism of DNA markers. We are using microsatellite DNA markers, at least five markers per chromosome, for selection of individuals in each step of backcrossing. At present, we have obtained second generations of the backcross for substitution of most chromosomes.

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F-b. Invertebrate Genetics Laboratory

(1) Determination of wing cell fate by the *escargot* and *snail* genes in *Drosophila*

Naoyuki FUSE, Susumu HIROSE[1] and Shigeo HAYASHI ([1] Division of Phenogenetics)

Insect appendages such as the wing and the leg are formed in response to inductive signals in the embryonic field. In *Drosophila*, cells receiving such signals initiate developmental programs which allow them to become imaginal discs. Subsequently, these discs autonomously organize patterns specific for each appendage. We have found that two related transcription factors,

Escargot and Snail that are expressed in the embryonic wing disc, function as intrinsic determinants of the wing cell fate. In *escargot* or *snail* mutant embryos, wing specific expression of Snail, Vestigial and LacZ regulated by *escargot* enhancer were found as well as in wild type embryos. However, in *escargot snail* double mutant embryos, wing development proceeded until stage 13, but the marker expression was not maintained in later stages, and the invagination of the primordium was absent. From such analyses, it was concluded that Escargot and Snail expression in the wing disc are maintained by their auto- and cross-activation. Ubiquitous *escargot* or *snail* expression induced from the *hsp70* promoter rescued the *escargot snail* double mutant phenotype with the effects confined to the prospective wing cells. Similar DNA binding specificities of Escargot and Snail suggest that they control the same set of genes required for wing development. We thus propose the following scenario for early wing disc development. Prospective wing cells respond to the induction by turning on *escargot* and *snail* transcription, and become competent for regulation by Escargot and Snail. Such cells initiate auto- and cross-regulatory circuits of *escargot* and *snail.* The sustained Escargot and Snail expression then activates *vestigial* and other target genes that are essential for wing development. This maintains the commitment to the wing cell fate and induces wing specific cell shape change. For the details, see Ref. 4.

(2) A Cdc2 dependent checkpoint maintains diploidy in *Drosophila*

Shigeo HAYASHI

DNA replication in G2 does not normally occur due to the checkpoint control. To elucidate its mechanism, functions of the *escargot* and *Dmcdc2* genes of *Drosophila* were studied. When *escargot* function was eliminated, diploid imaginal cells that were arrested in G2 lost Cyclin A, a regulatory subunit of G2/M cdk, and entered endocycle. *escargot* genetically interacted with Dmcdc2 that encodes a catalytic subunit of G2/M cdk. The mutant phenotypes of Dmcdc2 itself was similar to those of *escargot*; many of diploid cells in imaginal discs, salivary glands and the central nervous system entered endocycle and sometimes formed polytene chromosomes. Since mitotically quiescent abdominal histoblasts still required Dmcdc2 to remain diploid, the inhibitory activity of G2/M cdk on DNA replication appeared to be separable

from its activity as the mitosis promoting factor. These results suggest that in G2, *escargot* is required to maintain a high level of G2/M cdk that actively inhibit the S phase entry. For the details, see Ref. 3.

(3) Roles of Escargot and *D*E-Cadherin in the Formation of *Drosophila* Tracheal Network

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The *Drosophila* tracheal system is a network of tubular epithelium. It is derived from ten pairs of precursors that invaginate from ectoderm in the thoracic and abdominal segments. The precursors extend branches which migrate toward branches from other segment and fuse to form continuous tubes. At the site of fusion, a specialized cell called tip cell was identified. When thin branches of the dorsal branch and lateral trunk fuse, tip cells search their target by extrending filopodia which make a first contact. The homophilic cell adhesion molecule *D*E-Cadherin plays an essential role in the fusion (Ref. 5). Among several cell adhesion molecules examined, *D*E-Cadherin is the first molecule to be accumulated at the contact interface. Other cell surface molecules such as Fasciculin III and Crumbs were detected in later stages. Changes in the level and spacial pattern of *D*E-Cadherin accumulation closely corelated with the dynamic process of epitherial rearrangement during the fusion. *D*E-Cadherin is essential for the tracheal fusion, since tracheal branches failed to fuse in embryos mutant for *D*E-Cadherin gene *shotgun*. The tracheal tip cells specifically express the transcriptional regulator Escargot (Esg). In *esg* mutant, a subset of tracheal branch failed to express *D*E-Cadherin and did not fuse. In addition, tip cells in these branches extended unusually long cytoplasmic processes which migrated to search an alternative fusion partner. Such phenotypes were rescued by an *esg* transgene. Furthermore, heat inducible *D*E-Cadherin transgene also rescued the tracheal defects in *esg* mutant. These analyses demonstrated the essential role of *D*E-Cadherin in the tracheal fusion and identify *esg* as a regulator of cell adhesion and motility.

(4) The Early Development of *Drosophila* Imaginal Discs

Satoshi GOTO and Shigeo HAYASHI

The embryonic development of *Drosophila* imaginal discs provides an important experimental system to study pattern formation. The development of wing/leg primordia is thought to be composed of three steps. The first step is the detemination of the leg and wing primordia as a common precursor expressing the homeodomain protein *Distal-less.* This step requires the signaling molecule wingless. The second step is the segregation of wing primordium from the leg primordium. The wing primordium migrates dorsally within ectoderm expressing dpp–lacZ reporter, suggesting that *decapentaplegic* (*dpp*) might be involved in this step. The third step is the maintenance of the imaginal fates. We found that both the wing and leg primordia failed to form in the absense of Dpp receptor encoded by *thick vein* and *punt.* In less severe *tkv* and *punt* mutant background, only the wing primordium was affected, suggesting dpp signaling is required for the first two steps.

(5) A Nuclear GFP/β-Galactosidase Fusion Protein as a Marker for Morphogenesis in Living *Drosophila*

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A general, non-invasive method to trace morphogenesis in living *Drosophila* was developed. To label specific cells, green fluorescence protein (GFP) of jellyfish *Aequorea victoria* was expressed by the Gal4–UAS system. Green fluorescence from GFP fused to the nuclear localization signal was detectable in polytene larval tissue, but not in diploid tissue. Further fusion to bacterial β-galactosidase produced GFPN–lacZ, which fluoresced brightly in several diploid larval and embryonic tissues. GFPN–lacZ was used to trace dynamic cell movement during the formation of embryonic tracheal system. These results indicate that GFPN–lacZ can be used to mark specific cells to study cell movement and gene expression in living animal. For the detail, see Ref. 2.

(6) Control of *esg* Expression

Yoshimasa YAGI[2] and Shigeo HAYASHI ([2] Department of Biology, Faculty of Science, Nagoya University)

esg is expressed in spatially and temporally complex pattern during embryogenesis. To elucidate the regulatory mechanism of *esg* expression, we have been mapping cis-control elements in the upstream of *esg* gene. We have mapped testis specific enhancer whithin a 1 kb fragment in the vicinity of *esg* promoter. Control elements for expression in the nervous systems, gut imaginal cells, the leg and genital imaginal discs and the tracheal tip cells scatter within more than 20 kb region in the upstream from the *esg* promoter. These elements are candidates for targets of regulatory genes such as *wigless* and *dpp* which are involved in morphogenesis of many tissues expressing *esg*.

(7) *argos* is Reqired for Projection of Photoreceptor Axons During Optic Lobe Development in *Drosophila*

Kazunobu SAWAMOTO[1], Masataka OKABE[1], Teiichi TANIMURA[2], Shigeo HAYASHI, Katsuhiko MIKOSIBA[3] and Hideyuki OKANO[1] ([1] University of Tsukuba, [2] Kyushu University, [3] University of Tokyo)

The *Drosophila argos* gene encodes a secreted protein with an EGF motif, which acts as an inhibitor of cell recruitment in the developing eye and wing. We have analyzed the role of *argos* during optic lobe development. *argos* expression was observed in the optic lobes throughout the developmental stages. In *argos* mutants, neuropiles failed to develop normally, and photoreceptor axons did not project propery into the lamina. Ubiquitous expression of *argos*, under control of the hsp70 promoter, rescued the defects in optic lobes. We have found that glial cells failed to differentiate in the larval optic lobes of *argos* mutants. Correspondingly, in loss of function *repo* mutants, whose glial cells also failed to differentiate, photoreceptor axons showed impared projection pattern similar to the *argos* phenotype. These results suggest that glial cells play a role for guidance of photoreceptor axons. The loss of function *Star* mutation dominantly supressed the defect in the *argos* optic lobes, suggesting that these two genes act in an antagonistic fashion during optic lobe development. For the detail, see Ref. 1.

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F-c. Plant Genetics Laboratory

(1) Structural and Functional Analysis of the Genes Expressed During Early Embryogenesis in Rice

Yukihiro ITO

As the first step in understanding the molecular mechanism which regulates embryogenesis in rice, I isolated several cDNA clones and PCR fragments from cDNA libraries, which were constructed from mRNAs of rice embryos three days after fertilization using three different methods. The cDNA libraries constructed were the general lambda phage library, the lambda phage library in which cDNAs were amplified by PCR, and the solid-phase cDNA library in which cDNAs were fixed to a metallic support. I isolated several cDNA clones using cDNAs of homeobox genes of rice, maize, and *Arabidopsis* as probes from two lambda phage cDNA libraries, and several PCR fragments amplified from a solid-phase cDNA library using degenerated oligonucleotide primers corresponding to the conserved amino acid sequences of homeodomains. These results suggest that homeobox genes are probably involved in early embryogenesis in rice.

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F-d. Microbial Genetics Laboratory

(1) A New Gene Determining the Frequency of Cell Division in *E. coli*

Maki TADENUMA and Akiko NISHIMURA

We have isolated a novel mutant of *E. coli*, MT1, and found it to have the following characteristics. The MT1 had two mutations, *cfcM1* and X^-. The *cfcM1* gene mapped within several minutes of the *oriC* gene. The *cfcM1* mutation caused excess division per round of DNA replication at 41°C. As a result, a lot of mini cells with DNA and short filamentous cells with high ploidy were produced. However, the colony forming ability of the single mutant strain which carried the *cfcM1* or the X^- was normal at 41°C. Temperature sensitivity for growth was only shown for the double mutant, *cfcM1* and X^-. The *cfcM1* mutant continued to divide even when DNA replication was inhibited in the presence of nalidixic acid (7.5 μg/ml). We have subcloned the DNA region, which complemented the *cfcM1* mutation, into the miniF plasmid but could not do so into pSC101. Over production of CfcM is assumed to be toxic for cell.

(2) Control of Cell Division at Low pH

Hideki UKAI, Nahoko WATANABE, Keiko SUZUKI and Akiko NISHIMURA

We isolated a novel mutant, *cpcA58* (Control by pH and cAMP). The *cpcA58* mutant strain stopped cell division when cultured in a low pH medium containing cAMP at 43°C. The *cpcA58* mutant strain carrying the tetracycline (Tc) resistance gene did not show Tc resistance. Lack of Tc

resistance was shown even at pH 7.0 and was more marked in the presence of cAMP. Tc resistance is known to be acquired through efflux of the drag out of the cells by antiporter, TetA, coupled with H^+ influx in cells. The multicopy suppressor gene of the *cpcA58* mutation, *sunU*, was also isolated. The plasmid carrying the *sunU*$^+$ gene complemented the growth deficiency of the *acrA* strain cultured in the presence of the H^+ uncoupler. These results suggest that the *cpcA58* mutant might be defective in the H^+ efflux systems and ΔpH might be decreased in the *cpcA58* strain. Under these conditions, a low pH is assumed to lower the proton motive force. As a result, localization of some protein(s) required for cell division might be inhibited.

