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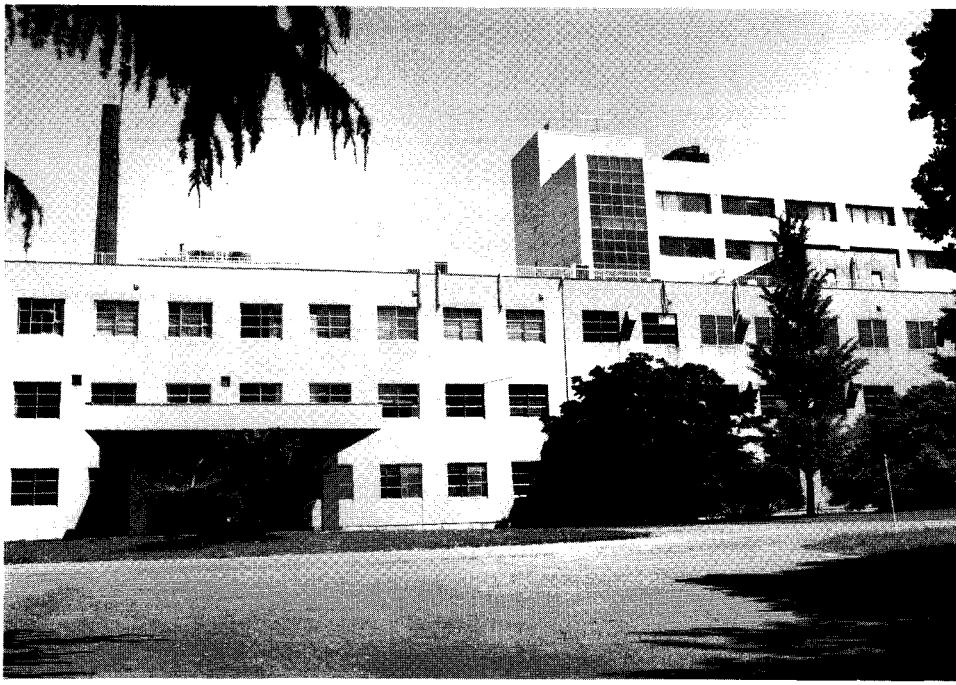
No. 45, 1994



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GENERAL STATEMENT

Our institute was established 45 years ago as a center for comprehensive genetics research. Outstanding accomplishments in the advancement of research on genetics, in particular population genetics, plant genetics and molecular genetics, by members of our institute have made it the center of 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 banks in the world that gather, annotate, store and distribute information on DNA sequences. DDBJ will continue to be active as a regional center for such activities. 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 successful future.

Dr. K. Moriwaki, the Deputy Director and the Head of Division of

Cytogenetics, retired at the end of March. I appreciate very much his assistance in the administration of this institue. He continues his research on mice at Fukuyama University. Dr. T. Seno succeeded in the role of Deputy Director.

We mourn the sudden loss of our honorary and honored member, Dr. M. Kimura, who died on the 13th of November at the age of 71. His works are monumental as they lay the foundation for modern population genetics and evolution biology.

In the past year, we have had many personnel changes. The Gene Function Research Laboratory was established in the DNA Research Center and Dr. Y. Tateno was promoted to professor and was transferred to the new laboratory from the Genetic Resources Laboratory, which Dr. M. Fujita joined as a research member. Dr. Y. Sato was transferred to Shizuoka University as associate professor and Dr. H. Nakashima to Kyushu University. Dr. S. Kaneda retired to work at the HSP Research Institute, Inc., and Dr. E. Moriyama to study at Harvard University. In addition to Dr. Fujita the following five people joined us as research members: Dr. H. Seino in the Division of Mutagenesis, Dr. Y. Ina in the Division of Population Genetics, Dr. T. Tenzen in the Division of Evolutionary Genetics, Dr. K. Izuhara in the Division of Human Genetics and Dr. T. Imanishi in the DNA Data Analysis Laboratory.

It is a pleasure to note that Dr. A. Ishihama was honored by the Cultural Award of the Chunichi Press for his studies on the regulation of transcription by RNA polymerase.

STAFF (as of December 31, 1994)

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Microbial Genetics Laboratory

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MORISHIMA, Hiroko; Professor, National Institute of Genetics

KATSURA, Isao; Professor, National Institute of Genetics

Adviser

MORIWAKI, Daigoro; Honorary member, National Institute of Genetics

PROJECTS OF RESEARCH FOR 1994

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 and YAMAGISHI)

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

Mechanism of mRNA capping (MIZUMOTO)

Transcription of the Sendai virus genome (MIZUMOTO)

Structural genetics: X-ray crystallography of proteins (MORIKAWA)

2. DEPARTMENT OF CELL GENETICS

Division of Cytogenetics

Genetic differentiation of mouse species (MORIWAKI, SHIROISHI* and MIYASHITA*)

Theoretical and experimental bases for chromosome evolution (IMAI)

Study of adrenal function in mouse development by transgenesis (GOTOH, SHIROISHI* and MORIWAKI)

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* Genetic stock research center.

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 cytoplasmic genes during subspecies differentiation of house mouse *Mus musculus* (YONEKAWA)
Cell-cycle regulation by cyclin and CDC2 kinase (YASUDA)

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)

4. DEPARTMENT OF POPULATION GENETICS

Division of Population Genetics

Theoretical studies of population genetics (OHTA, TAJIMA and INA)

Theoretical studies on the evolution of multigene family (OHTA)
Theory of gene genealogy (TAJIMA and INA)
Statistics for DNA polymorphisms (TAJIMA and 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 and SATO)
Genetic studies of speciation in rice (SANO and HIRANO)
Studies on plant gene expression (HIRANO and SANO)
Bio-archaeological analysis of plant remains (SATO)

Division of Applied Genetics

Molecular genetics of human genome imprinting mechanisms (SASAKI)
Domestication dynamics in (SHIMAMOTO)

6. GENETIC STOCK RESEARCH CENTER

Mammalian Genetics Laboratory

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

Invertebrate Genetics Laboratory

Molecular genetics of Drosophila melanogaster (HAYASHI)

Plant Genetics Laboratory

Microbial Genetics Laboratory

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

Genetic Resources Laboratory

Theoretical studies in molecular phylogeny (TATENO)

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

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)

Gene Library Laboratory

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

Gene Function Laboratory

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

8. RADIOISOTOPE CENTER

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

9. EXPERIMENTAL FARM

RESEARCH ACTIVITIES IN 1994

I. MOLECULAR GENETICS

Molecular Anatomy of RNA Polymerase Alpha Subunit from *Escherichia coli*: Functinal Map of the Amino-terminal Assembly Domain

Makoto KIMURA and Akira ISHIHAMA

The α subunit of *E. coli* RNA polymerase consisting of 329 amino acid residues plays a major role in assembly of the core enzyme. Analysis of carboxy-terminal deletion derivatives of α indicated that the amino-terminal two-thirds of α, down to amino acid residue 235, is involved in this assembly (Igarashi, K. *et al.* (1991) *J. Mol. Biol.* **218**, 1–6; Hayward, R. S. *et al.* (1991) *J. Mol. Biol.* **221**, 23–29). Detailed analysis of the assembly domain by making sets of terminal and internal deletion α mutants indicated that the minimal fragment with the full activity of subunit assembly is α(21-235) (Kimura, M. *et al.* (1994) *J. Mol. Biol.* **242**, 107–115). Insertion of Ala-Ser (AS) dipeptide made α inactive in dimerization, β- and/or β'-binding depending on the position of AS insertion (Kimura, M. and Ishihama, A. (1995) *J. Mol. Biol.* **248**, 710–718). For fine mapping, we then made a set of mutants with Ala substitution near the subunit-subunit contact regions. Results altogether indicate that for α dimerization, multiple contact sites are involved, which cluster within the whole sequence of the amino-terminal assembly domain, while the contact with the two large subunits, β and β', includes each two sites (Kimura, M. and Ishihama, A. (1995) *J. Mol. Biol.* **254**, in press).

To confirm the subunit-subunit contact sites on α thus determined using *in vitro* reconstitution system, we made the expression plasmids for mutant α with His-tag and analyzed subunit complexes isolated from cell extracts by Ni^{+}-affinity column chromatography (Kimura, M. and Ishihama, A., submitted for publication). Results of the *in vivo* analysis agreed well with the conclusion obtained from the *in vitro* studies.

Molecular Anatomy of RNA Polymerase Alpha Subunit: Analysis of the Carboxy-terminal Activation Domain by Class-I Factors and Upstream Enhancer Elements

Katsuhiko MURAKAMI, Nobuyuki FUJITA, Kazuyuki TAO*,
Tomasz HEYDUK** and Akira ISHIHAMA

The carboxy-terminal one-third of RNA polymerase α subunit plays an essential role in transcription activation by class-I transcription factors [we proposed to classify transcription factors on the basis of the contact site on RNA polymerase]. Mutant RNA polymerases containing carboxy-terminal truncated α carry the basic function of RNA synthesis but are inactive in cAMP receptor protein (CRP)-dependent transcription of *lac* (Igarashi, K. and Ishihama, A. (1991) *Cell* **65**, 1015–1022). A number of class-I transcription factors have since been identified, which all require the contact site I on the carboxy-terminal domain of α (reviewed in Ishihama, A. (1993) *J. Bacteriol.* **175**, 2483–2489). Later, this region was found to play a role in recognition of promoter upstream (UP) elements with enhancing activity of transcription (Ross, W. *et al.* (1993) *Science* **262**, 1407–1413). Amino acid substitution mutations of *rpoA* affecting the response to cAMP receptor protein (CRP) are mapped mostly within a narrow region between amino acid residues 265 and 270 [and a region between 298–300 for some mutations with weak effects] (Zou, C. *et al.* (1992) *Mol. Microbiol.* **6**, 2599–2605). Likewise, mutations affecting the response to OxyR, another class-I factor involved in transcription activation of the oxidative stress-response genes, were mapped in the same two regions in the vicinity of CRP site [although the mutations at 293–300 region influenced as much as the mutations at 265–270 region] (Tao, K. *et al.* (1993) *Mol. Microbiol.* **7**, 859–864; Tao, K. *et al.* (1995) *J. Bacteriol.*, in press).

For detailed analysis of the individual amino acids in this region, we have constructed two sets of mutant α, *i.e.*, the first-set mutants carrying a single Trp substitution at all the residues in the major CRP contact site region between residues 260–270, and the second-set mutant α carrying the substitution of Arg265 for all kinds of other amino acids. *In vitro* transcription assay using the reconstituted RNA polymerases from overexpressed and purified

* Radioisotope Center, University of Tokyo, Tokyo.
** Department of Biochemistry and Molecular Biology, University of saint Louis, St. Louis.

mutant α indicated that this region is involved in not only CRP contact but also *rrnB* UP element recognition, but the roles of individual amino acid residues in these two functions are different because some mutations affected only transcription activation by the UP element but not by CRP (Murakami, K. *et al.*, in preparation). Likewise, mutations affecting the response to DNA UP element do not always influence OxyR response (Tao, K. *et al.* (1995) *J. Bacteriol.*, in press). DNase I footprinting analysis showed significant decrease in the cooperative promoter binding of the mutant holoenzymes with CRP.

Molecular Anatomy of RNA Polymerase Alpha Subunit: Structure of the Carboxy-terminal Activation Domain

Tomofumi NEGISHI, Nobuyuki FUJITA, Yoshimasa KYOGOKU* and Akira ISHIHAMA

The α subunit of *E. coli* RNA polymerase is composed of two major structural domains, *i.e.*, the amino-terminal domain upstream from Arg235 and the carboxy-terminal domain downstream of Glu245, both being connected by a protease-sensitive inter-domain linker (Negishi, T. *et al.* (1995) *J. Mol. Biol.* **248**, 723–728). This structural organization is in good agreement with the functional map of this subunit, consisting of the amino-terminal assembly domain and the carboxy-terminal transcription activation domain by class-I transcrition factors (reviewed in Ishihama, A. (1993) *J. Bacteriol.* **175**, 2483–2489) and DNA UP elements (Ross, W. *et al.* (1993) *Science* **262**, 1407–1413).