(3) The Instability of Membranes Affect the Transcription of Cell Division Gene, *ftsZ*

Kana NISHIMORI and Akiko NISHIMURA

We analyzed the properties of temperature sensitive cell division mutants of *E. coli*, *ftsK830* and *ftsK1167*. They were alleles of the *kdsA* gene which is involved in the synthesis of lipopolyshaccharide (LPS). LPS is a major component of the outer membrane. Accoordingly, the barrier property of the outer membrane is supposed to be reduced in these *ftsK* mutants. Actually, sensitivity for some hydrophobic agents such as novobiocin was increased in the *ftsK830* mutants. However unexpectedly, some other agents such as methylene blue accelerated the growth of the *ftsK830* and *ftsK1167* mutants at which concentration the parent strain was sensitive. These results suggested that the instability of the membrane in the *ftsK* mutants might affect the expression of many genes rather than just the lack of protein localization into the outer membrane. Preliminary experiments showed that the *ftsZ*-mRNA in the *ftsK* mutant was half that of the parent when cultured at nonpermissible temperatures for 50 min.

(4) *sdiA* and *rpoS*-Mediated Dual Transcriptional Regulation of 7α-HSDH Gene, *hdhA*, in *Escherichia coli*

Kengo KANAMARU and Kyoko KANAMARU[1] ([1] Deep-Sea Microorganisms Research Group, JAMSTEC)

To investigate the cell-cell communication of *Escherichia coli*, we focused in the physiological function of a positive transcriptional activator of the *ftsQAZ* operon, SdiA. At least on the level of amino acids sequence, SdiA belongs to the LuxR family of regulators of cell-density dependent gene expression in several other gram-negative bacteria. We have found that super-multicopying of the *sdiA* gene causes the intracellular accumulation of 7α-hydroxysteroid dehydrogenase (7α-HSDH), encoded by the *hdhA* gene, which catalyzes the dehydration of primary bile acids to secondary bile acids. Interestingly, the transcription of *hdhA* was controlled by both *sdiA* and *rpoS*, the latter of which encodes a stationary phase-specific sigma factor. The trancriptional regulation of *hdhA* was very similar to the regulation of the *ftsQAZ* operon by these transcriptional factors. A more than 20-fold increase in total *hdhA*–mRNA level was observed in the cells of early stationary phase when compared with those of early log phase. *sdiA*-dependent transcription kept increasing in the log phase but decreased immediately before entering the stationary phase. Marked activation of *rpoS*-dependent transcription occurred only during early stationary phase. Deletion of *sdiA* did not affect *rpoS*-dependent transcription of *hdhA*, whereas, in contrast, deletion of *rpoS* resulted in a significant stimulation of *sdiA*-dependent transcription in the log phase without any change in transcription of *sdiA*. These results imply a link between the expressions of the 7α-HSDH and the cell division machinery encoded by *ftsQAZ*, and therefore an involvement of the *E. coli* adaptive response system to the intestinal environment.

(5) A Novel Member of the *cspA* Family of Genes, *cspG*, which is Induced Upon Cold-shock in *Escherichia coli*

Kyoko KANAMARU[1] and Kengo KANAMARU ([1] Deep-Sea Microorganisms Research Group, JAMSTEC)

Escherichia coli contains a major cold-shock protein, named CspA (or CS 7.4), whose production is predominantly induced at low temperatures. This

bacterium is known to possess five additional genes each encoding a protein highly homologous to CspA (referred as the CspA family). We have identified a new gene encoding a cold-shock inducible analogue of CspA/CspB. This newly cloned *cspG* gene is located at 22 min on the *E. coli* genetic map, at a distance from the other *cspA* family genes. Its gene-product (70 amino acids) is 73% and 77% identical to CspA (70 amino acids) and CspB (71 amino acids), respectively. Analyses of the *cspG–lacZ* transcriptional fusion gene and Northern hybridization revealed that *cspG* is a low temperature responsive gene. Its low temperature inducible promoter elements were determined, and the results indicate that the *cspG* sequence is highly similar to both the *cspA* and *cspB* sequences not only in the coding-regions but also in the 5′-upstream non-coding regions surrounding their own promoters.

Publication

None

F-e. Genetic Resources Laboratory

Compilation of Genetic Resources Information

Mari SAITO and Yukiko YAMAZAKI

The first trial phase of the Genetic Resources Databank Project has been initiated in this laboratory this year. The goal of this project is the collection, designation, construction, and online distribution of an Integrated Database, which contains genetic resources information and the relevant objects of different organisms, under a single logical data model.

Starting from local stock information such as mouse stocks maintained by the Mammalian Genetics Laboratory (T. Shiroishi) and drosophila stocks generated by the Invertebrate Genetics Laboratory (S. Hayashi), we have implemented the following databases available via the World Wide Web (WWW). At present we have compiled drosophila stocks of individual laboratories in Japan, local wheat germline stocks, and the cloning vector collection in cooperation with Yamamoto Y. (Kyoto Industrial University), H. Tsujimoto (Kihara Institute for Biological Research), T. Kawahara (Kyoto Germline Center) and S. Yasuda (Division of Microbial Genetics).

The data formats of these databases are minimally processed so that each database has its own format.

We also collaborated with the Wheat Networking Group, which is supported by National Science Foundation B, with aim of constructing an integrated wheat database called "KOMUGI-Wheat Network of Japan". We developed a data submission tool to collect data from each stock center and designed the database schema. The database, which will appear on the internet by the end of the fiscal year, is composed of nearly 50 entities including the biological and molecular biological features of each strain, as well as bibliographical information. The working group plans to continue the work on the database in several directions, such as incorporation of the wheat DNA repository database and of image data and cross referencing with related databases. The development of a data management system through which each researcher can constantly update their own data by connecting to the remote computer running the databases is also an ongoing project.

Rice Stocks Data maintained by several laboratories in Japan have been already compiled by T. Kinoshita (Hokkaido University) and H. Morishima (Division of Agricultural Genetics) over a period of several years and stored as hardcopy. Processing the hardcopy data to produce an electronic database is under way and the online database will soon be made accessible.

The next step will be providing a common model of these databases in order to accomplish inter-species searching. Although our current databases are organized as a relational database under Sybase DBMS, we plan to pursue the possibility of a new system like the unified relational and object-oriented system in the future.

Publication

None

F-f. Mammalian Development Laboratory

(1) Analysis of Cellular and Molecular Mechanisms in Development of Mouse Fetal Germ Cells

Norio NAKATSUJI and Yasuaki SHIRAYOSHI

Autonomous regulation of proliferation and growth arrest. Primordial germ cells (PGCs) isolated from mouse embryos show proliferation and growth arrest in culture, with a time course that is similar to the events *in vivo.* Such a growth pattern could be regulated autonomously or by somatic cells, which always co-exist with PGCs in culture. Mixed culture experiments using PGCs from 8.5 and 11.5 dpc embryos indicated no detectable interaction between PGCs and somatic cells at the two stages. We then carried out clonal cultures of PGCs, and examined the proliferation of and morphological changes in individual clones. Such clonal cultures did not reveal any subpopulations of PGCs in terms of growth rate and morphological changes. We concluded that there is an autonomous regulation of growth and cell shape change in PGCs, which occur as stochastical events but not strictly timed by the number of cell divisions. For details, see Ref. 3.

Roles of the gp 130 signal-transduction. The leukemia inhibitory factor (LIF) is a cytokine known to influence proliferation and/or survival of mouse PGCs in culture. We analyzed the functional role of gp 130-mediated signaling in PGC growth *in vitro.* A combination of IL-6 and soluble IL-6 binding subunit (sIL-6R), which is known to activate intracellular signaling via gp 130, fully reproduced the LIF action on PGCs. The addition of a neutralizing antibody against gp 130 in culture markedly blocked PGC survival. These results suggest the pivotal role of gp130 in PGC development. We further demonstrated that a combination of LIF with forskolin or retinoic acid, a potent mitogen for PGCs (for the details, see Ref. 1), caused continuation of proliferation of PGCs, leading to propagation of embryonic stem cell-like EG cells. For details, see Ref. 4.

Gene transfection of PGCs in vitro. We tested the transfection methods of electroporation, liposome-mediated transfection and the calcium phosphate (CaPO4) co-precipitation method. When PGCs were transfected with

plasmid pSV–LT by the CaPO4 co-precipitation method, transient expression of simian virus 40 large-T antigen was detected in an average of 18% of PGCs transfected. However, we could not detect any effects on the morphology or proliferation of PGCs. We also succeeded in detecting transient expression of the adenovirus 2 E1B 19 kDa protein, which is known to suppress cell apoptosis, in about 15% of PGCs transfected with plasmid pEF–E1B. In this case, its expression significantly retarded the disappearance of PGCs from the culture system and increased the number of rounded PGCs, which probably represent PGCs at more advanced stages of development and which can not survive in the presently available culture conditions.

(2) Molecular Analysis of Cell Differentiation and Morphogenesis in Postimplantation Mouse Embryos

Yasuaki SHIRAYOSHI and Norio NAKATSUJI

Determination of cell fate and cell differentiation are crucial events in morphogenesis and embryogenesis. We are trying to approach such problems from the molecular aspect, for example, by cloning mouse homologues of *Drosophila* genes which could play important roles in cell fate decision or cell differentiation. One area involves identifying important genes in the determination and differentiation of the central nervous system (CNS) during the postimplantation period. We constructed a cDNA library from 6.5–8.5 day old mouse embryos, in which neural plate and neural tube formation has been initiated. We screened this mouse cDNA library for important genes relating to CNS development such as the Notch neurogenic or ASC (acheate-scute complex) proneural genes in *Drosophila.*

We cloned one Notch related gene "int-3" from the 8.5 day cDNA library. The entire structure of the int-3 gene resembles that of the Notch or vertebrate Notch homologues, and the int-3 gene showed 50–60% identity to the related Notch homologues in its amino acid sequence.

We consider int-3 to be one of the mouse homologues of Notch. RT-PCR and *in situ* hybridization analysis revealed that int-3 was expressed in the blood vessels in 9.5–10.5 day old embryos. The expression pattern of int-3 coincided with the expression of receptor tyrosine kinase Flk-1, which is regarded as a major regulator of vasculogenesis and angiogenesis. These results suggested that int-3 might be involved in the formation of blood

vessels in early mouse embryos. We will study its roles in blood vessel construction either through ectopic expression or by altering the gene through the gene targeting method using ES cells and homologous recombination. Another area of interest is the molecular mechanism of sex differentiation in fetal gonads. We are currently trying to find genes which show specific expression patterns in male or female gonads.

(3) Establishment of Neuronal Stem Cell Lines for Analysis of Cell Lineages and Differentiation in the Mouse Central Nervous System Development

Norio NAKATSUJI, M. T. SHOKUNBI and Yasuaki SHIRAYOSHI

We dissected out the neural plate from mouse embryos at 8.0–9.5 dpc, which were transgenic for the temperature sensitive simian virus 40 (SV40) large tumor antigen (TAg) gene, tsA58, driven by the mouse H-2kb gene promoter (Immorto Mouse, Charles River Lab., USA). We cultured these cells at 33°C in a medium containing interferon-γ. We characterized many sublines after cell cloning of such established cell lines. Among them, a large number of cloned lines showed similar characteristics suggesting very primitive neuroepithelial cells, one of which was named the NES-8 cell line. Other cloned lines consisted of those at more advanced stages of development because they were more easily induced to differentiate into neurons *in vitro* than the NES-8 cells. We obtained only the NES-8 cell type from the neural plate at 8.0–8.5 dpc, but both the NES-8 and more advanced types from the neuroepithelium at 9.0–9.5 dpc.

While growing under TAg permissive conditions, NES-8 cells expressed nestin and both the RC1 and A2B5 antigens, which are markers of the neuronal stem or progenitor cells. Under TAg impermissive conditions (39°C without interferon-γ), NES-8 cells showed morphological changes and produced both bipolar and flattened cells. Such bipolar cells continued to express nestin and the RC1 antigen but lost A2B5 antigen expression. The flattened cells seemed to lose all three marker expressions. However, we failed to demonstrate expression of differentiation markers such as the neurofilament protein (NF) and the glial fibrillary acidic protein (GFAP) even after various trials to induce differentiation with aggregation culture, use of the poly-lysine coated culture dishes, deprivation of serum, and

addition of bFGF or PDGF.

In contrast, approximately half of the cell lines derived from the neuroepithelium at 9.0–9.5 dpc showed transformation into neuron-like cells with long processes, with concomitant expression of NF when transferred into TAg impermissive conditions. Therefore, we concluded that early neuronal stem cells in the neuroepithelium undergo an important step toward commitment for neuronal differentiation at 8.5–9.0 dpc, and such cellular characteristics can be studied by using cell lines established from these cells at the earliest stages of CNS development. We are currently carrying out transplantation experiments of NES-8 cells into embryonic brains to test the hypotheses that they are the earliest neuronal stem cells, and that they can be induced to produce many cell types of CNS when transferred into the environment of embryonic brains.

(4) Analysis of the Migration Pattern of Neuroblasts and Morphogenesis in Development of the Mouse Central Nervous System

Norio NAKATSUJI and M. T. SHOKUNBI

In collaboration with Dr. I. Nagata at Tokyo Metropolitan Institute for Neuroscience and Dr. K. Ono at Okayama University Medical School (present affiliation, Shimane Medical College), we are studying migration patterns of neuroblasts during histogenesis of the mammalian central nervous system (CNS). We found a new type of cell behavior, "perpendicular contact guidance", which is exhibited by CNS neurons, but not by the peripheral nervous system neurons on aligned parallel bundles of neurites. We studied migration patterns of neurons in explants of brain cortices, using fluorescence cell labeling. We are now analyzing the role of perpendicular contact guidance in the actual morphogenesis of brain cortices using various histological and morphological methods.

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G. DNA RESEARCH CENTER

G–a. DNA-Protein Inateraction Laboratory

(1) Kinetic Study on Transcription by *E. coli* RNA polymerase: 1. Irreversible Trapping in Abortive Cycle

Tomoko KUBORI and Nobuo SHIMAMOTO

The time course of synthesis of long and short (abortive) transcripts by *Escherichia coli* RNA polymerase was investigated under single round conditions *in vitro.* The synthesis of long RNA initiated at the λP_R (with an altered leader sequence) or at the *lacUV5* promoter was completed within 5 min, but abortive transcripts were continuously synthesized for at least 20 min. The results indicate the presence of transcription complexes that are capable only of abortive synthesis, and not of productive elongation. Consistently, only one in four of the RNA polymerase molecules that initially associated with the λP_R promoter synthesized long RNA. The enzyme reisolated from productive complexes synthesized both long and abortive transcripts, behaving just like original enzyme. This suggests that RNA polymerase is homogeneous with respect to its ability to synthesize the two types of product. Overall, the results indicate that many transcription complexes can be irreversibly trapped *in vitro* in an abortive cycle (Ref. 1).

We found abortive release is greatly activated in the presence of polymerase in excess of a promoter. When translocation of a polymerase on DNA is inhibited by another polymerase molecule that stacked in front at 32 nt from a promoter, a misincorporation at 6 nt and abortive release of 4–5 nt were greatly enhanced. The misincorporated transcripts were never elongated into long RNA even when the stacked molecule was removed.

(2) Single-Molecule Dynamics of Transcription Using Immobilized Operons: Sliding of *P. putida* CamR protein on DNA

Hiroyuki KABATA and Nobuo SHIMAMOTO

The sliding motion of RNA polymerase along DNA during promoter search has been proved by direct visualization of single molecules of the enzyme. To check the generality of sliding, we applied similar single molecule dynamics to a bacterial repressor protein, *P. putida* CamR, which is a homodimer molecule unable to relocate by the intersegment transfer mechanism, another relocation mechanism requiring two DNA binding sites. The protein was observed to slide along DNA. In the absence of its inducer, d-camphor, CamR molecules were trapped at three positions on a DNA, one was its cognate operator cloned in DNA, and the other two were likely to be homologous to the operator. The homologous regions turned out to be 400 and 4,000 times weaker binding sites than the cognate fragments by a gel-shift assay. But CamR stayed on them as long as the cognate site by the direct visualization. Immunoprecipitation assay showed a result similar to the direct visualization, indicating the limitation of the gel-shift assay.

In the presence of the inducer molecules trapped at the strong binding sites were significantly decreased, but the frequency of sliding increased. The result showed that the inducer destabilized only specific interactions but not sliding complexes. This shows difference in interactions maintaining specific complex and sliding complexes.

(3) Protein Footprint analysis of Transcription Complexes

Hiroki NAGAI, Tadashi TAKAGISHI and Nobuo SHIMAMOTO

Structural informations of engaged transcription complexes have been mainly obtained from DNA footprinting/Exonuclease III protection approaches. This fact means that the obtained information is based on the projected views of RNA polymerase onto DNA. Recently a novel and direct approach, analogous to DNA footprinting, for detecting inter-protein/protein-nucleic acid interactions is developed. We used this method, protein footprinting, to analyze structure of transcription complexes from *Echerichia coli.* We constructed and purified C-terminal HMK-tagged sigma and β subunits of RNA polymerase. HMK-tagged portions of these subunits were

end-labeled with 32P by heart muscle kinase *in vitro.* Reconstituted RNA polymerases were incubated with DNA template containing T7A1 promoter to form open complex, which were challenged by hydroxyl radical, and then cleaved sites were mapped by SDS-PAGE analysis. Results obtained so far indicated that a portion of sigma subunit (mapped to conserved subregion 3.2) in open complex was more protected from hydroxyl radical attack than in holoenzyme. Detailed analysis is now in progress.

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G–b. Recombinant DNA Laboratory

(1) Fluoride-resistant Mutants of the Nematode *Caenorhabditis elegans*

Minoru KAWAKAMI, Masaya TAKE-UCHI, Misako URASAKI, Takeshi ISHIHARA and Isao KATSURA

The fluoride ion is known to interact *in vitro* with components of signal transduction systems such as Ca^{2+}, protein phosphatases and trimeric G-proteins. We are isolating and characterizing fluoride-resistant mutants of *C. elegans* to discover a new signal transduction system.

Thirteen fluoride-resistant mutants that we isolated are recessive and map in five new genes, *flr-1* to *flr-5.* They are grouped into two categories: strongly resistant, slow-growing mutants (*flr-1*, *flr-3* and *flr-4*) and weakly resistant, normally growing mutants (*flr-2* and *flr-5*). Interestingly, the latter

mutations suppress the slow-growing phenotype of the former (Katsura, I. *et al.* (1994) Genetics **136**, 145–154). All those mutations seem to affect the nervous system, because some of them are defective in chemotaxis (See below), the slow-growing mutants have short defecation-cycle periods (Dr. J. H. Thomas, University of Washington), and all except the *flr-2* mutants have synthetic dauer-constitutive phenotypes (See section (3)).

To learn the molecular mechanism of the action of *flr* genes, we cloned the genes and cDNAs of *flr-1* and *flr-3*. The amino acid sequence deduced from a partial-length cDNA of *flr-1* has weak homology to that of the MEC-4 and DEG-1 ion channels of *C. elegans* and amiloride-sensitive ion channels of mammals. We obtained a full-length cDNA of *flr-3*, which seems to encode a kinase-like molecule that has all the protein kinase consensus sequences except that of subdomain I, the ATP-binding site.

By comparing the physical map ("contigs") with the genetic map, we found that the positions of *flr-4* mutations are confined to a genomic region of several hundred kb. Injection of cosmid clones in this region into *flr-4* mutants revealed a clone that can rescue the phenotypes of slow growth and short defecation-cycle period of *flr-4*. We are now subcloning it and trying to define the minimal DNA region that can rescue the phenotypes.

We investigated the expression pattern of *flr-3*, using a *flr-3–lacZ* fusion gene that contained the upstream 3.65 kb and downstream 2.75 kb of the transcriptional start point of *flr-3*. Expression was detected in most intestinal cells, beginning just before body elongation in the mid-embryonic stage and ending at the end of the L1 or the beginning of the L2 larval stage.

To study the effect of *flr* mutations on the nervous system, chemotaxis assays were performed for the reference alleles of all the *flr* genes. It was found that the *flr-2* mutant was abnormal in chemotaxis to many volatile and non-volatile compounds, *flr-5* to lysine, cAMP (attractive) and 1-octanol (repulsive), and *flr-4* to lysine. The *flr-1* mutant occasionally showed weak abnormality, but no abnormality was detected for *flr-3*.

We plan to continue the analysis of the *flr* genes to elucidate the molecular mechanisms of fluoride-sensitivity, growth-rate regulation and neural functions.

(2) A Gene that Controls Both Hatching and Cell Migration in *C. elegans*

Ryuichi HISHIDA, Takeshi ISHIHARA and Isao KATSURA

Mutants in the *hch-1* gene have abnormalities both in hatching and in cell migration (Hedgecock, E. M. *et al.* (1987) Development **100**, 365–382). They cannot digest protein components of the eggshell, and a neuroblast called QL moves anteriorly instead of posteriorly during larval development. After we isolated a transposon-insertion mutant in *hch-1* (K. Kondo (Soka University) and I. Katsura, unpublished results), we cloned the gene by the transposon-tagging method and the cDNA by using the genomic fragment as the probe. The cloned cDNA had the same length (3 kb) as the mRNA as detected by Northern analysis. It encoded a protein that consisted of a signal peptide, a Zn protease domain, an EGF domain and a CUB domain, and that resembled the Drosophila TOLLOID and the mammalian BPM-1, both of which act in cell-differentiation. However, the protein most closely related to *hch-1* is the sea urchin BP10/SpAN, which is reported to act in morphogenesis but not in hatching. We performed *in situ* hybridization of *hch-1* to embryos to detect expression before hatching. The results showed that expression starts just before body-elongation, continues through the comma stage and ends at the 1.5-fold or 2-fold stage, while no expression was detected at later embryonic stages. The hybridization signal was found on the dorsal and lateral sides of the middle and posterior part of embryos before elongation, but was limited to the lateral sides (probably seam cells) at the 1.5-fold stage. Both the morphogenetic protein BP10/SpAN and hatching enzymes (sea urchin HE6/SpHE and teleost HCE/LCE) were reported to be expressed in the middle of embryogenesis, considerably before hatching. Hence *hch-1* may be a hatching enzyme, or may act in the processing/secretion of hatching enzymes or in the differentiation of the cells that produce hatching enzymes.

We are going to analyze the role of each domain of *hch-1* and to investigate whether *hch-1* acts upstream or downstream to the homeotic gene *mab-5*, which also affects the direction of QL migration.