Upon prolonged incubation with proteases, the amino-terminal assembly domain is cleaved between Arg45 and Glu68 into two sub-domains Na and Nb. The subdomain Nb of 168 amino acids in length is then cleaved between Arg150 and Gly151 into two segments; the amino-terminal proximal segment of 11 kDa containing the β subunit-binding site (Kimura, M. and Ishihama, A. (1995) *J. Mol. Biol.* **254**, in press) was resistant to further digestion with trypsin, while the carboxy-terminal segment containing the β' subunit-binding site was digested rapidly upon continued proteolysis.

The carboxy-terminal domain for transcription activation is highly resistant to proteolysis, indicating that this fragment forms a highly structured

* Institute for Protein Research, Osaka University, Suita.

domain. We expressed and purified this domain and subjected to NMR spectoscopic analysis by means of ^{15}N-single and $^{15}N/^{13}C$-double labeling techniques. The three dimensional structure predicted from the NMR analysis is in good agreement with the results obtained from mutant studies and limited proteolysis (Jeon, Y. O. *et al.* (1995) *Science*, in press).

Promoter Selectivity Control of *Escherichai coli* RNA Polymerase: Intracellular Levels of Sigma Subunits

Miki JISHAGE, Akira IWATA* and Akira ISHIHAMA

Bacterial cells have the capacity to respond rapidly to eventual environmental changes by turning off and on the expression of sets of genes. For the changes in global gene expression pattern, the RNA polymerase is considered to play a key role by rapidly modulating its promoter selectivity (Ishihama, A. (1988)*Trends Genet.* **4**, 282–286). One major mechanism of the promoter selectivity control of RNA polymerase is the replacement of σ subunit, the promoter recognition factor, on core enzyme with the catalytic function of RNA synthesis. Up to now, six different molecular species of σ subunit have been identified in *Escherichia coli* (reviewed in Ishihama, A. (1988) *Trends Genet.* **4**, 282–286). However, little is known about the intracellular level of each σ subunit under various growth conditions of *E. coli*.

In order to determine the levels and control of individual σ subunits, we produced mono-specific polyclonal antibodies against each σ subunit and measured the concentrations of σ subunits in cell extracts by quantitative Western blot analysis. Results indicated that: i) σ^D (or σ^{70}), the princial σ subunit in exponentially growing cells, is maintained at a constant level throughout the growth change from log to stationary phase; ii) in contrast, the level of σ^S (or σ^{38}), the stationary-specific σ subunit, increases when cell growth enters into the stationary phase; iii) the level of σ^S increases furthermore by exposure of the stationary-phase cells to osmotic stress; and iv) even in the middle of exponentially growing phase, the level of σ^S transiently increases when cells are exposed to heat shock (Jishage, M. and Ishihama, A. (1995) *J. Bacteriol.*, in press). The intracellular localization of σ^S, *i.e.* free dissociated form or core enzyme-associated form, is being examined by

* Nippon Institute of Biological Science, Ohme, Tokyo.

glycerol gradient centrifugation.

Promoter Selectivity Control of *Escherichia coli* RNA Polymerase: Functional Comparison of Sigma Subunits

Shuichi KUSANO, Nobuyuki FUJITA and Akira ISHIHAMA

RNA polymerase core enzyme of *Escherichia coli* with the subunit composition of $\alpha_2\beta\beta'$ is functionally differentiated into different forms of holoenzyme by binding one of the multiple molecular species of σ subunit, the promoter recognition subunit (Ishihama, A. (1988) *Trends Genet.* **4**, 282–286). The switching of gene expression pattern is considered to be controlled by the replacement of core enzyme-associated σ subunit. Until now, six different species of σ subunit have been identified in *E. coli.* In order to understand the molecular mechanism of σ replacement, we have performed a systematic comparison of the activities between different σ subunits.

First, the level of σ subunits required for the maximum transcription in the presence of a fixed amount of core enzyme was compared between various σ subunits. Results indicated that the core-binding affinity of σ^S (or σ^{38}; the principal σ in statinary-phase cells) is 4- to 7-fold weaker than σ^D (or σ^{70}; the principal σ in exponentially growing cells), σ^H (or σ^{32}; the heat-shock gene-specific σ subunit) and σ^F (or σ^{28}; the flagella gene-specific σ subunit) (Kusano, S. *et al.* (1995) *J. Biol. Chem.*, in press). To confirm this prediction, we then measured directly the core-binding affinity. For this purpose, mixtures of a fixed amount of core enzyme and various amounts of σ subunits were subjected to gel-filtration HPLC and the core enzyme-bound σ subunits were determined. The core enzyme was saturated with σ^D by adding 2-fold molar excess over the core enzyme but to achieve the same extent of core binding, at least 6-fold molar excess of σ^S was needed, indicating that the core enzyme-binding affinity of σ^S is at least three fold weaker than σ^D. These findings are rather unexpected because the intracellular concentration of σ^S is not higher than that of σ^D even in the stationary-phase *E. coli* (Jishage, M. and Ishihama, A. (1995) *J. Bacteriol.*, in press). Thus, it appears that a stationary-specific factor(s) or condition(s) is involved in either efficient replacement of σ subunit from σ^D to σ^S or efficient utilization of $E\sigma^S$ holoenzyme for transcription of stationary-phase specific genes.

As an attempt to examine these possibilities, we analyzed the effect of DNA

superhelicity on the promoter selectivity by $E\sigma^D$ and $E\sigma^S$ holoenzymes. Results indicated that the optimum superhelical density for maximum transcription of *lac*UV5 and *osmY* promoters is lower for $E\sigma^S$ than $E\sigma^D$. This is in good agreement with the decrease in superhelicity of the chromosomal DNA in stationary-phase *E. coli*.

Molecular Anatomy of *Escherichia coli* RNA polymerase: Modes of RNA Polymerase-Transcription Factor Interaction (Progress in International Collaborative Studies)

Akira ISHIHAMA

A number of transcription factors have been identified in *E. coli*, which activate transcription by modulating the promoter selectivity of RNA polymerase via direct protein-protein contacts (reviewed in Ishihama, A. (1992) *Mol. Microbiol.* **6**, 3283–3288; Ishihama, A. (1993) *J. Bacteriol.* **175**, 2483–2489). In collaboration with more than 50 laboratories from 18 countries, a systematic mapping of the domain(s) on RNA polymerase subunits for activation by each of these transcription factors is being carried out. Based on the results of our collaborative studies, we have proposed a classification system of the transcription factors. This new system is based on the location of the activation domain on RNA polymerase subunits. Table 1 shows a list of the up-to-date classification. Among these transcrption factors, fine mapping of the contact sites on RNA polymerase has been done extensively for the class-I factors. Analysis of the protein structure is also being focussed on the C-terminal region of α subunit.

Beside transcription activation by the protein factors, upstream (UP) enhancer DNA elements make direct contacts with RNA polymerase and activates transcription. Thus, the C-terminal domain of α subunit is involved in recognition of not only class-I transcription factors but also DNA UP elements (Ross, W. *et al.* (1993) *Science* **262**, 1407–1413). DNA-α subunit interaction is now recognized as one of the attractive research subjects.

Followings are the major progress in this year's collaborations:

CRP (in collaboration with S. Busby and colleagues, School of Biochemistry, Univerisity of Birmingham, Birmingham): cAMP receptor protein (CRP) functions as both class-I and class-I transcription factors depending on the DNA site of CRP binding relative to promoter (Igarashi, K. and

Table 1. Classification of *E. coli* transcription factors

Class	Location of the activation domain	Transcription factor*
I	α subunit	Ada[1], AraC[2], CRP(*lac*)[3/4], CysB, δ(P4), FlhD/FlhC[5], Fnr, IHF[6], MelR, Ogr(P2), OmpR[7], OxyR[8], SoxS(zwf)[9], TyrR[10]
II	σ subunit	CRP(*gal*)[3/11], *cI*(λ)[13], Fis, MerR[13], NtrC[14], PhoB[15], SoxS(*sodA*)[9]
III	β subunit	DnaA[16], ppGpp[17]
IV	β' subunit	NusA, Rho

*Underlined, factors analyzed in this laboratory in collaboration with: [1]K. Sakumi and M. Sekiguchi, Kyushu Univerisity School of Medicine, Fukuoka; [2]R. Schleif and colleagues, John Hopkins Univeristy, Baltimore; [3]Hiroji Aiba and colleagues, Nagoya University, Faculty of Science, Nagoya; [4]A. Kolb and H. Buc, Pasteur Institute; [5]P. Matsumura and colleagues, University of Illinois, Chicago; [6]A. Oppenheim and colleagues, Hebrew Univerisity School of Medicine, Jerusalem; [7]Hirofumi Aiba and T. Mizuno, Nagoya Univerisity, Faculty of Agriculture, Nagoya; [8]K. Tao, University of Tokyo, Radioisotope Center, Tokyo; [9]R. E. Wolf, Jr. and colleagues, University of Maryland Baltimore County, Baltimore; [10]B. Lawly and A. J. Pittard, University of Melbourne, Melbourne; [11]S. Busby and colleagues, Univ. Birmingham, Birmingham; [12]G. N. Gussin, Univ. Iowa, Iowa City; [13]A. Ansari and T. O'Halloran, Northwestern Univ.; [14]S. Kustu and colleagues, University of California, Berkeley; F. G. Hansen and T. Atlung, Department of Microbiology, Technical University of Denmark, Lyngby; [17]D. Chatterji and colleagues, Centre for Cellular and Molecular Biology, Hyderabad.

Ishihama, A. (1991) *Cell* **65**, 1015–1022; Igarashi, K. *et al.* (1991) *Proc. Natl. Acad. Sci. USA* **88**, 8958–8962). In the case of transcription activation of *lac*, one molecule of the CRP dimer interacts with the C-terminal region of one molecule of the α subunit dimer [contact site I]. On the other hand, one molecule of the CRP dimer interacts with the C-terminal region of σ subunit for activation of *gal* transcription (Kumar, A. *et al.* (1994) *J. Mol. Biol.* **235**, 405–413). We found, however, that the other molecule of the CRP dimer bound to the *gal* promoter interacts with the C-terminal region of α (Attey, A. *et al.* (1995) *Ncleic Acids Res.* **22**, 4375–4380). This CRP-α interaction appears to shift the α contact site on the *gal* promoter to a putative UP element, ultimately leading to enhanced transcription.

FlhD/FlhC (in collaboration with P. Matsumura and colleagues, Department of Microbiology and Immunology, University of Illinois, Chicago): Transcription of the flagellar operons is ordered in a hierarchy, allowing coordinated expression of flagellar components in the order of asssembly. In the early step of flagellar operon transcription, the FlhD/FlhC complex plays a key role as a master regulatory factor which activates transcription of the

genes expressed in the late stage of flagellar formation. *In vitro* transcription studies using three FlhD/FlhC-dependent promoters, *fliA*, *flhB* and *fliL*, indicated that the mutant RNA polymerases containing C-terminal truncated α subunits were not activated by FlhD/FlhC complex (Liu, X. *et al.* (1995) *J. Bacteriol.* **177**, 5186–5188). Thus, we concluded that the FlhD/FlhC complex is a class-I transcription factor.

SoxS (in collaboration with R. E. Wolf, Jr. and colleagues, Department of Biological Sciences, University of Maryland Baltimore County, Baltimore): In response to the oxidative stress, *E. coli* activates the SoxRS regulon, which is comprised of at least 10 genes. For this coordinate expression, SoxS, a XylS/AraC family protein, plays as a transcription activator of the member genes. SoxS binds to "soxbox" DNA located near the promoter −35 sequence and is considered to make direct contact with the C-terminal domain of α subunit, because the mutant RNA polymerases consisting of C-terminal truncated α subunit were unable to transcribe SoxS-dependent *zwf* (glucose-6-phosphate dehydrogenase) and *fpr* (NADPH: ferredoxin oxidoreductase) (Jair, K.-W. *et al.* (1995) *Mol. Microbiol.*, in press). However, SoxS-dependent transcription of *fumC* (oxygen stable fumarase), *micF* (antisense regulator RNA of *ompF*), *nfo* (endonuclease IV) and *sodA* (manganese superoxide dismutase) was indifferent to the truncation of α subunit C-terminus. Thus, like the cAMP receptor protein (CRP or CAP), SoxS is an "ambidextrous" activator.

Gene Cloning and Characterization of RNA Polymerase II Subunits from the Fission Yeast *Schizosaccharomyces pombe*: Cloning of the Genes Encoding Small Subunits

Hitomi SAKURAI, Hisako SUZUKI and Akira ISHIHAMA

Knowledge of the structure and function of RNA polymerase II is essential for understanding the molecular mechanisms of transcription regulation in eukaryotes. Purified RNA polymerase II from the fission yeast *Schizosaccharomyces pombe* contains more than 10 polypeptides as analyzed by SDS-gel electrophoresis. Up to the present time, we have cloned the genes coding for the subunits 1 (RPB1), 2 (RPB2), 3 (RPB3) and 5 (RPB5) (Azuma, Y. *et al.* (1991) *Nucleic Acids Res.* **19**, 461–468; Kawagishi, M. *et al.* (1993) *Nucleic Acids Res.* **21**, 469–473; Azuma, Y. *et al.* (1993) *Nucleic Acids Res.*

21, 3749–3754; Miyao, T. *et al.*, in preparation). The *rpb1* and *rpb2* genes were isolated using the corresponding genes from the budding yeast *Saccharomyces cerevisiae* as the hybridization probes. For cloning of the *rpb3* and *rpb5* genes, we determined amino acid sequences for parts of the isolated subunit proteins. The genes were PCR-amplified using primers designed based on the protein sequences determined in this study or the published amino acid sequences of the corresponsind genes from the budding yeast *Saccharomyces cerevisiae* or human. In addition to these four subunits, we have determined partial amino acid sequences for subunits 6, 7, 10 and 11. Results showed sequence similarity to the corresponding subunits of *Saccharomyces cerevisiae* RNA polymerase II. The knowledge of amino acid sequences can now be used for cloning of the genes coding for these proteins.

Gene Cloning and Characterization of RNA Polymerase II Subunits from the Fission Yeast *Schizosaccharomyces pombe*: Thermolabile RNA Polymerase II with Mutations in Subunit 3

Kiyoshi YASUI, Yoshinao AZUMA*, Masahiro YAMAGISHI and Akira ISHIHAMA

For analysis of the structure-function relationship of RNA polymerase II subunits from *S. pombe*, we constructed a number of temperature-sensitive mutants with mutations in the *rpb3* gene encoding subunit 3. For this purpose, we first PCR-amplified the *rpb3* gene in the presence of manganese ion to induce inaccurate DNA synthesis. The mutagenized *rpb3* gene was transformed into *S. pombe* and the recombinants were screened for temperature-sensitive cell growth. The *ts* mutants were classified into two groups: group 1 mutants stopped cell growth immediately after temperature up-shift, while group 2 mutants exhibited delayed response to the growth arrest at high temperatures. RNA polymersae II partially purified from Ts54, one of the group 2 mutants, was thermo-labile *in vitro* as measured by non-specific transcription assay (Azuma, Y. *et al.* (1995) *J. Biochem.* **118**, 216–220). These results suggested that RPB3 plays roles not only in enzyme assembly but also in transcription regulation. The RNA polymerase II mutants isolated in this study can be used as reference strains for *in vivo* studies of transcription regulation.

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Gene Cloning and Characterization of RNA Polymerase II Subunits from the Fission Yeast *Schizosaccharomyces pombe*: Structure and Function of Subunit 5 (RPB5)

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Masahiro YAMAGISHI and Akira ISHIHAMA

The *rpb5* gene coding for subunit 5 of *S. pombe* RNA polymerase II was amplified by PCR using primers degenerated from the knowledge of amino acid sequences of the subunit 5 from *S. cerevisiae* and human (Miyao, T. *et al.*, in preparation). In parallel, we determined the amino acid sequence of parts of the isolated RPB5 polypeptide. The amino acid sequence of the polypeptide predicated from the cloned gene agreed well with the sequence of RPB5. In addition, the predicted amino acid sequence is 56 and 44% identical with that of the RNA polymerase subunit 5 from budding yeast and human, respectively. In particular, the homology is high at the carboxy-terminal proximal region. We then concluded that the cloned gene represents the *S. pombe rpb5* gene encoding RNA polymerase II subunit 5.

Recently, it was found that the trans-acting factor from hepatitis B virus directly interacts with the subunit 5 of human RNA polymerase II (Murakami, S. *et al.* (1995) *EMBO J.* **14**, 143–150). This finding raises a possibility that cellular factors with the regulatory functions of transcription also bind to and form complexes with this subunit. To test this possibility, we are trying to identify *S. pombe* proteins specifically associating with RPB5.

Molecular Anatomy of Influenza Virus RNA Polymerase: Analysis of Subunit Functions

Makoto KOBAYASHI**, Tetsuya TOYODA, Susumu NAKADA***,
Kiyohisa MIZUMOTO**** and Akira ISHIHAMA

RNA polymerase of influenza virus is composed of three viral coded P proteins, PB1, PB2 and PA, and is involved in all step reactions in both transcription and replication (reviewed in Ishihama, A. and Nagata, K. (1989) *CRC Crit. Rev. Biochem.* **23**, 27–76; Ishihama, A. and Barbier, P.

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(1993) *Arch. Virol.* **134**, 235–258). The subunit composition was first determined by purification of the RNA polymerase from virus particles (Honda, A. *et al.* (1989) *J. Biochem.* **107**, 1021–1026) and later confirmed by reconstitution of the enzyme from three P protein subunits which were purified from individually expressed in insect cells after infection of the recombinant baculoviruses carrying the cloned cDNAs (Kobayashi, M. *et al.* (1992) *Virus Res.* **22**, 235–245).

In order to determine the function(s) of each subunit, we examined possible activities of viral RNA synthesis in nuclear extracts prepared from insect cells infected with various combinations of the recombinant baculoviruses. RNA synthesis activity was measured using 84mer short model RNA templates, each carrying 5′- and 3′-terminal conserved sequences of the genome RNA segment 8. Results indicated that: i) nuclear extracts expressing only PB1 subunit was able to catalyze viral RNA synthesis in the presence of ApG primer, but the activity was very low in its absence; ii) the nuclear extracts containing both PB1 and PB2 exhibited the 84mer model RNA-directed globin mRNA-primed RNA synthesis, producing transcripts of about 100 nucleotides in length; and iii) the nuclear extract containing all three P proteins exhibited the activity of complete replication, *i.e.*, minus-strand model RNA-dependent plus-strand RNA synthesis and plus-strand RNA-dependent minus-strand RNA synthesis in the absence of primers (Kobayashi, M. *et al.*, submitted for publication). These observations altogether indicate that: PB1 is a catalytic subunit of the influenza virus RNA polymerase; PB2 is required for mRNA-primed transcription; but the complete set of P proteins is required for the complete cycle of replication. Essentially the same results were obtained when recombinant vaccinia viruses carrying the cDNA for influenza virus P proteins were infected into HeLa cells (Toyoda, T. *et al.*, submitted for publication).

Molecular Anatomy of Influenza Virus RNA Polymerase: Analysis of Subunit-Subunit Contact Sites

Djanybeck ADYSHEV*, Tetsuya TOYODA, Akira IWATA**, Susumu UEDA** and Akira ISHIHAMA

Influenza virus RNA polymerase is composed of three viral coded proteins, PB1, PB2 and PA, each playing a different role(s) in transcription and replication. For the expression of intrinsic subunit activities *in vitro*, the assembly of all three P proteins is required (Kobayashi, M. *et al.* (1992) *Virus Res.* **22**, 235–245), while *in vivo*, individual subunits and binary complexes consisting of two P proteins exhibit low levels of the activities (see above). Thus, subunit-subunit contacts are required for the expression of intrinsic subunit functions or at least for the enhancement of the activities.

In order to understand the mechanism of subunit assembly and the functinal and structural changes of individual subunits during the assembly, attempts were made this year to determine the subunit-subunit contact sites between the three P proteins. For this purpose, we prepared sets of amino-terminal and carboxy-terminal deletion mutants for each P protein. cDNA of the mutant P proteins were expressed in various combination. In order to examine binary complex formation, two kinds of the cDNA clones, one deletion mutant P protein and the other intact P protein were simultaneously transfected, and expressed P proteins were immuno-precipitated using mono-specific anti-P protein antibodies. Results indicated that both PB2 and PA can bind to PB1, and that the PB2-binding site is located at the carboxy-terminal region of PB1 while PB1-binding site is located at the amino-terminal region of PB2.

Molecular Anatomy of Influenza Virus RNA Polymerase: Mapping of Nucleotide-Binding Sites

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8-Azido GTP (8-N_3 GTP) was demonstrated to be polymerized into RNA

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by influenza virus-associated RNA polymerase at about one tenth the rate of GTP incorporation. The K_m value for the azido analogue of GTP in primer-dependent RNA synthesis (transcription) was 94 μM whereas K_m for the natural substrate, GTP, was 6.7 μM. Upon exposure of a mixture of 8-N_3 [α-^{32}P]GTP and influenza virus RNP to UV light, the PB1 subunit was selectively radio-labeled. The photo-labeling of PB1 was strongly competed by GTP and to lesser extents by other nucleoside 5′-triphosphates. These results altogether support the prediction that the substrate-binding site (S site) is located on the PB1 subunit. 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 S site but also the primer- and product-binding site (P site).

Molecular Dissection of Influenaza Virus Nucleoprotein (NP): Identification of RNA-binding Region

Makoto KOBAYASHI*, Tetsuya TOYODA and Akira ISHIHAMA

Influenza virus nucleoprotein (NP) is associated with the genome RNA, forming ribonucleoptorein (RNP) cores. Isolated NP binds to viral RNA at an interval of about 20 nucleotides in a cooperative manner. To identify the amino acid sequence on NP involved in this RNA binding, we performed Northwestern blot analysis using a set of amino- and carboxy-terminal deletion mutants of NP produced in *Escherichia coli.* The RNA-binding region was mapped between amino acid residues 91 and 188, a stretch of residues that contains a sequence highly conserved among A-, B- and C-type influenza viruses (Kobayashi, M. *et al.* (1994) *J. Virol.* **68**, 8433–8436). A similar sequence was found in the RNA-binding domain of a plant virus movement protein and of ribosomal protein S9.

Growth Control of Influenza Virus by Membrane Protein (M1)

Jiro YASUDA** and Akira ISHIHAMA

Analysis of fast-growing reassortants (AWM viruses) of A-type influenza virus produced by mixed infection of a fast-growing WSN and a slowly

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growing Aichi strain indicated that RNA segment 7 including the M1 and M2 genes plays a role in the control of influenza virus growth at an early step of infection (Yasuda, J. *et al.* (1993) *Arch. Virol.* **68**, 8141–8146). In order to determine which of the two M genes, M1 or M2, encoded by RNA segment 7 is resonsible for the growth rate control, one recombinant WSN virus (CWA) clone possessing a chimeric M gene (WSN M1–Aichi M2) was generated by using an improved reverse genetics and transfection system. The recombinant CWA virus retained the phenotype of both large plaque formation and early onset of virus growth (Yasuda, J. *et al.* (1994) *J. Virol.* **68**, 8141–8146). This indicates that the WSN M1 protein is responsible for rapid virus growth.

Molecular Dissection of Mouse Mx1 Protein with Anti-Influenza Virus Activity

Testuya TOYODA, Yukiyasu ASANO and Akira ISHIHAMA

Mouse Mx1 protein is an interferon-inducible nuclear protein, which renders the expressing cells resistant to influenza virus infection. Previously we identified that Mx1 protein is a GTP-binding protein with GTPase activity and forms self-asseblies (Nakayama, M. *et al.* (1991) *J. Biol. Chem.* **266**, 21404–21408; Nakayama, M. *et al.* (1992) *Virus Res.* **22**, 227–234). The motifs for the GTPase and self-assembly have been mapped using a set of deletion and amino acid substitution Mx1 mutants (Nakayama, M. *et al.* (1993) *J. Biol. Chem.* **268**, 15033–15038).