(3) Analysis of the Head Neural Circuit of *C. elegans* as Studied by Formation of Dauer Larvae

Norio SUZUKI, Misako URASAKI, Takeshi ISHIHARA and Isao KATSURA

If larvae of *C. elegans* just after hatching are in a crowded state and given a limited food supply, they deviate from the normal life cycle and develop to enduring, non-feeding larvae called dauer larvae. The developmental decision to become dauer larvae is regulated by the head neural circuit, with the smell of bacteria and the pheromone as the inputs, which are sensed by amphids, a pair of sensory organs in the head. Since the assay of dauer formation is much less time-consuming than behavioral assays such as chemotaxis assays, we are analyzing the head neural circuit by detecting dauer formation as the output. We found that more than 40 genes are involved in the synthetic dauer-constitutive (syn Daf-c) phenotype, *i.e.*, the phenotype in which combinations of two mutations cause worms to develop to dauer larvae irrespective of environmental conditions. The synthetic nature of this phenotype, we think, is based on the structure of the neural circuit, in which many neurons are positioned in parallel or in a network, only a part of which can be blocked by a single mutation. If this is the case, the specific combinations of mutations for the phenotype should contain information for the structure of the functional neural network on which the dauer regulatory signal is transmitted and processed.

We found that the pattern of combinations for the syn Daf-c phenotype is very specific and can be explained by assuming three parallel pathways, if we exclude *flr* mutations. We plan to obtain further information by investigating whether known dauer-defective mutations can suppress the syn Daf-c phenotype of various double-mutations.

We isolated 44 new mutations that show the syn Daf-c phenotype in combination with the *unc-31*(*e169*) mutation, which causes abnormalities in body movement, pharyngeal pumping, mechanosensation and egg-laying. We mapped them by sequence-tagged site markers and are performing complementation tests to learn whether they are alleles of known genes. We also examined the penetration of the fluorescent dye diO into amphids and phasmids (sensory organs in the tail) to investigate possible structural defects in those organs. Thirty-three mutants were normal, five slightly abnormal and six clearly abnormal. The results together with the results of mapping

show that a majority of the new mutations are located in unknown genes and that this method is useful for the isolation of new neural mutations. For further characterization of the mutants we plan to look for behavioral abnormalities and, with the help of markers described in (4) below, abnormalities in the structure of the neural system and differentiation of neurons.

(4) Construction of GFP Markers for Various Neurons in *C. elegans* and Their Use for Mutant Isolation

Takeshi ISHIHARA, Manabi FUJIWARA and Isao KATSURA

To analyze the formation and function of the neural circuit in *C. elegans*, we made 15 types of worms in which specific sets of neurons emit fluorescence. They were prepared by integration (into the worm genome) of DNA constructs in which the cDNA of GFP (jellyfish green fluorescent protein; Chalfie, M. *et al.* (1994) Science **263**, 802–805) is fused with various *C. elegans* neural promoters. Three of the promoters were selected by the "promoter trapping" method (Hope I. (1991) Development **113**, 399–408), and twelve by looking at the genome DNA sequence. The number of cells that express GFP ranged from only one pair to a great majority of the neurons. The fluorescence was localized to cell nuclei and to processes and cell bodies, if the SV40 nuclear localization signal and a *C. elegans unc-76* genomic fragment, respectively, were inserted into the DNA constructs.

Six of the 15 promoters showed expression in interneurons. We are studying functions of four of the six genes, which code for three putative glycine (or GABA) receptors and a metabotropic glutamate receptor. Gene disruption experiments are continuing.

To learn the functions of specific neurons we plan to kill them by introducing DNA constructs in which those promoters are connected to cell-death genes (*mec-4*(*d*), *ced-3* or *ced-4*). We also plan to isolate mutants abnormal in neural morphology using the GFP markers. Using a marker that is expressed in most neurons, we have already performed such experiments and obtained three mutants in neural cell lineage or cell migration.

Publications

None

G–c. DNA Synthesis Laboratory

(1) Crystallographic Study of $\alpha_3\beta_3$ Sub-complex of F1–ATPase: Structural Analysis of Supramolecule

Yasuo SHIRAKIHARA

F1–ATPase is a catalytic sector of the membrane bound ATP synthase which plays a central role in energy conversion in mitochondria, chloroplasts and bacteria, generating ATP from ADP and inorganic phosphate using energy derived from a trans-membrane electro-chemical potential. The $\alpha_3\beta_3$ sub-assembly of F1–ATPase is an active complex with 20–25% of the ATPase activity of intact F1, and has similar catalytic properties to those of F1. Both F1–ATPase and the $\alpha_3\beta_3$ sub-assembly have been challenging targets for crystallographic study because of their large sizes *e.g.* 380 kDa for F1 and 320 kDa for the $\alpha_3\beta_3$ sub-assembly.

The nucleotide-free $\alpha_3\beta_3$ sub-assembly from *Bacillus* PS3 F1 formed the crystals which diffracted to 3.2 Å resolution. We solved the structure of the subcomplex by the molecular replacement technique using bovine F1 structure as a search model (manuscript in preparation). The current model has R factor of 16.2% (free R factor 32.0%) and has a reasonable stereochemistry *e.g.* the root mean square deviations from ideal values of 0.01 Å for bond lengths, and 1.5° for bond angles. The structure was solved in collaboration with Andrew Leslie, Jan Pieter Abrahmas and John Walker at MRC Laboratory of Molecular Biology, Cambridge, UK.

The crystal structure of the $\alpha_3\beta_3$ sub-assembly was symmetrical: that is, the three α and three β subunits have identical conformations. This was in contrast to the asymmetry observed in the nucleotide-bound bovine F1 structure. In PS3 structure the β subunits adopted a conformation essentially identical to that of the nucleotide-free β subunit in bovine F1. This suggests that in F1 the nucleotide occupancy as well as the interactions with the γ subunit determine the conformation of the nucleotide-free β subunit. In spite

of the difference in nucleotide ligation, the α subunit had a similar conformation in both structures.

(2) Crystallographic Study of Transcription Factors, PhoB and CamR

Yasuo SHIRAKIHARA

PhoB Protein is a transcriptional activator for the genes in the phosphate regulon of *E. coli*, such as *phoA* and *pstS*, that are induced by phosphate deprivation. PhoB acts by binding to the *pho box* in the promoter region, which is the consensus sequence shared by the regulatory regions of *phoA*, *phoB*, *phoE* and *PstS*. The activity of PhoB is regulated by phosphorylation by PhoR. The C terminal domain of PhoB has a DNA binding ability, while the N terminal domain has a regulatory role governed by phosporylation by PhoR protein.

We previously crystallized the C terminal domain of PhoB. Crystals diffracted to at least 2.4 Å resolution, but had a minor disorder in the crystalline lattice. Using a new C terminal fragment which is shorter by 8 amino acid residues than the fragment above, we got crystals with no appreciable disorder. Also we got crystals of the intact PhoB protein. This work has been done with Kozo Makino, Research Institute for Microbial Diseases, Osaka University.

CamR protein is a repressor that regulates transcription of the cytochrome P-450cam hydroxylase operon of *Pseudomonas putida*. Expression of the *camDCAB* operon and the *camR* gene is regulated through interaction of the CamR protein with the single operator located in the overlapping promoter region between the *camDCAB* operon and the *camR* gene. D-camphor is an inducer which allows those genes to be transcribed. CamR is a homodimer with a molecular mass of 40 kDa.

CamR was crystallized from polyethyleneglycol solution with a typical size of 0.5 mm × 0.4 mm × 0.3 mm in the presence or absence of D-camphor. However, recent crystallization experiments have failed to grow such large crystals, probably due to variability of protein preparations. While trying to improve the quality of the CamR protein preparation, we searched for new crystallization conditions. In stead of polyethyleneglycol, Na-K-phosphate was found to give crystals of the similar sizes in the presence of D-camphor. This work has been done with Hironori Aramaki, Daiichi College of Pharm-

aceutical Sciences.

Publications

None

G-d. Gene Library Laboratory

(1) Expression map of the *C. elegans* genome

Yuji KOHARA, Tomoko MOTOHASHI, Hiroaki TABARA, Akiko SUGIMOTO, Hisako WATANABE, Masako SANO and Akiko MIYATA

Aiming to ultimately understand the network of gene expression in the development of the nematode *C. elegans*, we are trying to construct an expression map of the 100 Mb genome by identifying and characterizing all of its cDNA species, whose number is estimated to be around 13,000.

Altogether 38,000 cDNA clones were picked up randomly from 3 different cDNA libraries (from a mixed-stage population, from size-fractionated (>2 Kb) cDNA of a mixed stage population and from embryos), stored and gridded. To avoid unnecessary redundancy of analyses, cDNA clones that belong to previously identified abundant cDNA species were screened by probing the cDNA grids and removed. cDNA inserts of individual clones were PCR-amplified using vector primers and then subjected to the following analyses.

(1) Tag sequencing: Single reads were made from both 5′-ends (using vector primers) and 3′-ends (using anchored oligo-dT primers in order to minimize the effect of a long poly-A stretch) on ABI sequencers. 3′-tag sequences were compared with each other using FASTA to classify the cDNA clones into unique groups (genes). Thus far, about 16,000 clones gave 11,104 clean 3′-tag sequences, which were classified into 4,279 unique cDNA species (assigned group names CELK00001 through CELK04279). This analysis also detected many pairs of clones which appeared to be generated by alternative splicing or differential poly-A addition. BLASTX search showed that 44% of the cDNA groups gave significant similarities (blastx score >100).

(2) Mapping onto the genome: Using the ordered YAC filters, 1,416

cDNA groups have been mapped onto the genome; central regions of autosomes turned out to be much denser for cDNA than other regions. Using the genomic sequences kindly provided by the Sanger Centre (1,001 cosmids including 172 cosmids at a shotgun phase), 1,341 cDNA species out of the 4279 species were mapped ("mapping *in silico*").

(3) Analysis of expression pattern: We are interested in gene expression patterns particularly during embryogenesis. For this purpose we have developed a method of *in situ* hybridization of the standard mult-well format on whole mount embryos, and we have started a systematic in situ analysis using the set of representative clones of the identified cDNA groups. Thus far, 594 cDNA species were analyzed in this way, providing a specific pattern of expression at a frequency of 1/5.

(2) A Multi-well Version of *in situ* Hybridization on Whole Mount Embryos of *C. elegans*

Hiroaki TABARA, Tomoko MOTOHASHI and Yuji KOHARA

We have developed an efficient procedure for *in situ* hybridization of a multi-well format on *C. elegans* embryos for large scale screening of gene expression patterns in this organism. Each hybridization well contains embryos at various stages throughout embryogenesis. The validity of the method was confirmed by results with control genes whose expression patterns had been reported; *glp-1* in very early embryos, *myo-2* in pharyngeal muscle and *unc-54* in body-wall muscle. Several collagen genes and a pepsinogen gene were also examined to establish a set of lineage specific markers.

(3) *Pos-1*, a Gene Which Shows Localization of Its mRNA to the P Lineage During Early Cleavage of *C. elegans* Embryos

Hiroaki TABARA, Tomoko MOTOHASHI, Craig MELLO[1], Jim PRIESS[2] and Yuji KOHARA ([1]Cancer Center, University of Massachusetts, Worcester, MA, USA, [2]Fred Hutchinson Cancer Research Center, Seattle, WA, USA)

We have long searched for localized maternal mRNA in early embryos of the worm using a range of approaches to differential screening. In the course

of the systematic analysis of *in situ* hybridization with a set of classified cDNA clones which have been generated by our cDNA project, we found that the clone, yk61h1, of the cDNA group CELK01662 (=YK1662 in ACEDB) showed an asymmetric distribution of its mRNA in 2-cell stage embryos. Closer examinations on the behavior of the mRNA in gonads and embryos using *in situ* hybridization showed that (1) the mRNA started to appear at the turn of gonad, (2) it was distributed evenly in oocytes, (3) it was segregated posteriorly as the first cleavage proceeded, (4) the mRNA remained in P4 but disappeared in other somatic blastmeres (perhaps through a mechanism of degradation). *In situ* analysis using *nop-1* embryos which lacked pseudo-cleavage suggested that the segregation of the mRNA occurred during the first cleavage. Northern hybridization showed that the mRNA (1.3 Kb) was detected in embryos and adults but not in L1 larva. We named this gene *pos-1* (posterior segregation), and have analyzed it further.

Full sequencing of the cDNA clone showed that the *pos-1* mRNA has the trans-spliced leader SL1 and a strong homology to the zinc-finger region of the mammalian Tsi11 growth factor inducible genes. This zinc-finger motif is also found in the maternally expressed *C. elegans* gene *pie-1*. Interestingly, the position of *pos-1* turned out to be very close to a second maternal gene, *skn-2*. The *skn-2* gene, like *pie-1*, is required for proper development of the P lineage, and *skn-2* and *pie-1* mutants exhibit dominant genetic interactions. These genetic interactions and the physical and genetic proximity of *pos-1* and *skn-2* led us to invetigate the possibility that *pos-1* = *skn-2*. The following evidence supports this hypothesis: (1) injection experiments using *pos-1* anitsense RNA caused phenocopies of *skn-2*, and (2) a missense mutation was found in the zinc finger region of *pos-1* in a *skn-2* mutant (zu148).

The phenotypes of *skn-2* mutants were examined closely. The lack of germ line was confirmed by the fact that anti-P granules antibodies did not stain late embryos of *skn-2*; it stained two germ line precursor cells Z2 and Z3 in wild type embryos. Although *skn-2* embryos are lethal, some somatic lineages seem to differentiate properly; we found the differentiation of hypodermis, gut and pharynx in *skn-2* embryos. As to the function of *pie-1*, a genetically related gene to *skn-2*, it was hypothesized that the *pie-1* protein repressed the transcription of somatic genes in the germ line, based on the observation that the mRNA of some zygotic genes, *e.g. pes-10*, which was detected in all somatic cells but not in the germ line, was detected also in the germ line of *pie-1* embryos. We perfomed *in situ* experiments with such

zygotic genes including *pes-10* and *4–3* on *skn-2* embyros. However, the mRNA of *4–3* was not detected in the germ line of *skn-2* embryos.

Antibodies against the N-terminal part of the *pos-1* protein were raised and the distribution of the *pos-1* protein was examined. The expression of the protein was coincident with the pattern of the localized mRNA. Essentially no expression of the protein was detected in 1-cell embryos or AB blastmeres in 2-cell embryos. The expression of the protein was detected in P1, P2, P3 and P4. Very interestingly, the expression of the *pos-1* protein was cytoplasmic in contrast to other genes conatining the Tis11 zinc finger motif.

Based on these facts and preliminary observations about other genes containg the Tis11 zinc finger motif, we hypothesize that the function of the *pos-1* proteins is to stabilize or activate the translation of mRNA of other factors which are necessary for establishing the fate of the germ line.

Publication

1. Barnes, T., Kohara, Y., Coulson, A. and Hekimi, S.: Meiotic recombination, noncoding DNA and genomic organization in *Caenorhabditis elegans*. Genetics, **141**, 159–179, 1995.

H. CENTER FOR INFORMATION BIOLOGY

H–a. DNA Data Analysis Laboratory

(1) Domain Evolution of Serine Protease and Its Inhibitor Genes

Takashi GOJOBORI, Toshinori ENDO and Kazuho IKEO

The evolution of serine protease and its inhibitor was discussed with special reference to domain evolution. It is now known that most proteins are composed of more than one functional domain. Because serine proteases such as urokinase and plasminogen and some of their protease inhibitors are made of various functional domains, these proteins are typical examples of the so-called mosaic proteins.

When kringle domains in serine proteases and a Kunitz-type protease inhibitor domain in the amyloid β precursor protein in Alzheimer's disease patients were examined by the molecular evolutionary analysis, the phylogenetic trees constructed showed that these functional domains had undergone dynamic changes in the evolutionary process. In particular, these domains are considered to be evolutionarily movable.

It was concluded that these functional domains evolved independently of each other and that they have been shuffled to create the existent mosaic proteins. This conclusion leads us to the reasonable speculation that these functional domains must have been minigenes, possibly at the time of primordial life or the origin of life. We may call these minigenes "ancestral minigenes". For more details, see Ref. 7.

(2) Sequence Divergence Estimation

Takashi GOJOBORI and Kazuho IKEO

Methods for estimating sequence divergence are crucial for studies of molecular evolution. In particular, the number of nucleotide (or amino acid) substitutions is a good quantity to use in representing the degree of sequence divergence when two homologous sequences are being compared. Thus,

knowledge of the number of nucleotide (or amino acid) substitutions is important for computing evolutionary rate and for constructing phylogenetic trees at the DNA level. We reviewed statistical methods for estimating the number of nucleotide and amino acid substitutions, putting particular emphasis on their practical usage and usefulness. For more details, see Ref. 10.

(3) Different Evolutionary Histories of Kringle and Protease Domains in Serine Proteases: A Typical Example of Domain Evolution

Kazuho IKEO, Kei TAKAHASHI[1] and Takashi GOJOBORI ([1]Department of Physiology, Shimane Medical University, Isumo)

With the aim of elucidating the evolutionary processes of the kringle and protease domains in serine proteases which are involved with the system of blood coagulation and fibrinolysis, we constructed phylogenetic trees for the kringle and protease domains, separately, by use of amino acid sequence data. The phylogenetic trees constructed clearly showed that the topologies were different between the kringle and protease domains. Because both domains are coded by single peptides of serine proteases, this strongly suggests that the kringle and protease domains must have undergone different evolutionary processes. Thus, these observations imply that serine proteases evolve in a way such that each domain is a unit of evolution, exemplifying a typical mode of domain evolution. A possible relationship between the domain evolution and the exon shuffling theory were also discussed from the viewpoint of gene evolution. For more details, see Ref. 8.

(4) Evolution of Genes and The DNA Database-Implications for Bioinformation

Takashi GOJOBORI, Toshinori ENDO and Kazuho IKEO

Needless to say, the DNA sequence database has tremendous value in many contexts. Data retrieval has been very useful not only in academic circles but also in bioindustries. The DNA database can also be used to study the molecular evolution of genes. We conducted a large-scale analysis of molecular evolution, using the DDBJ DNA sequence database. The results obtained showed that homologous domains exist among remotely related

gene groups. This suggests that present genes may have emerged as "mosaic genes" from different domains of their ancestral gene by shuffling of homologous domains during evolution. This concept of "mosaic genes" is very useful and important not only for biotechnology but also for bioremediation. For more details, see Ref. 11.

(5) Large-Scale Search for Genes on Which Positive Selection May Operate

Toshinori ENDO, Kazuho IKEO and Takashi GOJOBORI

We conducted systematic search for the candidate genes on which positive selection may operate, on the premise that for such genes the number of nonsynonymous substitution is expected to be larger than that of synonymous substitutions when the nucleotide sequences of the genes under investigation are compared with each other. By obtaining 3,595 groups of homologous sequences from the DDBJ, EMBL, and GenBank DNA sequence databases, we found that seventeen gene groups can be the candidates for the genes on which positive selection may operate. Thus, such genes are found to occupy only about 0.5% of the vast number of gene groups so far available. Interestingly enough, nine out of the seventeen gene groups were the surface antigens of parasites or viruses. This work is described in more details in Molecular Biology and Evolution. For more details, see Ref. 13.

(6) Large-Scale Search for Within-Gene Regions Where Positive Selection May Operate

Toshinori ENDO, Kazuho IKEO and Takashi GOJOBORI

To determine what is the proportion of genes on which positive selection operates, we searched for genes on which positive selection may operate from a large number of DNA sequences in DDBJ, EMBL and GenBank DNA databases. We also attempted to identify the within-gene regions which responsible for positive selection. For this purpose we developed a window search method which quickly identifies the within-gene regions possibly responsible for positive selection on the basis of estimation and comparison of the numbers of nonsynonymous substitution with that of nonsynonymous

substitutions for each window in a homologous DNA alignment. From 3,595 homologous gene groups which we previously obtained using homology search, we found that 192 genes (5.3%) of the gene groups examined were the candidates on which positive selection may operate. Among these genes, the most major category was the gene group for the surface proteins of parasites and viruses. The candidates also contained the gene groups for secretory proteins, various kinds of enzymes, toxins and so on. These results suggest that there may be various reasons for positive selection. These results were presented at the Society of Molecular Biology and Evolution Meeting 1995 at Hayama, the meeting of Genetics Society of Japan at Okayama and the annual meeting of the Molecular Biology Society of Japan at Nagoya.

(7) Evolutionary Relationship of Hepatitis C, Pesti-, Flavi-, Plantviruses, and Newly Discovered GB Hepatitis Agents

Ken-ichi OHBA[1], Masashi MIZOKAMI[1], Johnson Y. N. LAU[2], Etsuro ORITO[1], Kazuho IKEO and Takashi GOJOBORI ([1]Second Department of Medicine, Nagoya City University Medical School, Kawasumi, Mizuho, Nagoya, [2]Section of Hepatobiliary, Department of Medicine, University of Florida, Gainesville, FL, USA)

Two flavivirus-like viruses, GB virus-A (GBV-A) and GB virus-B (GBV-B), were recently identified in the GB hepatitis agent, and are known to be distinct from the hepatitis A to E viruses. The putative helicase domain of GBV-A and GBV-B was found to have amino acid sequence homology with that of hepatitis C virus (HCV), and to be distantly related to pestiviruses, flaviviruses, and plant viruses. A phylogenetic tree constructed showed that GBVs and HCV were closely related to each other and they were clustered with pestiviruses, flaviviruses and plant viruses in that order. This finding may lead us to an interesting speculation that HCV and GBV may have originated from some of insect and plant viruses. For more details, see Ref. 15.

(8) Classification of Hepatitis C Virus into Major Types and Subtypes Based on Molecular Evolutionary Analysis

Ken-ichi OHBA[1], Masashi MIZOKAMI[1], Tomoyoshi OHNO[1], Kaoru SUZUKI[1], Etsuro ORITO[1], Yasuo INA, Takashi GOJOBORI and Johnson Y. N. LAU[2]
([1] Second Department of Internal Medicine, Nagoya City University Medical School, Kawasumi, Mizuho, Nagoya, [2] Section of Hepatobiliary Diseases, Department of Medicine, University of Florida, Gainesville, FL, USA)

Molecular evolutionary analysis was applied to determine the number of hepatitis C virus (HCV) types and subtypes based on all the HCV nucleotide sequences available from the DNA data banks (DDBJ, GenBank (NCBI), EMBL) and the literature. There was an excellent concordance among the types and subtypes assigned based on different HCV genomic regions. Only one HCV isolate was assigned to different HCV types based on the 5′ non-coding (NC) and envelope I (E1) regions. The 5′ NC region was well conserved and could be used to assign only types and not subtypes. From the sequence data available, there were 13 subtypes based on the core region and 14 subtypes based on the E1 and non-structural protein 5 (NS5) regions. For more details, see Ref. 12.

(9) Patterns of Amino Acid Substitutions in the Third Variable Envelope Region of HIV Within Single Hosts

Yumi YAMAGUCHI and Takashi GOJOBORI

The third variable envelope region (V3) is one of the major epitopes of HIV. We intended to elucidate the evolutionary mechanism of molecular evolution of the V3 region within a human body. We collected the nucleotide sequence data of HIV clones which were isolated from single hosts at several time points after infection. We then estimated the rates of synonymous and nonsynonymous substitutions for the V3 region of HIV within single hosts at each period of time. In some periods of time since infection, the rate of nonsynonymous substitution was significantly higher than that of synonymous substitution. We also estimated frequencies and types of amino acid substitutions that occurred at each amino acid site in the V3 region. As a result, we found that amino acid substitutions dominantly occurred at several amino acid sites where the substitutions are known to be responsible for

production of antigenic variation and determination of the viral phenotypes. These observations indicate a strong possibility that positive selection is operating in the V3 region of HIV within single hosts by changing amino acids at particular sites.