In order to address possible relationship between these biochemical activities and the antiviral activity, we constructed cell lines expessing intact or mutant Mx1 proteins and examined for the growth of influenza virus in transfected cells. Results indicate that: i) one mutant Mx1 (S50I) with Ser to Ile substitution at residue 50 within the GTP-binding motif showed a low activity of GTP hydrolysis *in vitro*, formed apparently linear large aggregate bundles in expressed cell nuclei, and lost the anti-viral activity; and ii) another mutant Mx1 (C71S) with Cys to Ser substitution at residue 71 within the self-assembly domain, did not form self-assemblies *in vitro*, showed uniform diffuse distribution in expressed nuclei, but retained the anti-viral activity. Thus, we concluded that the GTPase activity is essential for the

anti-influenza acitivity (Toyoda, T. *et al.* (1995) *J. Gen. Virol.* **76**, 1867–1869).

DNA Replication/Repair is Impaired in a Temperature-Sensitive Ubiquitin-Activating Enzyme Mutant of Mouse Cells, without the Accompanied Enhanced Mutation Frequency— A Possible Involvement of Deoxyribonucleotide Metabolism

Fumiko AOKI, Yusuke WATAYA* and Takeshi SENO

A mouse cell mutant, tsFS20 with the temperature-sensitive ubiquitin-activating enzyme E1, was arrested in a S-phase at the nonpermissive temperature (39°C) (Ayusawa D. *et al.* (1992) *Cell Struct. Funct.* **17**, 113–122), where cellular DNA synthesis was inhibited, but not total RNA and protein synthesis. DNA synthetic activity decreased to 30% after 4 h incubation at 39°C and was near 0% of the initial level in the mutant cells after 16 h. When the DNA synthetic activity in vitro was measured after the 4 h-arrested cells were permeabilized by lysolecithin, the activity had recovered to a normal level. It also recovered to near normal level in the 16 h arrested cells. Thus, the inhibition of DNA replication in this mutant seems to be reversible. DNA synthesis in the permeabilized cells was also not temperature-sensitive. To explain these results, the possibility has been raised that the enzyme system involving a reduction of ribonucleotides is affected in this mutant. In support of this supposition, the mutant showed increased sensitivity to hydroxyurea, a specific inhibitor of ribonucleotide reductase. Also, the mutant showed a significant decrease in intracellular pools of dTTP and dCTP when cells were cultured at a nonpermissive temperature. This decrease in the nuclotide pool seemed to be specific for the mutation in the ubiquitin-activating enzyme, since a mouse cell mutant tsFT20 with temperature-sensitive DNA polymerase α, arrested in the S-phase at a nonpermissive temperature, and wild-type cells arrested in the S-phase in the presence of a DNA synthesis inhibitor, aphidicolin, did not show a decrease in the deoxyribonucleotide pool. Hence, targeted ubiquitination in relation to the mutant phenotype may not be involved on component(s) of DNA replication machinery, but rather on protein(s) involving reduction of nucleotides as

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suggested above.

tsFS20 cells showed an increased sensitivity to the alkylating agent MNNG. Interestingly, the increased sensitivity to MNNG was not accompanied by an increased mutation frequency as measured at the HPRT locus. These results suggest that the ubiquitin system is involved in the DNA repair mechanisms and more specifically in the induced mutation fixation processes. This phenotype has not been reported for mammalian mutants, but is reminiscent of the *S. cerevisiae rad6* mutant in which a mutation in the ubiquitin-conjugating enzyme E2/UBC2 has been identified. Attempt to complement the mutant phenotype by transfection of cloned cDNA encoding the human homologue of RAD6 have failed so far.

Perturbation of Nucleolar Dissolution in the G2-M Transition in a Mouse Cell Mutant with Temperature-Sensitive Ubiquitin-Activating Enzyme E1: Possible Involvement of HSP70 and Proteasome

T. SUDHA,* Masazumi SAMASHIMA, Hideo TSUJI,
Fumiaki YAMAO and Takeshi SENO

Ubiquitin stress created by a shift-up of temperature in a mouse cell mutant, ts85 with the temperature-sensitive ubiquitin-activating enzyme E1, brings about cell cycle arrest at the G2 phase accompanied by abnormal integrity of the nucleolus represented by a U-shaped or ring-shaped arrangement of nucleolar lobes. More severe fragmentation of the lobes was observed when the mutant cells were synchronized at the G2 phase before the temperature shift-up. A cloud of unidentified electron-dense particles (70 nm in diameter) was observed in regions surrounded by the nucleolar lobes (Sudha, T. (1995) *Chromosome Res.* **3**, 115–123). The particles grew in number when cells in the G2-phase were subjected to a temperature-shiftup. Also, among several proteins tested, immuno-histochemical signals for inducible type HSP70 and proteasome were concentrated and colocalized in the region. It should be mentioned that such local concentrations of HSP70 and proteasome in nucleoli were also observed for canonically heat-shocked wild-type cells. Following the release of the arrested mutant cells into the M phase, nucleolar materials persisted in prophase and prometaphase chromosomes which brought about a peculiar bouquet-like figure of metaphase-

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chromosomes. In such odd chromosomal figures, condensation and separation of nucleolar organizer regions seemed to be incomplete as shown by FISH with rDNA and NOR-Ag staining. The mutant phenotype was complemented by transfection with a cloned cDNA for the E1 enzyme (Imai, N. *et al.* (1992) *Gene* **118**, 279–282). Hence, the ubiquitin system in association with HSPs seems to play a key role in the dissociation processes of the nucleolus during G2-M transition.

Ubiquitin-activating Enzyme, E1 Is Phosphorylated in Mammalian Cells by the Protein Kinase CDC2

Yukiko NAGAI, Sumiko KANEDA, Takeshi SENO and Fumiaki YAMAO

The ubiquitin-activating enzyme (E1) is the first enzyme in the pathway leading to the formation of ubiquitin-protein conjugates. E1 was found to be phosphorylated in cells of a mouse mammary carcinoma cell line, FM3A. 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* with Cdc2 kinase-mediated phosphorylation of E1, affinity-purified from the same cell line. Cdc2 kinase is a key enzyme playing a pivotal role in G2/M transition in the cell cycle. 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. These findings suggest that E1 is a target of Cdc2 kinase in the cell, implying that the ubiquitin system may be dynamically involved in cell cycle control through phosphorylation of this key enzyme.

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

Fumio OSAKA, Hiroaki SEINO, Takeshi SENO and Fumiaki YAMAO

By screening the cDNA gene products expressed in *E. coli* cells with their ability to bind activated ubiquitin, we have isolated cDNAs for a family of ubiquitin-conjugating enzymes (UBC) of *S. pombe*. Among more than

20,000 clones, twenty were found to encode protein with UBC-like activity, and subsequent sequence analysis identified four types of genes. We concluded that these four were ubc genes and designated them, ubcP1, ubcP2, ubcP3 and ubcP4. Their gene products form a thiolester bond with activated ubiquitin *in vitro* in the presence of the ubiquitin-activating enzyme. A UBC domain structure which included cysteine residue forming a thiolester bond with ubiquitin was found in their sequences and is characteristic to all known UBC's. ubcP1 and ubcP2 which were predominantly isolated in the screening were found to encode the homologue of Ubc4 and Ubc2/Rad6 in budding yeast, *Saccharomyces cerevisiae*, respectively. The other two, ubcP3 and ubcP4 were novel ubc genes. ubcP4 was found to be essential for growth, and was arrested at the G2 period of the cell cycle when its expression was abolished. The terminal phenotypes of the cells in which the ubcP4 gene was repressed under the control of the nmt promoter were not unique but very close to those found when there is overexpression of the dominant negative cdc2 kinase mutant. The genetic and biochemical analysis of the gene and its product, including the identification of the target protein of this ubiquitin pathway are now underway.

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

Tsutomu KISHI, Motoyasu UI* and Takeshi SENO

When G1 yeast cells reach a critical size, they not only initiate DNA replication but they also form buds and duplicate their spindle pole bodies. Three genes (CDC4, CDC34 and CDC53) are specifically needed to enter S phase. Mutants defective in each of these three genes showed three characteristics; they failed to initiate DNA replication, fail to separate their spindle pole body, and they budded repeatedly.

CDC34 has already been shown to code for ubiquitin conjugating enzyme E2 (Ubc3). Therefore, degradation of some specific target proteins has been suggested to be essential for the initiation of DNA replication. One of the target proteins was shown to be Sic1 which functions as an inhibitor of B-type cyclin dependent kinase activity. Deletion of SIC1 in $cdc34^{ts}$ strain allowed the cells to enter the S phase at its restriction temperature. However, they

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failed to undergo nuclear division and accumulated as large budded cells with 2N DNA content. These evidences suggested that Cdc34 regulated not only initiation of DNA replication by promoting the degradation of Sic1, but also nuclear division. However, the function of Cdc34 on nuclear division is not known. We are trying to identify the target proteins to be ubiquitinated.

(1) Detection of target proteins of Cdc34 by *in vitro* system

Cdc34 was expressed and purified from *E. coli.* First, the protein was mixed with labeled ubiquitin and crude yeast extract prepared from the G1 phase of the cell cycle. However, detection of ubiquitinated protein by Cdc 34 was not possible. Then, we prepared the extract from yeast nuclei. Using this nuclear extract, we detected one major signal (ubiquitinated histone) and one faint signal (a protein about 65 kDa). As histone is not an *in vivo* substrate, we are trying to deplete histone from the nuclear extract.

(2) Isolation of suppressor sensitive mutants of the cdc34 strain

We deleted SIC1 from $cdc34^{ts}$ strain. As reported, this strain failed to undergo nuclear division, and were arrested with a large bud. Several suppressor mutants were isolated and cloning of the revertant genes is under way.

Cell Cycle Regulation by the Ubiquitin System in Eukaryote

Hiroaki SEINO, Tatsuo YAGURA*, Fumiaki YAMAO and Takeshi SENO

Ubiquitin is activated by the ubiquitin-activating enzyme, E1, and ubiquitin is transferred from the E1 enzyme to one member of a family of isozymes called ubiquitin-conjugating enzyme E2, which catalyzes the conjugation of ubiquitin to specific target proteins. There is increasing evidence that each E 2 enzyme has a specific target protein(s). Detailed analysis of pleiotropic phenotypes of mutant strains of mammalian cells which have a mutation on the structure gene coding the E1 enzyme suggest that the ubiquitin system is involved not only in G2/M phase regulation, but also in G1 and S phase regulation. To identify the ubiquitin pathway(s) involved in each phase of the cell cycle, we examined ubiquitin transfer from E1 to E2 *in vitro* using extracts of various temperature-sensitive E1 mutants isolated from the mouse cell line, FM3A. Ubiquitin transfer to an E2 protein of approximately 35 kDa was found to be reduced specifically in the extract of the mutant cell line,

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tsFS20, which was predominantly arrested in the S phase at a restrictive temperature (39°C). Furthermore, the activity of this E2 protein was found to increase during the S phase in the extract of wild-type cells. These results suggest that this 35 kDa E2 protein is involved in progression through the S phase. We purified this protein from cell extract by using affinity column chromatography and deduced a partial amino acid sequence. Cloning of a cDNA encoding this protein and characterization of its properties are under way.

Crystallographic Studies of Proteins Involved in DNA Repair

Kousuke MORIKAWA

In view of short term survival, DNA must be chemically stable in order to maintain a genetic record, even though the evolution of organisms requires some alteration of genes. Adequate stability of genetic records on DNA is maintained not only through an extremely accurate mechanism for copying DNA sequences but also through precise and sophisticated mechanisms for repairing the many accidental lesions in DNA that occur spontaneously within cells.

By making a rough classification, the mechanisms of DNA repair can be divided into two groups, recombinational repairs and excision repairs. We have determined the crystal structures of two key enzymes which are involved in these two DNA repair mechanisms. The structure of the enzyme involved in recombinational repair has been determined in a DNA-free state, while that for excision repair has been analyzed in a complex state with a DNA substrate duplex containing a pyrimidine dimer (PD). These structures, both refined to atomic resolution, reveal how the respective enzymes interact with a key intermediate in recombinational repair and with damaged bases to catalyze DNA excision repair.

1. Holliday junction resolvase from *E. coli* (RuvC protein).

In all organisms, homologous recombination is a crucial process not only for generation of genetic diversity but also for repair of damaged chromosomes. The Holliday junction is a universal intermediate produced in the process of homologous DNA recombination. We elucidated the crystal structure of the RuvC Holliday junction resolvase (19 kd) from *E. coli.* Together with results from extensive mutational analyses, the refined struc-

ture at 2.5 Å reveals that the catalytic center, comprising four acidic residues, lies at the bottom of a cleft that nicely fits a DNA duplex (Ariyoshi, M. *et al.* (1994) *Cell* **78**, 1063–1072). The structural features of the dimeric protein, with a 30 Å spacing between the two catalytic centers, provide a substantially defined image of the Holliday junction architecture. Thus, based on the docking examination of the RuvC protein with DNA, a new model of the Holliday junction was proposed. This model, which consists of two antiparallel quasicontinuous DNA duplexes, is similar to the stacked X structure (vin Kitzing, E. *et al.* (1990) *Nucl. Acids Res.* **18**, 2671–2673) but has longer extended linkers with at least two nucleotides. The folding topology in the vicinity of the catalytic site exhibits a striking similarity to that of RNaseHI from *E. coli.* More recently, the RNaseH-like fold also has been found in HIV integrase (Dyda, F. *et al.* (1994) *Science* **266**, 1981–1986), suggesting that it forms a ubiquitous and important super family (Yang, W. and Steitz, T. A. (1995) *Structure* **3**, 131–134).

2. DNA excision repair enzyme in a complex with a DNA substrate

T4 endonuclease V (16 kd) is a pyrimidine dimer specific excision repair enzyme encoded by the bacteriophage T4 gene, and it catalyzes the first reaction step in the excision repair pathway for a pyrimidine dimer. This enzyme possesses two distinct catalytic activities: a pyrimidine dimer (PD) glycosylase and an apurinic-apyrimidinic lyase.

We determined the crystal structure of an active site mutant enzyme in a complex with a DNA substrate containing a thymidine dimer at 2.75 Å resolution. This enzyme contains Gln in place of Glu23, which is a catalytic residue for glycosylase reactions (Morikawa, K. *et al.* (1992) *Science* **256**, 523–526). The abolishment of glycosylase activity in crystallization drops allowed us to obtain crystals of the enzyme complexed with the substrate. The atomic model of the complex has revealed the detailed catalytic mechanism and the major factors, by which the enzyme recognizes PD specifically.

The bound DNA adopts a unique structure which is sharply kinked at the central dimer position with a 60° inclination. Thus, the DNA duplex is divided to two regions, both of which retain the DNA-B form. The adenine base complementary to the 5′ side of the dimer, is completely flipped out from the B-DNA duplex and trapped into a cavity on the protein surface. This striking local distortion disrupts the base pair, whereas the adjacent base pair within the dimer can be retained in spite of its substantial deformation. A shortened phosphodiester bond distance at the dimer site is recognized by

four basic residues. These structural features implicate a general mechanism of how damaged DNA is recognized by repair enzymes.

Kinetic Study on Transcription Using Immobilized Operons: Branched pathway in early elongation

Tomoko KUBORI and Nobuo SHIMAMOTO

In transcription experiments *in vitro* a template DNA fragment was fixed to acrylamide beads at the tight binding between avidin and biotin. The immobilized DNA was a good template in transcription by *E. coli* RNA polymerase. This technique enabled us to separate transcription complexes from free components with brief centrifugation. In addition, dead-end complexes which lost elongation activity can be assayed by rapidly diluting a reaction mixture with a new substrate solution. We studied the relationships between active transcription complexes, dead end complexes, and abortive release.

In the *E. coli* transcription system, 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 preceding molecule stuck at 32 nt from a promoter, a misincorporation at 6 nt was found in addition to further enhancement of abortive release. The misincorporated transcripts were never elongated into long RNA even when the stacked molecule was removed. Thus misincorporation was checked in the early steps in elongation.

The immobilized DNA was preincubated with RNA polymerase holoenzyme and packed in a 5 μl column. Reactions were started by eluting the column with a buffer containing substrates. The kinetics of releases of abortive transcripts and the sigma factor were thus studied.

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 proved by direct visualization of single molecules of the enzyme. To address whether sliding motion is common to other DNA binding

proteins, 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, and the movement is common to the proteins so far examined. In the absence of its inducer, d-camphor, CamR molecules were trapped at three positions on a λ DNA, one was its cognate operator cloned on λ DNA, and the other two were likely to be regions 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. Another binding assay using an affinity column immobilizing CamR protein showed a result similar to the direct visualization, indicating the limitation of the gel-shift assay.

Addition of the inducer, d-camphor, significantly decreased trapped molecules, but the frequency of sliding increased. The result showed that the inducer destabilized specific interactions but not those for sliding. The interaction in the specific complex is different from that during sliding.

Functional and Structural Study of *E. coli* Single-Stranded DNA Binding Protein

Hiroki NAGAI, and Nobuo SHIMAMOTO

E. coli Single-Stranded DNA Binding Protein (SSB) is a 19 kda protein and essential to replication and recombination. In physiological conditions, it exists as a tetramer and binds to both single-stranded DNA and a group of mRNAs which have a homologous RNA element, termed SSB box. Both of the bindings are cooperative and the protein molecules form clusters on such nucleic acid molecules. These bindings suggest a coupling between replication and translation through the exchange of SSB between replication forks and mRNAs. Because the translation initiation region of the ssb gene contains three putative SSB boxes, it is possible that the expression of ssb gene is regulated autogenously. We investigated the expression from *ssb–lacZ* fusion genes in cells overproducing or depleting SSB protein. The results showed, however, that cellular amounts of SSB protein hardly affect the expression of *ssb–lacZ* fusion genes, suggesting that autogenous regulation of

the ssb gene expression through putative SSB boxes is unlikely.

In vitro Transcriptional Analysis of the *Bacillus subtilis* Sporulation Gene

Masaya FUJITA and Yoshito SADAIE

Interconversion of transcription apparatus with different promoter selectivity among RNA polymerases of a single species is considered to be the major mechanism of global control of transcription in bacteria. At least five sporulation specific sigma factors have been identified in *Bacillus subtilis* at present. Replacement of the sigma subunit leading to the generation of multiple forms of the holo enzyme and reversible interaction of the RNA polymerase with one of the transcription factors are the main determinant of regulation of sporulation gene expression.

In this study, we constructed *in vitro* transcription system with EσA, EσE, and EσH. Under our standard reaction conditions, the order of promoter strength of sporulation genes was determined. Several sporulation genes were analyzed with this system in the presence or absence of some transcription factors, such as SpoIIID or Spo0A. For example, the *abrB* gene encoding a transcriptional regulator was transcribed with EσA and repressed by Spo0A. Transcription of the *spoVE* gene was initiated with EσE and repressed by SpoIIID. The SpoIIID binding site was located around the -10 region of the spoVE promoter by DNase I footprint analysis.

II. MICROBIAL GENETICS

Recognition of the Minus Strand DNA Replication Origin of the Filamentous Phage by *E. coli* RNA Polymerase

Zhi Wen GUAN, Nahoko HIGASHITANI, Atsushi HIGASHITANI, and Kensuke HORIUCHI

Synthesis of the minus strand DNA of filamentous phage f1 is initiated by a RNA primer, which is synthesized by the host RNA polymerase holoenzyme. The minus strand origin contains two hairpin structures called [B] and [C]. Footprinting and mutational analyses have indicated that the region containing the two hairpins is specifically recognized by RNA polymerase in the presence of SSB (single-stranded DNA binding protein).

We previously constructed a number of deletion and insertion mutations in each of the hairpins and base substitution mutations in hairpin [B], and analyzed them for the origin function (Higashitani, N. *et al.* (1990) *annual rep.* **41**, 31–32, and Higashitani, N. *et al.* (1992) *annual rep.* **43**, 34–35). In the present study, we constructed and tested base substitutions in hairpin [C]. From all the results obtained, it appears that the origin DNA may take a secondary structure as shown in Fig. 1 and may function through a mechanism similar to transcriptional initiation.

For most regions within the stem of hairpin [B], double-strandedness is required for active origin function. A specific nucleotide sequence is not required except for a short region, which is about 35 bases away from the start site of the primer RNA when the secondary structure of the origin is as depicted in Fig. 1. Thus, this particular region may correspond to the -35 region of transcriptional promoters. The base sequence of this region includes 5′-TTTACG which can be found in lists of -35 regions of weak promoters of *E. coli*. In hairpin [C], we constructed base substitutions in a region that corresponds to the -10 region in relation to the primer RNA start site. In the wild type origin, this region carries a sequence, 5′-AAAAAA, in the upper strand as shown in Fig. 1, and 3′-TTTTT in the lower strand. When the 3′-TTTTT was replaced with 3′-GGGGG, the origin was active. When 5′-AAAAAA was replaced with 5′-CCCCCC, the origin was inactive. When both of the substitutions were introduced into a single

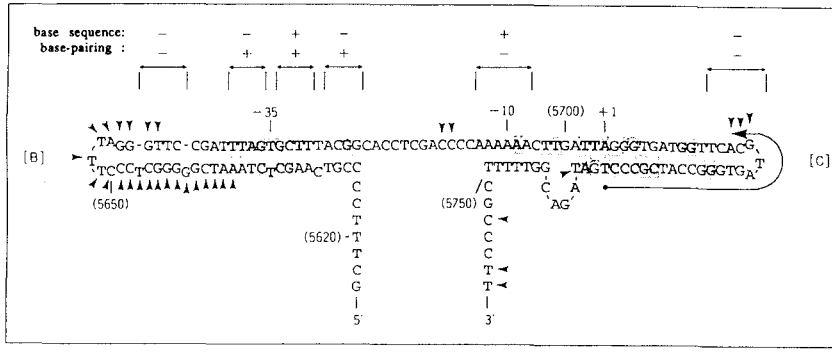

Fig. 1. A possible secondary structure of hairpins [B] and [C] at the minus-strand origin. The long arrow indicates primer RNA. The shaded nucleotides and the arrowheads represent regions that are protected from nuclease digestion and regions that become hypersensitive to nucleases, respectively, upon binding of the RNA polymerase holoenzyme. Nucleotide numbers are shown in a manner analogous to that generally used for transcriptional promoters. The Requirement of the DNA sequence and that of the base-paired structure for the origin function are indicated by +, while − indicates the absence of the requirement.

origin, it was inactive. Thus, it is specific bases and not paired strands that are required in this region for the origin function. In addition, it has been reported that the nucleotide sequence at the region around −10 to +1 of a transcriptional promoter could be replaced by unpaired sequences that lead to the formation of bubble-like structures without loss of RNA synthesizing activity (Tripatara and deHaseth (1993) *J. Mol. Biol.* **233**, 349–358).

These results suggest structural and functional similarity between the minus strand origin and transcriptional promoters. The recognition mechanism of the RNA polymerase holoenzyme at the minus strand origin may be related to that in transcriptional promoters. Then, the recognition mechanism of the −10 region by sigma (σ) factor may involve a single-stranded DNA structure with specific base sequences.

The *psv* Gene Coding for Penicillin-Binding Protein 7 of *Escherichia coli*

Hiroshi HARA, Noriko ABE*, Masayo NAKAKOHJI*,
Yukinobu NISHIMURA*, and Kensuke HORIUCHI

We identified the gene encoding penicillin-binding protein (PBP) 7 of *E. coli*, a peptidoglycan endopeptidase, in the course of our study on periplasmic protease Prc, which was originally found to cleave the C-terminal 11 residues from the precursor form of PBP3. A Δ*prc* mutant lacking this protease shows thermosensitive growth at low osmolarity. Its suppressor (*spr*) mutations also caused thermosensitivity at low osmolarity when placed in the *prc*$^+$ background. They were all located around *fruA* at 48 min on the chromosome map, not in the gene for PBP3. A gene that could correct the thermosensitive defect of an *spr* mutant was cloned from the ordered cosmid library, and the gene turned out to be a multicopy suppressor of *spr*. The cloned gene and the *spr* gene were mapped about 1 min apart from each other.