(10) Molecular Epidemiology of Human Immunodeficiency Virus Type 1

Yumi YAMAGUCHI and Takashi GOJOBORI

Human immunodeficiency virus type 1 (HIV-1) that possibly came from Africa is now distributed all over the world. Previous phylogenetic analyses showed that almost all HIV-1 isolates from developed countries and Haiti form a distinct cluster known as "subtype B". Those studies suggested that one type of HIV-1 variants in Africa was introduced to developed countries and Haiti. We intended to elucidate the course of transmission of HIV-1 to all over the world by phylogenetic analysis. We collected nucleotide sequences of HIV-1 from international DNA databases. We constructed phylogenetic trees of HIV-1 using amino acid sequences in a part of the envelope glycoprotein including the third variable envelope (V3) region. We found that HIV-1 isolates from Russia and Romania form each new cluster. Our results suggest the possibility that course of transmission of HIV-1 to Russia and Romania might differ from that to most developed countries. Our results also support the possibility that HIV-1 might be introduced to India and Thailand directly from Africa.

(11) Molecular Evolution of HIV-1 and its Implication to Mother-To-Child Transmission

Kazunari TAKAHASHI and Takashi GOJOBORI

Transmission of human immunodeficiency virus type 1 (HIV-1) takes place, with a high frequency, among drug users, sexual partners, and mother-to-child pairs. Our study was conducted with the aim of elucidating the evolutionary modes of HIV-1 at the time when it is transmitted to a new host. We used the nucleotide sequences of HIV clones isolated at the time of the mother-to-child transmission. These data were obtained from the three DNA databases of DDBJ, EMBL, and GenBank. We then constructed phylogene-

tic trees by the neighbor-joining method. The phylogenetic tree constructed clearly showed how particular HIV clones in a mother were transmitted to her child.

(12) Molecular Evolution of Marburg and Ebola Viruses

Yoshiyuki SUZUKI and Takashi GOJOBORI

Marburg and Ebola viruses, members of the nonsegmented negative-stranded RNA viruses, constitute the genus *Filovirus*, the sole member of the family *Filoviridae* which is one of the constituents of the order *Mononegavirales.* These viruses are known to be the etiological agents of the haemorrhagic fever with high mortality rates and have been grouped into the "biosafety level 4" agents. From the molecular evolutionary points of view, it is of importance to elucidate the origin and evolutionary mode of these viruses. In this study, the rate of nonsynonymous substitutions for Marburg virus was estimated to be 4.3×10^{-4} per nonsynonymous nucleotide site per year, which was of almost the same order of magnitude with other RNA viruses. When this rate was applied to the degrees of sequence divergences the divergence time between Marburg and Ebola viruses was estimated to be more than 300 years ago, possibly several thousand years ago. Moreover, the pattern of nucleotide substitutions for Marburg virus indicated that the purifying selection has operated on the evolution of this virus. The results for the rate of nonsynonymous substitutions and the divergence time between Marburg and Ebola viruses were presented on the 43rd Annual Meeting of the Society of Japanese Virologists at Okayama, Japan in October, 1995.

(13) Molecular Phylogenetic Trees Based on Minimum Description Length Principle

Fengrong REN[1], Hiroshi TANAKA[1], Norio FUKUDA[1] and Takashi GOJOBORI
([1] Tokyo Medical and Dental University, Tokyo)

Ever since the discovery of a molecular clock, many methods have been developed to reconstruct the molecular phylogenetic trees. In this study, we dealt with this problem from the viewpoint of an inductive inference and apply Rissanen's minimum description length principle to extract the mini-

mum complexity phylogenetic tree. Our method describes the complexity of molecular phylogenetic tree by three terms which are related to the tree topology, the sum of branch lengths, and the difference between the model and the data measured by logarithmic likelihood. Five mitochondrial DNA sequences from human, common chimpanzee, pygmy chimpanzee, gorilla and orangutan were used for investigating the validity of this method. It is suggested that this method is superior to the traditional method in that it still shows the good accuracy even near the root of phylogenetic trees. For more details, see Refs. 1 and 2.

(14) NRSub: a Non-Redundant Database for *Bacillus subtilis*

Guy PERRIÈRE[1], Ivan MOSZER[2] and Takashi GOJOBORI ([1]Laboratoire de Biométrie, Génétique et Biologie des Populations, Université Clause Bernard-Lyon 1, France, [2]Unité de Régulation de l'Expression Génétique, Institut Pasteur, 28, France

In the context of the international project aimed at sequencing the whole genome of *Bacillus subtilis*, we have developed a non-redundant, fully annotated database of sequences from this organism. Starting from the *B. subtilis* sequences available in the EMBL, GenBank and DDBJ collections, we have removed all encountered duplications and then added extra annotations to the sequences (*e.g.* accession numbers for the genes, locations on the genetic map, codon usage, *etc.*). We have also added cross-references to the EMBL, MEDLINE, SWISS-PROT and ENZYME data banks. The present system results from merging of the NRSub and SubtiList databases and the sequence contigs used in the two systems are identical. NRSub is distributed as a flatfile in EMBL format (which is supported by most sequence analysis soft ware packages) and as an ACNUC database, while SubtiList is distributed as a relational database under 4th Dimension. It is possible to access the data through two dedicated World Wide Web servers located in France and Japan. For more details, see Ref. 14.

(15) Analysis of the Structure of the *Escherichia coli* Genome by Seeing the Distance between Homologous Genes

Hidemi WATANABE and Takashi GOJOBORI

A hypothesis has been proposed for the evolution of the *Escherichia coli* genome that the genome has increased in size not by gradual addition but through at least two times genome doublings. Although this hypothesis was originally presented on the basis of the regular distribution of functionally related homologous genes in the genome, it seems rather unconvincing because the number of the genes used was very small and the regularity was not evaluated by any statistical method. To date, the genome of *E. coli* has been sequenced around three quarters. Thus we tried to test the hypothesis with all the genes already sequenced. The frequency distribution of the physical distances between all homologous gene pairs was computed. In this computation, the orientation of the genes of each pair in the genome was considered, and thus two distributions were obtained for the pairs of genes in the same orientation and those in different orientations. Several characteristic features are found in these distributions: Two dominant peaks are detected in the distribution of the pairs in the same orientation whereas no obvious peak is not found in the distribution of those in different orientations. One of the two peaks shows that tandem repeats of homologous genes in the same orientation are predominant among all the homologous gene pairs. The other peak is found around 18 min distance. In both of the two distributions, no peak is found around 25 or 50 min distance. Therefore, it is concluded that gradual additions and duplications of local regions rather than recent two times genome doublings have been contributed to the evolution of genome size in *E. coli.*

(16) Analyses of the Structures of Microbe Genomes on the Basis of the Homologous Relationships between Genes

Hidemi WATANABE, Hirotada MORI and Takashi GOJOBORI

To test two different hypotheses on the evolution of microbe genomes that microbe genomes are evolutionarily stable and that the variety of their sizes is mainly brought by genome doubling during evolution, the genome structures of *Haemophilus influenzae*, *Mycoplasma genitalium*, *Escherichia coli* and

Bacillus subtilis were compared by use of the DNA sequences of their genomes. In these comparisons, the locations of orthologous genes were examined between different genomes. Comparisons of orthologous genes guarantee that the differences revealed by the comparisons mostly reflect the changes in genome structure after the speciation of the species compared. We invented two new measures for the degree of conservation of relative gene locations and for the difference in gene number. The comparisons by the new measures primarily revealed striking evidence that genome rearrangements have frequently occurred in microbe genomes during their evolution even after the relatively recent speciation between *E. coli* and *H. influenzae.* Quite exceptionally are only a few regions conserved, *e.g.* the S10 region, the cluster of genes for cell division, and the ATP synthase operon. This indicates that even the clusters consisting of genes functionally related to each other, *e.g.* operons, are not preserved through evolution. An additional analysis led to us to suggest that the common ancestral genome of *E. coli* and *H. influenzae*, having genomes of 4.7 and 1.8 megabases, respectively, must have been as large as the *E. coli* genome, and then the genome of *H. influenzae* has been shrunk.

(17) Monophyletic Origin and Unique Dispersal Patterns of Domestic Fowls

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With the aim of elucidating in more details the genealogical origin of the present domestic fowls of the world, we have determined mitochondrial DNA(mtDNA) sequences of the D-loop regions for a total of 21 birds which belong to red junglefowl (*Gallus gallus*) comprised of three subspecies (6 *Gallus gallus gallus*, 3 *Gallus gallus spadiceus* and 3 *Gallus gallus bankiva*) and 9 representing diverse domestic breeds (*Gallus gallus domesticus*). We also sequenced 4 green junglefowl (*Gallus varius*), 2 Lafayatte's junglefowl

(*Gallus lafayettei*) and 1 grey junglefowl (*Gallus sonneratii*). We then constructed a phylogenetic tree for these birds by the use of nucleotide sequences, choosing the Japanese quail (*Coturnix coturnix japonica*) as an outgroup. We found that a continental population of *G. g. gallus* was the real matriarchic origin of all the domestic poultries examined in the present study. It is also of particular interest to note that there were no discernible difference among *Gallus gallus* subspecies; *G. g. bankiva* being a notable exception. This was because *G. g. spadiceus* and a continental population of *G. g. gallus* formed a single cluster in the phylogenetic tree. *G. g. bankiva*, on the other hand, was a distinct entity, thus, deserving its subspecies status. It implies that a continental population of *G. g. gallus* sufficed as the monophyletic ancestor of all domestic breeds. We also discussed a possible significance of the initial dispersal pattern of the present domestic fowls, utilizing the phylogenetic tree. For more details, see Ref. 16.

(18) Isolation and Characterization of cDNA Clones for Japanese Quail (*Coturnix Japonica*) Major Histocompatibility Complex (*MhcCoja*) Class I Molecules

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Two cDNA clones (*QF41* and *QF63*) corresponding to the MHC class I genes of the Japanese quail (*Coturnix japonica*) were isolated by screening a liver cDNA library with the chicken class I cDNA (B–F) as a probe. The overall amino acid sequence identity between the Japanese quail and chicken MHC class I α chain was from 74% to 78%, with the most divergence in the transmembrane (TM) and cytoplasmic region (CY). Between the *QF41* and *QF63* cDNA clones, there was only 79% amino acid identity with the highest homology (93%) in the functionally conserved $\alpha 3$ domain. PCR analysis using genomic DNA as a template with the *QF41*- and *QF63*-specific primers revealed the different and unique exon/intron organization around the $\alpha 1$ and $\alpha 2$ domain regions. Taken together, these results suggest that the Japanese

quail has at least two distinct expressed MHC class I loci, designated *Coja-A* and *Coja-B*. For more details, see Ref. 6.