Analysis of the sequence of this multicopy suppressor gene revealed the existence of three motifs conserved among PBPs and β-lactamases in the gene product. [^{14}C]penicillin-binding experiments showed it was a PBP of about 31 kDa that had high affinity to *N*-formimidoylthienamycin and was converted to a PBP of about 30 kDa by protease OmpT. The product was thus identified as PBP7, and the gene designated as *psv*. PBP7 seemed to be degraded by protease Prc, because the band of [^{14}C]penicilloyl-PBP7 was more intense in a Δ*prc* mutant than in the wild type. Disruption of the *psv* gene on the chromosome through the insertion of a drug resistance gene caused no apparent defect in cell division and growth.

A Cell Division Inhibitor SulA of *Escherichia coli* Directly Interacts with FtsZ through GTP Hydrolysis

Atsushi HIGASHITANI and Kensuke HORIUCHI

The FtsZ protein plays a central role in bacterial cell division and is one of the earliest factors involved in septum synthesis (Lutkenhaus, J. (1990) *Trends Genet.* **6**, 22–25). It forms a ring-like structure at the division site prior to invagination of the cell wall (Bi, E. and Lutkenhaus, J. (1991) *Nature* **354**, 161–164). The purified FtsZ protein displays binding activities

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for GTP and GDP, and GTPase activity (RayChaudhuri, D. and Park, J. T. (1992) *Nature* **359**, 251–254). The putative binding domain for GTP contains an amino acid sequence that is highly conserved among eukaryotic tubulins (de Boer, P. *et al.* (1992) *Nature* **359**, 254–256). Like tubulins, FtsZ assembles upon incubation with GTP *in vitro* into a filamentous structure (Mukherjee, A. and Lutkenhaus, J. (1994) *J. Bacteriol.* **176**, 2754–2758) that may be comparable to the ring-like structure formed *in vivo*.

DNA damage in bacteria results in induction of at least 15 genes named SOS genes, which include those involved in DNA repair (Walker, G. C. (1987) in *E. coli* and *S. typhimurium*, *ASM 1346–1357). One of these genes, sulA*, is a cell-division inhibitor and prevents premature segregation of the damaged DNA into daughter cells during the DNA repair process (Gottesman, S. *et al.* (1981) *J. Bacteriol.* **148**, 265–273). Genetic evidence suggests that the target of the SulA protein is FtsZ, because a class of mutations in *ftsZ* (*Rsa*, *sulB*, or *sfiB*) which confer resistance to overproduction of SulA has been isolated (Bi, E. and Lutkenhaus, J. (1990) *J. Bacteriol.* **172**, 5602–5610). In a recent study, induction of SulA blocked the formation of the ring-like structure of FtsZ (Bi, E. and Lutkenhaus, J. (1993) *J. Bacteriol.* **175**, 1118–1125). On the other hand, it has not been determined whether FtsZ and SulA interact directly with each other, and if they do, whether the interaction requires any other factor(s). In this study, we show direct interaction between purified FtsZ and SulA *in vitro*.

Direct interaction was examined *in vitro* using SulA, which was fused to the maltose binding protein (MBP-SulA; Sonezaki, S. *et al.* (1995) *Appl. Microbiol. Biotechnol.* **43**, 304–309), and purified FtsZ. Since MBP-SulA binds to amylose resin and is eluted from it with 10 mM maltose, it is expected that FtsZ can be co-eluted with MBP-SulA if a stable complex is formed between the two proteins prior to application to the resin. During incubation at 37°C for 30 min, FtsZ did form a complex with MBP-SulA. When MBP-SulA was omitted, or MBP-SulA was replaced by MBP, FtsZ was not detected in the eluate. These results indicate that a complex was formed by direct interaction of FtsZ and SulA.

In order to determine if any co-factor(s) are required for complex formation, the effects of Mg and Ca ions, and various nucleotides on complex formation were tested. Complex formation required both GTP and Mg ions. Ca ions could not be substituted for Mg ions. Various nucleotides, ATP, CTP, UTP, ADP, GDP, and GTPγS all failed as substitutes for GTP.

Complex formation proceeded progressively with time until the reaction was completed after 20 min of incubation at 37°C. At 20°C for 20 min, complex formation was about 13% complete, and less than 3% complete at 4°C. A good correlation between complex formation and GTP hydrolysis in each rection was observed. These results suggest that the complex formation of FtsZ and SulA requires GTP hydrolysis.

GTPase activity was only dependent upon FtsZ, and not on SulA. The GTPase activity of FtsZ was not affected by the addition of MBP-SulA. Furthermore, specific GTPase activity of the complex that was eluted from amylose resin was identical to that of FtsZ alone. These results indicate that the GTPase activity of FtsZ is not affected by formation of the complex with SulA.

The amount of FtsZ found in the complex was proportional to the amount of FtsZ added to the reaction, and was saturated when the molar ratio of FtsZ to MBP-SulA reached about one to one. This suggests that the complex was formed in a molar ratio of approximately one to one of the two proteins.

It is likely that the complex formation we reported here reflects the *in vivo* mechanism by which SulA inhibits cell division.

Isolation and Characterization of Mutations that Suppress the Effect of a Cell-Division Inhibitor SulA in *Escherichia coli*

Atsushi HIGASHITANI and Kensuke HORIUCHI

The *sulA* gene of *Escherichia coli* codes for a cell division inhibitor which is induced upon SOS response. In order to determine the interaction between SulA and other proteins, we isolated suppressor mutants that survive overproduction of SulA (*sso* phenotype). We have constructed a plasmid, pSulA 5, which carries the wild-type *sulA* gene under *lacUV5* promoter control (Higashitani, A *et al.* (1995) *Biochem. Biophys. Res. Comm.* **209**, 198–204). *E. coli* cells harboring this plasmid fail to grow in the presence of IPTG. Dominant mutants that were able to grow on IPTG-containing plates arose spontaneously at a frequency of about 10^{-6}. Twelve such mutants were independently isolated and named *sso* mutants. The chromosomal positions of the mutations were mapped by a method previously reported (Higashitani, A. *et al.* (1994) *Nucl. Acids Res.* **22**, 2426–2427), and the results indicated that they could be classified into two alleles, *pcnB* (class I) and *ftsZ* (class II).

The *pcnB* gene has been reported to be involved in the copy number control of ColE1-related plasmids (Liu, J. and Parkinson, J. S. (1989) *J. Bacteriol.* **171**, 1254–1261). In the class I *sso* mutants, the copy number of plasmid pSulA5 was about 10% of that of wild-type cells. Thus, the level of the SulA protein in the presence of IPTG may not be so high in these mutants, and this may explain why the mutants can grow on IPTG plates. The *ftsZ* gene plays a central role in cell division (for review, Lutkenhaus, J. (1990) *Trends Genet.* **6**, 22–25), and the class II mutants are probably similar to previously isolated mutants in *ftsZ* (*Rsa*, *sulB*, or *sfiB*) which confer resistance to overproduction of SulA. In our recent studies, direct interaction between the SulA protein and the FtsZ protein was found *in vitro*. These results suggest that FtsZ may be the only target of SulA, and we may not be able to obtain other suppressor mutants that would bypass the loss of function of FtsZ.

Molecular Mechanisms that Determine the Timing of Cell Division in *Escherichia coli* K12

Akiko NISHIMURA

To elucidate the molecular mechanisms of the timing of cell division in *E. coli* K12, we isolated novel mutants, *cfcA*, *B*, *C*, *D*, *E*, and *F*, (Nishimura, A. (1989) *Mol. Gen. Genet.* **215**, 286–293) in which DNA replication and cell division are uncoupled. These mutations partially suppressed both division arrest and lethality induced by treatments that inhibit DNA replication. Under permissive conditions for DNA replication, the *cfc* mutants divide 1.3 to 1.5 times more frequently per round of DNA replication. Complementation tests and sequencing analyses showed that the *cfcA* gene encodes the α subunit of glycine tRNA synthetase. From genetic and physiologic analyses of the *cfcA1* mutant, the *cfcA* gene was found to be specifically involved in the regulation of the cell cycle by a pathway different from stringent response or SOS induction. The locus of the *cfcB1* mutation was found in the *apaH* gene mapped at 1 min of the *E. coli* genetic map. The *ApaH* gene encodes diadenosine tetraphosphate (AppppA) hydrolase, and *apaH*$^-$ mutants showed a $\geq$16 fold increase in the cellular level of AppppA. AppppA may be a pleiotropic signal for cell growth and DNA replication (review: Zamecnik, P. (1983) *Anal. Biochem.* **134**, 1–10). AppppA is formed *in vitro* by aminoacyl tRNA synthetases in a reaction in which an enzyme-bound

aminoacyladenylate intermediate donates AMP to ATP (Goerlich, O. *et. al.* (1982) *Eur. J. Biochem.* **126**, 135–142). However, so far, neither aminoacyl tRNA synthetase mutants, inhibitory treatments of aminoacylation, nor structural mutants of tRNA has been known to overproduce AppppA *in vivo*. The *cfcA1*/*glyS* mutation was suppressed by a high copy number plasmid carrying the *apaH*$^+$ gene. HPLC analyses showed that the intracellular level of AppppA in *cfcA1* and *cfcB1* cells was about 100-times higher than in *cfc*$^+$ cells. This suggests that *cfcA1*, the mutant of glycine tRNA synthetase, could synthesize AppppA *in vivo* and that the AppppA nucleotide is involved in the determination of the timing of cell division in a manner that couples cell division to DNA replication. We are currently analyzing other *cfc* mutants to search for targets of AppppA.

A New Gene Which Couples Cell Growth and Cell Division in *Escherichia coli* K-12

Hideki UKAI, Naoko WATANABE, and Akiko NISHIMURA

Our research focused on the regulatory mechanisms of cell division in *E. coli*. We hypothesized that a cell has mechanisms to coordinate cell growth with cell division. Recently we identified a novel gene, *sunU*, which uncouples cell division and cell growth. Multiple copies of the *sunU* gene partially suppressed the decline of cell growth, but not the defect of cell division, of *ftsE*, *cpcA58*, and *ftsU349* mutants. *FtsE* gene has the concensus sequence for the ABC-transporter. The *cpcA58* mutation stopped cell division at 42°C in media of pH 5.8 containing cAMP. The phenotype was more marked in the presence of Ca^{2+}. The *cpcA58* mutant which harbors the tetracycline resistance gene shows less resistance than *cpcA*$^+$ *mutants. This indicates that the cpcA58* mutation causes a defect in the antitransport system of tetracycline resistance. The *FtsU* gene has a 'gearbox' promoter, and the mutation stopped cell growth and cell division at 42°C showing high ploidy. A possible explanation is that all three mutations may be related to an ion pump, and the *sunU* gene may couple the ion pump and cell division.

The Role of the SecA protein of *Bacillus subtilis* in Sporulation

Yoshito SADAIE and Masaya FUJITA

We investigated the expression and role of the *secA* gene of *Bacillus subtilis* during early stages of sporulation as it plays a crucial role in sporulation and sporulation associated events as well as in protein secretion and cell septation. Its RNA transcript and protein product were measured with S1 nuclease mapping and immunoblotting, while a temperature sensitive *secA* mutation was used to examine the role of the SecA in the expression of the component genes of phosphorelay and sigma cascade. The start site of the synthesis of mRNA was 110 bases upstream of the initiation codon for the *secA* gene, preceeded by −10 and −35 sequences atypical of σ^A promoters. The amount of *secA* mRNA decreased upon sporulation, while a constant amount of SecA protein was recovered throughout early sporulation stages after a temporal decrease around T0.5 (30 min after the end of the logarithmic phase of growth). A temperature shift experiment revealed an arrest of sporulation by the temperature-sensitive *secA341*(*ts*) mutation at 37°C up to the T3 stage, and that the transcriptional expression of *abrB*, *spo0H*, *kinA*, *spo0A*, *spoIIG*, and *spoIIIG* genes monitored with *lacZ* activity was blocked by the *secA341* mutation at 37°C except for *abrB* and *spo0H* genes. Since these blocked genes have promoters depending on σ^H and/or phosphorylated Spo0A, these results suggest the SecA protein is necessary for the functioning of the product of the *spo0H* gene.

III. MAMMALIAN GENETICS

Genetical Analysis of *rim2* Coat Color Mutation

Tomoko SAGAI, Sigeharu WAKANA*[1], Kazuo MORIWAKI
and Toshihiko SHIROISHI

rim2 is a single recessive mutant that arose spontaneously in one of the recombinants, B10.A(R201) strain, which has a recombinational breakpoint at the LMP-2 hotspot. The mutant is characterized by diluted pigmentation of the entire body hair, ears and tail. In particular, the amount of pigment in the eyes at birth is markedly reduced from that of the original wild type strain. In addition to hypo-pigmentation, eye defects such as cataracts, eyelessness and small eyes are frequently observed. To search for the *rim2* mutant gene, we carried out chromosomal mapping using micro-satellite markers. Backcross mating with MSM strain revealed that the *rim2* locus was tightly linked with several microsatellite markers located on the chromosome 13.

At the near side of the *rim2* loci, an old coat color mutation, *pearl*, has been mapped. A mating cross of *rim2* and *pearl* mutants showed that they are allelic. It is known that the *pearl* and many other hypo-pigmented mouse mutants cause a platelet storage pool deficiency (SPD). In humans, SPD is a recessive autosomal disease associated with hemorrhagic symptoms and phenotypically heterogeneous syndromes. Chediak-Higashi Syndrome(CHS) and Hermansky-Pudlak Syndrome(HPS) are the most intensely studied of these diseases. The *rim2* and the hypopigment mutants are expected to serve as models for heterogeneous human SPDs.

On the basis of a linkage analysis of 1550 backcross mice, we obtained several YAC clones including the marker sequences tightly linked to the *rim 2* mutation. Construction of YAC contig is in progress.

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Meiotic Recombination at the Lmp2 Hotspot in the Mouse Major Histocompatibility Complex Is Tolerant of Substitutional Base Mismatches

Masayasu YOSHINO, Tomoko SAGAI and Toshihiko SHIROISHI

Recombination is widely considered to linearly depend on the length of the homologous sequences. It was reported that 11% mismatch decreased the rate of phage-plasmid recombination 240-fold. It was found that two single nucleotide mismatches that reduce the longest uninterrupted stretch of homology from 232 bp to 134 bp resulted in a 20-fold reduction in gene conversion of plasmid DNA in mouse L cells.

In this study we were interested in determining whether or not a very high degree of sequence identity between homologous chromosomes enhances recombinational events even in mouse meiosis. We made a cross between B 10.A(R209) strain with the MHC haplotype of Kwm^7–Ab^k–$Cyp21^d$–D^d and B 10.BR strain with the MHC haplotype of K^k–Ab^k–$Cyp21^k$–D^k, both of which have the serologically identical *k* haplotype from the *Lmp2* hotspot to the recombinational breakpoint of the *a* haplotype located between the *Int3* and *Cyp21* genes in the major histocompatibility complex (MHC) region on mouse chromosome 17. The physical length of this serologically identical segment is roughly estimated to be about 300 kb. We screened the *K*–*D* recombinants by cytotoxicity test using anti-K^{wm7} and anti-D^d antibodies. The resultant *K*–*D* recombinants were genotyped by RFLP analysis using the *Lmp2* hotspot DNA as a probe and the *Cyp21* probe and the recombinational frequency around the hotspot was estimated.

We obtained twenty-two *K*–*D* recombinants from 749 offspring and eighteen breakpoints of these were confined to the chromosomal segment spanning from the polymorphic DraI site which is located 1.2 kb proximal to the *MT*-consensus sequence at the *Lmp2* hotspot to the polymorphic *EcoR*V site detected by the *Cyp21* probe. Nine of the 18 *Lmp2* hotspot-*Cyp21* recombinational breakpoints were proximal to the polymorphic *Msp*I site at the 1174 nucleotide position and they were confined to the 2.4 kb DNA segment overlapping the *Lmp2* hotspot. The remaining nine of the 18 *Lmp2* hotspot-*Cyp21* recombinational breakpoints were distal to the polymorphic *Msp*I site at the 1174 and they were confined to the 300 kb serologically identical region spanning from the hotspot. Since there was no polymorphic restriction site in the serologically identical region from the breakpoint at the *Lmp2* hotspot to

the breakpoint at the *Int3–Cyp21* interval, the distal end of the breakpoints could not be determined in this chromosomal segment. The remaining four breakpoints of the 22 *K–D* recombinants were located in the *Cyp21–Tnfa* interval.

Previous fine mapping of the recombinational breakpoints at the *Lmp2* hotspot from the crosses of B10.A (MHC type K^{k}–Ab^{k}–$Cyp21^{d}$–D^{d}) and B10.MOL–SGR (MHC type K^{wm7}–Ab^{wm7}–$Cyp21^{wm7}$–D^{wm7}), and B10.BR (MHC type K^{k}–Ab^{k}–$Cyp21^{k}$–D^{k}) and B10.A(R218) (MHC type K^{wm7}–Ab^{wm7}–$Cyp21^{aw18}$–D^{d}) revealed that the breakpoints were equally distributed in regions both proximal and distal to the *Msp*I site (Shiroishi *et al.*, 1991). In the cross of B10.BR (K^{k}–Ab^{k}–$Cyp21^{k}$–D^{k}) and B10.A(R209) (K^{wm7}–Ab^{k}–$Cyp21^{d}$–D^{d}), the breakpoints were again equally divided into the two segments. The five sites of single base nucleotide mismatch were located in the (B10.A × B10.MOL–SGR)F_1 at the 1243, 1261, 1377, 1388 and 1649 nucleotide positions, while these sites are matched and identity spans along 300 kb distal to the hotspot in a cross of B10.BR and B10.A(R209). Therefore, these results indicate that the distribution of the breakpoints was not biased toward the more homologous region distal to the *Msp*I site in the middle of the hotspot and that recombination was not elevated in the same region.

The recombinational frequency in the *Lmp2* hotspot and *Cyp21* interval in the [B10.BR × B10.A(R209)]F_1 female mice in which there is identity over 300 kb around the *Lmp2* hotspot was 3.2% and this is almost equal to the 2.8% frequency at the *Lmp2* hotspot in the [B10 × B10.A(R209)]F_1 female mice. In males, the frequency in the *Lmp2* hotspot and *Cyp21* interval was 1.5% in the [B10.BR × B10.A(R209)]F_1 and this is almost equal to the 2.2% frequency at the *Lmp2* hotspot in the [B10 × B10.A(R209)]F_1. The results with both sexes indicate that the long identity of the chromosomal segment did not enhance recombination around the *Lmp2* hotspot during meiosis.

The rate of mitotic recombination in mammalian cells has been thought to primarily depend on the amount of uninterrupted homology. In the present study, no enhancing effect on meiotic recombination around the hotspot was observed in crosses, in which two homologous chromosomes have long identity spanning 300 kb, suggesting that meiotic recombination around the hotspot in the mouse MHC is tolerant of substitutional base mismatches. Since mitotic and meiotic recombination have been thought to be mechanistically distinct in yeast, the present results may reflect some differences between mitosis and meiosis.

Genetic Analysis of Mouse Mutants Affecting Limb Pattern Formation

Hiroshi MASUYA, Tomoko SAGAI, Kazuo MORIWAKI and Toshihiko SHIROISHI

Positional signaling along the anteroposterior axis of the developing vertebrate limb is provided by the zone of polarizing activity (ZPA) located at the posterior margin. It is postulated that *Sonic hedghog* (*Shh*), a homologue of the segment polarity gene *hedgehog* in *Drosophila*, mediates ZPA activity. The downstream activity of the ZPA is thought to be performed by genes such as *Hoxd* and *bone morphogenic protein-2* (*Bmp-2*), which may direct positional specification during pattern formation in vertebrate limbs. It is also known that some members of the FGF family expressed in the AER can functionally replace the AER. It has been evident for a long time that ZPA stimulates signaling of the AER, while the posterior portion of the AER maintains functional ZPA in the posterior mesoderm. In vertebrate tetrapods, there are many congenital abnormalities affecting the pattern formation of limbs. Preaxial polydactyly which causes extra digit(s) on the side of digit 1 is one of the major abnormalities.

In this study, we carried out a morphological analysis of mouse mutants with preaxial polydactyly. It revealed that a new mouse mutant, *Recombination induced mutant 4* (*Rim4*), and two old mutants, *Hemimelic extra toes* (*Hx*) and *Extra toes* (*Xt*), exhibit mirror-image duplication of the skeletal pattern of the digits. *In situ* hybridization of the embryos of these mutants indicated ectopic expression of *Shh* and *fibroblast growth factor-4* (*Fgf-4*) genes at the anterior margin of limb buds. The new mutation, *Rim4*, was mapped to chromosome 6 with linkage to *HoxA* but was segregated from *HoxA*. No linkage to other known polydactylous mutations was detected. In this mutant, ectopic expression of *Hoxd-11* gene which is thought to be the downstream of ZPA was also observed at the anterior margin of the limb buds. All results demonstrate the presence of an additional ZPA at the anterior margin of limb buds of these mutants. Thus, it appeared that there are multiple endogenous genes that regulate the spatial localization of the ZPA in the developing mouse limb bud.

When C57BL/10J-*Rim4*/+ mice were crossed with other laboratory strains, the frequency of mice with the *Rim4* phenotype was variable, depending on the strain with which the *Rim4* was crossed. The penetrance was incomplete in heterozygotes, ranging from 0% in crosses with MSM strain to almost 100% in crosses with NZB strain. The difference in

penetrance indicates that the *Rim4* phenotype is controlled by allelic forms of the *Rim4* locus or other genes, which are not linked to the *Rim4*.

Linkage Analysis of the Skeleton Mouse Mutant, *Tail short* (*Ts*)

Toshihiko SHIROISHI, Kikue UCHIDA, Peter KOOPMAN[*1], Akihiko MITA, Sigeharu WAKANA[*2] and Kazuo MORIWAKI

Tail short (*Ts*) is an old skeletal mutant (Morgan, 1950). Heterozygotes for *Ts* have shorter kinked tails and smaller bodies than normal mice. Other skeletal abnormalities of various kinds scattered over the whole body were also observed. Homozygous embryos die at about the time implantation of their litter mates occurs. Abnormalities in homozygous mice can be traced back to $3_{1/2}$ days post coitum (p.c.) through several criteria including low cell number and absence of cavitation. All these indicated the indispensable function of the *Ts* gene not only in skeletal development but also in the early development of mice. The *Ts* gene was mapped to the teromeric region of chromosome 11. It was reported that mutant mice fell short of the Mendelian expectation by 30–40% in $Ts/+ \times +/+$ mating. This indicated that some fraction of mice heterozygous for *Ts* were lethal at the time of birth, rendering genetic analysis difficult. We have started a fine linkage analysis of *Ts*, toward positional cloning of this gene. We conducted backcross mating between $Ts/+$ and MSM inbred strain derived from Japanese wild mouse. Mice heterozygous for the *Ts* from this cross were less defective and litter-size was relatively large. Furthermore, penetrance of the *Ts* phenotype was almost complete in this cross. In the last year, two candidate genes for the *Ts* mutation were cloned and mapped to the syntenic region of human chromosome 17q24.3–q25.1. Sox9, a Sry-related gene, was identified as a candidate gene for human campomelic dysplasia (*CMPD1*) displaying features similar to *Ts* (Foster *et al.*, 1994). The M33 gene, a human homologue for a polycomb gene which modifies expression of Hox genes, was mapped to the same region of the human chromosome. To elucidate whether either Sox9 or M33 is a candidate for the *Ts* mutant gene, we analyzed linkage of *Ts* phenotype and these two genes on the *Ts*/MSM backcross panel. It appeared that *Ts* was segregated from both Sox9 and M33, excluding these two genes

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as candidates for the *Ts* mutant gene. The order of the genes is; centromere–sox9–(3.8cM)–*Ts*–(17.0cM)–M33. We are continuing to make a fine linkage map around the *Ts* for positional cloning of this gene.