(19) Determination of HLA Class II Alleles by Genotyping in a Manchu Population in the Northern Part of China and Its Relationship with Han and Japanese Populations

Liao GENG[1], Tadashi IMANISHI, Katsushi TOKUNAGA[2], Dingliang ZHU[3], Nobuhisa MIZUKI[4], Shan XU[5], Zhencheng GENG[3], Takashi GOJOBORI, Kimiyoshi TSUJI[1] and Hidetoshi INOKO[5] ([1]Department of Transplantation Immunology, Tokai University School of Medicine, Kanagawa, [2]Department of Research, Japanese Red Cross Central Blood Center, Tokyo, [3]Institute of Genetics, Fudan University, China, [4]Department of Ophthalmology, Yokohama City University School of Medicine, Kanagawa, [5]Department of Genetics Information, Division of Molecular Life Science, Tokai University School of Medicine, Kanagawa)

The genetic polymorphism of the HLA class II loci was investigated in a Manchu population resident in the northern part of China and compared with those of other Asian populations including Japanese and Han. In 8 DQA1 alleles, the most frequent allele was DQA1*03 with the gene frequency of 25.5%. Of 15 DQB1 alleles tested, 11 were observed and the most common allele was DQB1*0301 with the gene frequency of 24.5%. Among 19 DPB1 alleles, 11 were detected and DPB1*0501 (43.8%) was the most frequent allele as observed in other Asian populations such as Japanese, Chinese and Korean. Of 43 DRB1 alleles tested, 21 were detected and DRB1*0901 (14.0%), *1501 (11.0%), *1201 (11.0%), *07 (9.0%) and *1401 (9.0%) were highly predominant and account for the high frequencies of DR9, DR2, DR5, DR7 and DR6. In the DRB3 gene (DR52), DRB3*0202 (18.0%) was the most frequent. With respect to the DRB4 gene (DR53), the gene frequency of DRB4*0101 was 35.0%. Of 3 DRB5 alleles detected, DRB5*0101 (11.0%) was highly predominant. Comparison of HLA class II allele frequencies in Manchu with those in Japanese and Han Chinese populations (South & North) detected some significant differences and genetic divergence between these Oriental populations. The dendrogram constructed by the neighbor-joining (NJ) method based on the allele frequencies of DQA1, DQB1, DPB1 and DRB1 of 10 representative populations

over the world suggested that Manchu is the closest, but at the same genetic distance to both Northern and Southern Han Chinese. For more details, see Ref. 5.

(20) Distribution of Shared Polymorphisms and Its Relation to Gene Conversion in the Major Histocompatibility Complex Genes

Tadashi IMANISHI

Human major histocompatibility complex (MHC) genes are highly polymorphic, and numerous MHC alleles are thought to be maintained by balancing selection. On the other hand, there seem to be three mechanisms to produce new MHC alleles: point mutation, recombination, and gene conversion. However, importance of gene conversion has been controversial, partly because its molecular mechanism is not yet clearly demonstrated. In this study, I analyzed statistically the nucleotide sequence data of human MHC loci to detect inter-locus gene conversion among MHC genes. By comparing the consensus sequences of HLA–A, B, C, DPB1, DQB1, DRB1, and DRB5, I found that a significantly large number of DNA polymorphisms are shared between different, duplicated MHC loci at orthologous nucleotide sites. The shared polymorphisms were observed in the hyper-variable exons of both class I (HLA–A, B, and C) and class II (HLA–DRB1, DQB1, and DPB1) loci. The shared polymorphisms between MHC loci can be accounted for primarily by frequent inter-locus gene conversion of very short gene fragments. Positive natural selection might have operated in order to maintain the shared polymorphisms that were produced by gene conversion. Moreover, it was suggested that the shared polymorphisms have ancient origins. For example, the shared polymorphisms observed between DRB1 and DRB5 implied that inter-locus gene conversion events took place immediately after the gene duplication of these loci. These results were reported at the third international meeting of the Society for Molecular Biology and Evolution and at the annual meeting of the Japanese Society of Molecular Biology.

(21) YAMATO AND ASUKA: DNA Database Management System

Hajime KITAKAMI[1], Tadasu SHIN-I, Kazuho IKEO, Yoshihiro UGAWA[2], Naruya SAITOU, Takashi GOJOBORI and Yoshio TATENO ([1]Hiroshima City University, Hiroshima, [2]National Institute of Agrobiological Resources, Ibaraki)

We have realized effective join processing with the newly developed DDBJ-schema which is easy to use, because its structure looks like a grammatical structure of the flat-file format. The DDBJ-schema was successfully implemented as a relational schema with three parts. It is possible to provide the SQL-service for on-line and E-mail users. Moreover, we showed that the schema allowed us to implement two types of interfaces which were named YAMATO and ASUKA. We also implemented a tool to convert EMBL and NCBI/GenBank-formats into the relational format with the DDBJ-schema. Finally, we proposed a structured SQL-programming method. The restructuring and tree search were implemented in the programming method. We have succeeded in a trial restructuring data with the GenBank-schema into those with the DDBJ-schema by use of the structured-SQL programming method. We are planning to move the current system to the newly developed system at DDBJ. For details, see Ref. 9.

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H-b. Laboratory of Gene Function Research

Molecular Evolution of a protein Seemingly Responsible for Epoxidation in Eukaryote

Yoshio TATENO

Fosfomycin (FOM) is an antibiotic discovered in cultures of Streptomyces. It has phosphite in the molecular structure and is characterized by a C–

P bond and an epoxy ring. FOM inhibits the initial reaction in biosynthesis of peptidoglycans in a broad spectrum of Gram-positive and Gram-negative bacteria, and thus is used as an antibiotic. FOM is now chemically synthesized by epoxidation of cis-propenylphosphonic acid (cPA) and isolation of the L-form compound in the chemical products. The isolation of the L-form, however, is not necessary, if the epoxidation process is attained by microbial conversion which was devised 25 years ago. If a gene of an enzyme involved in the microbial conversion is identified, the converting activity may be markedly increased by gene manipulation. The gene or the enzyme has not been identified yet.

The present study is to identify and search out the gene encoding epoxidase, which is to convert cPA into FOS in *Penicillium decumbens*. If the gene is available, the converting activity can be markedly increased by gene manipulation. We first screened *Penicillium decumbens*. The wild strains of the species so far examined, however, showed low converting activities, and to increase the activity through mutation and selection was difficult without identifying the responsible gene. The use of genetic engineering for enhancing the activity was another idea, but this again could not easily be realized for the same reason.

Thus we took an approach to identifying the corresponding product instead of the gene. By carrying out two-dimensional electrophoresis for cell-free extract of *P. decumbens* including cPA, we found possible proteins with a molecular weight of 31 kD but different isoelectric points. On the basis of sequence information of the proteins, we could find a gene that enhanced the converting activity when it was placed in a proper environment.

To confirm our observation further, we searched for the genes in the DDBJ database which encode enzymes catalyzing similar activities as the epoxidation in question. We found five such genes in prokaryote, and aligned the *Penicillium* gene with those sequences after translation. The alignment showed that the six sequences shared several residues where tryptophan resided. There were some more residues implying that the six sequences were homologous.

(The present study is still underway in collaboration with Dr. H. Watababe at NIG, Dr. K. Murakami at Meiji Seika Co. Ltd., and his colleagues.)

Publications

1. KITAKAMI, H., SHIN-I, T., IKEO, K., UGAWA, Y., SAITOU, N., GOJOBORI, T. and TATENO, Y.: YAMATO and ASUKA: DNA Database Management Systems. In Proceedings of the Twenty-Eighth Hawaii International Conference on System Sciences, pp. 72–80, L. Hunter and B. D. Shriver, eds., IEEE Computer Society Press, Los Alamitos, California, 1995.
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H–c. Laboratory for Gene-Product Informatics

(1) Prediction of Protein Three-Dimensional Structures by A 3D–1D Compatibility Method

Ken NISHIKAWA

After a number of unsuccessful trials over the years, the protein structure prediction from the sequence information has been actualized by the structure (3D) and sequence (1D) compatibility approach. The prediction program we developed, called COMPASS [Ref. 1], has been applied to a number of proteins of unknown structure [Ref. 2]. We have obtained five convincing predictions so far. Two of them, for spermidine/putrescine-binding protein and β subunit of F1–ATPase, were already verified in comparison with their X-ray structures later solved. Another application to sphingomyelinase (SMase) from *Bacillus cereus* was performed recently [Ref. 3]. Our COMPASS program detected its structural similarity with bovine DNase I with a high score, while the sequence identity between them was around 10%, as low as the complete random matching. Apparently, these two enzymes seem quite different, one for cleaving DNA and the other for phospholipid (sphingomyelin). However, they are common as phosphodiesterases, cleaving the phosphoester bond next to phosphorus atom. The 3D–1D alignment shows that several residues functionally important in DNase I are also conserved in SMase. These results indicate that the 3D–1D

compatibility approach could successfully identify evolutionary distant relationships.

(2) Protein Stability Change Caused by Single Amino Acid Substitutions: Estimation by The 3D-Profile Method

Ken NISHIKAWA

The 3D profile table, utilized in aligning the structure with any sequence in the 3D–1D compatibility approach, has interesting features. The 3D profile is expressed as a $20 \times N$ table, where N is the number of residue positions of a structure. Placing each of the 20 amino acids at each position, its compatibility with the surroundings is quantitatively estimated and listed in the table. A slight modification provides a rearranged profile table, which represents the relative stability of all possible point mutations, all at once, against the wild type protein. A comparison was made between stability changes computed from the profile table and changes in the melting temperature experimentally obtained, for about 100 mutants of ribonuclease H. The results showed a fairly good correlation between the computations and experiments [Ref. 5], indicating that the 3D–1D compatibility function we developed was realistic.

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I. Radioisotope Center

Transcriptional Analysis of the *Bacillus subtilis* Sporulation Gene

Masaya FUJITA and Yoshito SADAIE

Nutrient deprivation in *Bacillus subtilis* leads to a process of differentiation that results in endspore formation. Sporulation takes place in a sporangium consisting of two cellular compartments known as the forespore and the mother cell. More than 50 specific genes are expressed sequentially in both compartments during sporulation. Their transcriptions are initiated by at least five RNA polymerase sigma factors specific for sporulation, and enhanced or repressed by transcription factors which appear during sporulation. To understand the molecular mechanism of promoter selectivity with these sigma factors as well as the interaction between RNA polymerase and the sporulation specific transcription factors, we constructed an *in vitro* transcription system with purified *B. subtilis* RNA polymerase whose components were overproduced in *Escherichia coli*. The genes for σA, σH, σE, σF, σG, σK, β, β', and a were cloned into a T7 RNA polymerase expression system. Over expressed proteins were purified and mixed to reconstitute an active RNA polymerase holoenzyme. We also developed a simple, rapid procedure for reconstitution of the *B. subtilis* RNA polymerase holoenzyme from crude cell extract. A hexahistidine-tagged recombinant subunit protein was purified from T7 expression system and incubated with crude *B. subtilis* cell extract. The resulting reconstituted RNA polymerase was purified by Ni-ion-affinity chromatography. Using this procedure, some proteins interacting with RNA polymerase were purified. We are now identifying these proteins.

Publication

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ABSTRACTS OF DIARY FOR 1995

Biological Symposium

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433rd–Feb. 16	A reanalysis of human mtDNA strongly supports the out-of-Africa hypothesis (David Penny)
434th–Mar. 8	Roles of σ^S, σ^{70}, N-HS and HU proteins in osmotic regulation of proU transcription in *E. coli* (J. Gowrishankar)
435th–Mar. 24	Sox-9 and the molecular genetics of skeletal development (Peter Koopman)
436th–Mar. 27	An evaluation of genetic distances for use with microsatellite loci (Marcus W. Feldman)
437th–May 12	Function and regulation of sigma factors in *Escherichia coli* (Carol A. Gross)
438th–May 15	Crystal structure of T7 RNA polymerase (Bi-Cheng Wang)
439th–May 29	Combining protein secondary structure and evolution (Jeffrey L. Thorne)
440th–July 19	Urokinase-type plasminogen activator gene regulation in kidney cells: coupling of hormonal regulation and cell-specific gene expression (Yoshikuni Nagamine)
441st–Aug. 28	Location, structure and function of the target of a transcription activator protein (Richard H. Ebright)
442nd–Sept. 1	The elongin complex: A multi-subunit regulation of elongation by RNA polymerase II (Joan W. Conaway)
443rd–Sept. 1	Mechanisms mediating transcription factor remodeling of chromatin structure (Jerry L. Workman)
444th–Aug. 28	Recent investigation on synonymous substitutions (Giorgio Bernardi)
445th–Aug. 28	Tetraploid origin of mammals (Susumu Ohno)
446th–Aug. 28	Molecular evidence for human evolution (Simon Easteal)
447th–Aug. 28	Genetic variation of human Alu insert polymorphisms

	(Mark Stoneking)
448th–Aug. 28	Tracking male migrations in Asia (Michael Hammer)
449th–Aug. 28	New advances of genes mapping (Clay Stephens)
450th–Sept. 20	GATA transcription factor control of hemotopoietic and neuronal cell fates (James Douglas Engel)
451st–Sept. 18	The mussel mtDNA: A new mode of cytoplasmic DNA transmission and evolution (E. Zouros)
452nd–Oct. 5	Negative control of transcription initiation in bacteria (Hyon Choy)
453rd–Oct. 26	Towards sequencing the human MHC (Stephan Beck)
454th–Nov. 7	*Hedgehog* genes and the patterning of the vertebrate embryo (Cliff Tabin)
455th–Nov. 17	The role of neutral mutations in evolution: Molecular mechanisms (Miroslav Radman)
456th–Nov. 21	Positional cloning and characterization of genes for deafness in the mouse and human (Stephen D. M. Brown)
457th–Nov. 27	Genetic analysis of transmission ratio distortion in inter-specific cross (Xavier Montagutelli)
458th–Nov. 22	Molecular evolution of color vision in vertebrates (Syozo Yokoyama)
459th–Dec. 1	Searching mechanism for homologous DNA by RecA protein (Masayuki Takahashi)
460th–Dec. 11	Structural aspects of phage Mu transpososome (Kiyoshi Mizuuchi)
461st–Dec. 8	New developments of biological utilization of the DNA sequence database (David Lipman)
462nd–Dec. 14	Protein-directed molecular interactions during genetic recombination (Stephen C. West)
463rd–Dec. 15	From genome information to protein function and structure (Chris Sander)

Mishima Geneticists' Club

442nd–Jan. 11	Specific gene expression in embryonic cells (Ohtsura Niwa)
443rd–Jan. 11	Developmental control of the DNA replication-related genes in *Drosophila* (Akio Matsukage)

444th–Jan. 11 Genetic recombination—from molecular to chromosomal level (Tomoko Ogawa)
445th–Jan. 19 Analyses of cell differentiation and pathways of signal transduction in *Drosophila* (Leo Tsuda)
446th–Jan. 19 Pattern formation of *Drosophila melanogaster*—The imaginal discs and the nervous system (Satoshi Goto)
447th–Jan. 30 Structure and function of a tobacco protein kinase NPK 15 (Yukihiro Ito)
448th–Jan. 25 Map construction of rice chromosome (Ken-Ichi Nonomura)
449th–Jan. 26 Cis element controlling s-phase specific transcription of plant histone genes (Norihiro Ohotsubo)
450th–Jan. 26 Acetyl CoA carboxylase in higher plants (Tomokazu Konishi)
451st–Feb. 13 Processing of Sindbis virus non-structural polyproteins and regulation of viral RNA replication (Yukio Shirako)
452nd–Feb. 16 Regulation of DNA replication by the p21 inhibitor of CDK—analysis of SV40 DNA replication in vitro (Shou Waga)
453rd–Feb. 28 X protein of human hepatitis B virus: Molecular structure and mechanisms of trans-activation (Kiyoshi Murakami)
454th–Mar. 1 Target genes of mouse Hox gene products (Daihachiro Tomotsune)
455th–Mar. 2 Genomic imprinting: What is the target for modification? (Tsuyoshi Koide)
456th–Mar. 15 Study of mouse P53 gene by two-steps gene targeting (Yoichi Gondo)
457th–Apr. 3 Cell migration and cell adhesion molecules in central nervous system (Katsuhiko Ono)
458th–Mar. 27 Structure of a mini-collagen gene encoding a nematocyst capsule protein from a reef-building coral and its use for phylogenetic analysis (Wenqiao Wang)
459th–Apr. 5 Approach on the biomolecule database construction based on the experimental science (Yukiko Yamazaki)
460th–May 1 X-ray crystallographic analysis of F1–ATPase from a

	thermophilic bacterium (Yasuo Shirakibara)
461st–Apr. 26	Prediction of protein three-dimensional structures based on database (Ken Nishikawa)
462nd–May 19	NMR studies on the structure of transcription factors and their interaction with nucleic acids (Yoshimasa Kyogoku)
463rd–May 24	NMR studies of protein structure: SH2 domain and Trp repressor (Toshio Yamazaki)
464th–June 1	Structure and function of yeast chromatin as revealed by in vivo foot printing (Shigeo Tanaka)
465th–June 8	Dynamics of folding of DNA long chain (Kenichi Yoshikawa)
466th–June 12	Molecular dissection of F-plasmid SopB protein (Ryo Hanai)
467th–June 22	Function of the basement membrane component laminine during *Drosophila* development (Yasumitsu Takagi)
468th–June 27	Ecology of influenza viruses (Hiroshi Kida)
469th–June 28	Mouse mutation, *jumonji*, produced by gene-trap and its function in embryogenesis (Takashi Takeuchi)
470th–June 30	Construction of phylogenetic trees using a maximum likelihood method and its speed-up through parallel processing (Hideo Matsuda)
471st–July 12	Function of cytokine common γ chains (Masataka Nakamura)
472nd–Aug. 7	From information biology to database (Hideaki Sugawara)
473rd–Aug. 15	Solution structure of virus particles: study by UV-resonance Raman (Shoji Kaminaka)
474th–Sept. 6	Residues of yeast farnesyltransferase that may be involved in protein substance recognition (Hiroshi Mitsuzawa)
475th–Oct. 4	Evolution of tertiary structures of proteins (Hideaki Moriyama)
476th–Oct. 18	Structure and physiological functions of protocadherins (Shintaro Suzuki)
477th–Oct. 25	Evolution of RNA-binding domains (Kaoru Koba-

	yashi)
478th–Nov. 10	Molecular phylogeny of HIV and HTLV (Tomoyuki Miura)
479th–Nov. 20	Interaction between SWI proteins and TFIID (Tsutomu Ohta)
480th–Dec. 18	Mechanism of transcriptional activation by heat shock factor on reconstituted chromatin (Gaku Mizuguchi)
481st–Dec. 26	Study on rice chromosomes–Molecular and genetic analysis of fission yeast chromosomes as a model (Ken-Ichi Nonomura)

FOREIGN VISITORS IN 1995

Dec. 1, 1991–Feb. 28, 1995	Thangirala Sudha, Indian Association for Down's Syndrome, India
Oct. 5, 1993–Mar. 31, 1995	Dzhanybek M. Adyshev, Institute of Biochemistry and Physiology, Kyrgys Academy of Sciences, Kyrgyz
Mar. 10, 1994–Mar. 30, 1995	Hak Soo Suh, Yeungnam Univerity, Korea
Apr. 1, 1994–Mar. 31, 1995	Perriere Guy Bernard, URA Centre National de la Recherche Scientifique, France
June 9, 1994–	H. W. Cai. Beijing Agriculture Univcersity, China
Sept. 1, 1994–Mar. 30, 1995	John R. Wakeley, University of California at Berkeley, U.S.A.
Jan. 26, 1995	Michael D. Baron, Institute for Animal Health, U.K.
Feb. 16	David Penny, Massey University, New Zealand
Feb. 16–Mar. 12	Jayaraman Gowrishankar, Centre for Cellular and Molecular Biology, India
Mar. 18–21	Hans Bode, University of California at Irvine, U.S.A.
Mar. 24–25	Peter Koopman, University of Queensland, Australia
Mar. 26–27	Marcus W. Feldman, Stanford University, U.S.A.
Apr. 3–4	Walter Doerfler, Institut für Genetik, Germany
Mar 12–13	Carol A. Gross, University of California at San Francisco, U.S.A.
May 14–16	Bi-Cheng Wang, University of Georgia, U.S.A.
May 14–16	Orna Amster-Choder, Hebrew University Hadassah Medical School, Israel
May 14–16	Mordechai Choder, Hebrew University Hadassah Medical School, Israel
May 29	Jeffrey L. Thorne, North Carolina State University, U.S.A.
June 21–July 14	Dipankar Chatterji, Centre for Cellular and Molecular Biology, India
June 28–30	Casey Bergman, Cornell University, U.S.A.
June 29–30	Michael Brenner, National Institute of Neurological Disorders and Stroke, NIH, U.S.A.

July 19	Yoshikuni Nagamine, Friedrich Miescher Institute, Switzerland
Aug. 17–18	Noboru and Tamiko Sueoka, University of Colorado at Boulder, U.S.A.
Aug. 17–21	James F. Crow, University of Wisconsin, U.S.A.
Aug. 27–29	Greg Gibson, University of Michigan, U.S.A.
Aug. 27–29	Grey Gibson, Duke University, U.S.A.
Aug. 27–30	Elizabeth Waters, University of Arizona, U.S.A.
Aug. 28	Giorgio Bernardi, Institut Jacques Monod, France
Aug. 28	Simon Easteal, The Australian National University, Australia
Aug. 28	Michael Hammer, University of Arizona, U.S.A.
Aug. 28	Susumu Ohno, Beckman Research Institute of The City of Hope, U.S.A.
Aug. 28	Mark Stoneking, Pennsylvania State University, U.S.A.
Aug. 28	Clay Stephens, National Cancer Institute, NIH, U.S.A.
Aug. 28–29	Richard H. Ebright, Rutgers University, U.S.A.
Aug. 29–30	Spencer V. Muse, Pennsylvania State University, U.S.A.
Aug. 31– Sept. 1, 1995	Jerry L. Workman, Pennsylvania State University, U.S.A.
Sept. 1	Joan W. Conaway, Program in Molecular and Cellular Biology, Oklahoma Medical Research Foundation, U.S.A.
Sept. 17–19	E. Zouros, Dalhousie University, Canada
Sept. 20–21	James Douglas Engel, North-Western University, U.S.A.
Oct. 5	Hyon Choy, National Cancer Institute, NIH, U.S.A.
Oct. 8–16	William B. Provine, Cornell University, U.S.A.
Oct. 8–16	Tomoko Steen, Emory University, U.S.A.
Oct. 26	Stephen Beck, DNA Sequencing Laboratory, Imperial Cancer Research Fund, U.K.
Oct. 30–31	Joe Inselburg, Dartmouth Medical School, U.S.A.
Nov. 7	Cliff Tabin, Harvard Medical School, U.S.A.
Nov. 17	Miroslav Radman, CNRS Institut Jacques Monod, France
Nov. 21	Stephen D.M. Brown, St. Mary's Hospital Medical School, U.K.

Nov. 22	Syozo Yokoyama, Syracuse University, U.S.A.
Nov. 27	Xavier Montagutelli, Institut Pasteur, Fraance
Dec. 1	Masayuki Takahashi, Institut Curie, Orsay, France
Dec. 8	David Lipman, National Center for Biotechnology Information, NIH, U.S.A.
Dec. 9–12	Kiyoshi Mizuuchi, Laboratory of Molecular Biology, NIDDK, NIH, U.S.A.
Dec. 13–14	Stephen C. West, Clare Hall Laboratories, Imperial Cancer Research Fund, U.K.
Dec. 15	Chris Sander, EMBL-EBI, Cambridge, U.K.

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