Gene Mapping Toward Positional Cloning of the *Rim3* Gene

Hiroshi MASUYA, Shigeharu WAKANA*1, Tomoko SAGAI,
Kazuo MORIWAKI and Toshihiko SHIROISHI

Rim3 is a hairless mutant derived from an intra-MHC recombinant, which shows hyperkeratinozation in the cornea and probably throughout the whole body. In the present study, we extended gene mapping of the *Rim3*, using crosses between C57BL/10J-*Rim3* and MSM strains. As a result, the *Rim3* gene was tightly linked to a microsatellite marker *D11Mit14.* on chromosome 11. The *Krt-1* (*Keratin type1*) locus has been mapped to the same position. It is known that *Krt-1* is a multi gene family consisting of more than 10 genes related to each other. It is likely that one of the members of *Krt-1*, which is expressed in the cornea, is affected by the *Rim3* mutation. We carried out PCR-screening of YAC library provided from Research Genetics, Alabama, USA, using *D11Mit14* primers, and identified a single positive clone. A search for a candidate gene for *Rim3* is now in progress using this YAC clone.

Meiotic Recombination on DNase I-hypersensitive Sites in the Mouse Major Histocompatibility Complex

Ken-ichi MIZUNO, Tomoko SAGAI, Kazuo MORIWAKI
and Toshihiko SHIROISHI

In murine major histocompatibility complex (MHC), meiotic recombination does not occur at random in an array of DNA. We can serologically detect meiotic recombinations in the MHC using antibodies for *H2K* and *H2D* marker loci, which are physically separated by more than 1 Mb of distance. Almost all breakpoints of the recombinants are clustered within limited regions. At present, four clusters of recombinational breakpoints in MHC class II are known and the clusters are called hotspots. One of them was identified at the downstream of the *Lmp2* gene and is designated the

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Lmp2 hotspot. The *Lmp2* hotspot was mapped within a 2 kb segment of DNA and meiotic recombination occurs at an extremely high frequency, about two percent, in this hotspot. However the molecular nature which determines the location of recombinational hotspots is not well understood.

It is also well known that hotspots exist during meiotic recombinations in the lower eukaryote *Saccharomyces cerevisiae* and, moreover that transient double-strand DNA breaks (DSBs) occur at these hotspots, leading to the initiation of recombination. Detailed analysis of chromatin structure around the hotspots using endonucleases, such as DNase I, indicated that the distribution of DSBs overlaps with that of constitutive DNase I-hypersensitive sites (DHSSs). It seems likely that features of the chromatin structure, which is established prior to meiosis, play a key role in determining the sites at which recombination is initiated. It is conceivable that DSBs occur at sites where nucleosomes are disrupted and the DNA is accessible to the enzymes involved in the recombination. In this context, we have analyzed the chromatin structure around the hotspots in the mouse MHC. The results of the study indicated an absence of DHSSs within the hotspot. There were clear DHSSs in a region proximal to the hotspot instead. Although we have obtained the data indicating that features of the chromatin structure during mouse meiosis do not play a critical role in determining the sites of recombinational hotspots, the contribution of the chromatin structure to meiotic recombination is still controversial in the mouse.

If DHSSs determine the initiation sites of recombination but not the resolution sites of recombinations around the hotspots, it is possible that there are gene conversions and recombinations in the vicinity of the DHSSs observed in the region proximal to the hotspot. We attempted to examine whether these events occur at the DHSSs around the hotspot in the mouse MHC. B10.A(R209) strain shows recombination at the hotspot during male meiosis and B10.MOL–SGR dose not. These strains were crossed with C57BL/10J strains and the F1 progeny were backcrossed with C57BL/10J strain. Genomic DNA were prepared from the tails of the backcrossed progeny (BC2) and were examined by Southern analysis and PCR. Now we are typing the mice for five loci (*H2K*, *H2D*, *Lmp2* hotspot and, the proximal and distal flanking sites of a mouse meiosis specific DHSS located proximal to the *Lmp2* hotspot).

IV. DEVELOPMENTAL GENETICS

Sexual Differentiation Potency of Multi-Potent Stem Cells in Hydra

Chiemi NISHIMIYA-FUJISAWA and Tsutomu SUGIYAMA

Interstitial stem cells of hydra consist of at least two subpopulations. One is the germline-restricted stem cells differentiating only into sperm or eggs (Littlefield, 1985). The other is the multi-potent stem cells capable of differentiating into both somatic (nerve, nematocyte, gland cell) and gametic cells (probably through germline-restricted cells) (Bosch and David, 1987). "Pseudo-epithelial hydra" contain only the former but not the latter cells. The stem cells in these animals are all localized only in and above, but not below the budding zone. Evidence exists which suggests that germline-restricted cells are also absent below the budding zone in normal hydra. Therefore, the foot tissue in normal hydra probably contains only multi-potent but no germline-restricted cells. Under the assumption that this view is correct, sexual differentiation potency of the stem cells present in the foot tissue of normal hydra was examined.

The experiment was done using 5 male (nem-1, SSC·m, nB-2, ms-1 and SSE) and 3 female (nem-1·f-1, SSC and SSB) strains of *Hydra magnipapillata*. Nem-1 (male) had been female in the past. Strain nem-1·f-1 (female) arose from nem-1 (male) and SSC·m (male) from SSC (female) through spontaneous sex reversal. Foot tissue was cut out from the 8 strains, allowed to regenerate, and the regenerates were induced to differentiate sexually. Sex phenotype was identical between the regenerates and their corresponding parents for the 3 female and 2 of the 5 male strains (ms-1 and SSE). However, it was not identical for the remaining 3 male strains. The foot regenerates of the 3 strains (nem-1, SSC·m and nB-2) all produced eggs instead of sperm.

The results obtained suggest that multi-potent stem cells present in the foot tissue of the 3 strains (nem-1, SSC·m and nB-2) differentiate into eggs (presumably via egg-restricted stem cells), although sexual phenotype is male in these strains. This observation can be interpreted as follows:

The 3 strains were originally females, and their multi-potent stem cells differentiated into eggs via egg-restricted stem cells. In the past, sex reversal

took place from female to male in these strains. This occurred at the level of germline-restricted cells through transdifferentiation from egg-restricted to sperm-restricted cells. This type of transdifferentiation was observed to occur in pseudo-epithelial hydra containing the egg-restricted stem cells. Transdifferentiation in the germline-restricted cells reversed the sexual phenotype of the 3 strains without altering the original potency of the multi-potent stem cells to differentiate into eggs.

Hydra Regeneration from Recombined Ectodermal and Endodermal Tissue. I. Cell rearrangement

Yasuyuki KISHIMOTO, Motohide MURATE and Tsutomu SUGIYAMA

Hydra regeneration can occur from dissociated and reaggregated cells (Noda, 1971; Gierer *et al.* 1972). In the early stage of this process, a sphere is formed which consists of an ectodermal cell layer outside, an endodermal cell layer inside, and an empty space in the center. This observation shows that hydra ectodermal and endodermal epithelial cells together have the ability to self-organize themselves into a two-layered epithelial sheet. In this study, a new system was developed to examine the interaction and self-organization of the ectodermal and endodermal epithelial cells into the two-layered epithelial sheet.

Procaine treatment (Epp *et al.* 1979) was used to separate the ectodermal and endodermal layers of *Hydra magnipapillata* (strain 105). In some experiments the separated tissue was dissociated into single cells and then reaggregated to produce ectodermal or endodermal cell aggregates. When two pieces of tissue or aggregate derived from the two layers were placed in direct contact with each other, a firm adhesion was quickly established between them. This was followed by a rapid spreading of the ectodermal epithelial cells as a thin layer over the endoderm as in "epiboly" in developing embryos of fish, amphibians, etc. Spot-labeling with DiI was used to follow cell rearrangement taking place in the epibolic process. On the contact surface of the recombined ectoderm-endoderm, cells initially located inside migrated to the surface to intercalate themselves among the cells originally present in the contact surface. Continuous cell intercalation occurring on both sides of the contact surface produced a rapid increase of the contact surface, eventually resulting in a complete covering of the entire endoderm by

a thin ectodermal layer in about a day. The structure produced in this way, which closely resembled the sphere produced during regeneration from mixed cell aggregate, later developed into a new hydra.

An extract prepared from hydra membrane fraction specifically inhibited the epiboly, but not the initial adhesion or later regeneration.

These observations suggest that the major driving force for the epiboly in hydra is the cell intercalation occurring at the contact surface.

The epiboly probably requires an exchange of signals between the ectodermal and endodermal cells. And the active factor in the membrane extract may be involved in the signal exchange mechanism in some way.

Hydra Regeneration from Recombined Ectodermal and Endodermal Tissue. II. Electron Microscopic Study

Motohide MURATE, Yasuyuki KISHIMOTO, Tsutomu SUGIYAMA, Hiromi TAKAHASHI-IWANAGA* and Toshihiko IWANAGA*

In the epibolic process in recombined hydra ectoderm-endoderm, extensive cell rearrangement takes place near the contact surface of the two tissue (preceding abstract). Electron microscopy was used in this study to examine structural changes that occur in cells and tissue at various stages of the epiboly.

Soon after recombining, major structures of the ectoderm and endoderm were similar to those originally present in normal hydra. One new feature, however, was present at the contact surface between the two tissue. The ectodermal epithelial cells extended onto the endodermal surface many membranous or bulbous processes resembling lamellipodia in migrating fibroblast cells.

By 4–6 hours, some ectodermal epithelial cells moved onto, and formed a thin layer on, the endoderm. At the advancing edge, these cells extended long processes which formed multi-layers on the endoderm. In the endoderm, many cells lost their typical epithelial characteristics, turning into a mass of irregularly-shaped cells with large empty spaces between them. Large vacuoles initially present were missing in these cells.

At 10–20 hours, the ectodermal and endodermal cells started to regain cuboidal or columnar shape. These cells, now containing large vacuoles,

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were organized in layered structure, in characteristic 2 layers in some parts, but also in multi-layers in others. The layer formation started at the initial contact site, gradually moved to surrounding areas, and eventually covered the entire tissue.

Studies reported in this and preceding abstracts have revealed two important differences in the behaviors of the ectodermal and endodermal epithelial cells. One was flexibility of the overall tissue shape. The ectoderm was flexible, changing from the initial small spherical shape to the final thin layer covering the endoderm. In contrast, the endoderm made relatively much smaller change, from the initial rod-shape to a near sphere. This difference may be produced by the differential adhesiveness of the two epithelial cell types (Steinberg, 1970). The other was cell organization in the tissue. The ectodermal cells maintained the epithelial sheet organization throughout. In contrast, the organization was once lost and later produced again in the endodermal cells. Thus, the ectodermal cells are persistent in maintaining tissue organization, but flexible in overall tissue shape. The endodermal cells are opposite in both properties. These two contrasting characteristics in the two epithelia may play important roles in hydra morphogenesis.

Isolation of Cytoskeletal Molecules in Hydra by Epitope Selection

Masayuki HATTA, Masahiko SAGAGUCHI[1], Yoshihisa KOBAYAKAWA[2], Osamu KOIZUMI[3] and Tsutomu SUGIYAMA

Marker molecules were isolated for fine description of hydra cell structures and examination of their changes in morphogenesis, with emphasis on isolating molecules associated with cell membrane or cytoskeletal structures.

Epitope selection was used for isolation. A cDNA expression library was screened with antisera raised against an insoluble fraction of hydra homogenate. 200 clones were isolated. Each clone was grown individually on a petri-dish. The fusion-protein expressed by each clone was lifted onto a membrane, which was incubated with the antisera. The bound antibodies were recovered from the membrane, and then used to examine localization of the antigen in hydra cells and tissue using indirect FITC staining.

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Three clones were isolated. (1) Clone-4 is a homolog of ABP-280 (actin binding protein, non-muscle filamin) initially found in erythrocytes. The antibody stains cell-cell boundaries of the ectodermal cells, particularly in the area of adherence and septate junctions. The stain disappears when cells are dissociated, suggesting that the molecule is involved in maintaining the stability of cell adhesion apparatuses and epithelial integrity. (2) Clone-1 is a myosin heavy chain homolog. The muscle processes of ectodermal epithelial cells are specifically stained. Stained fibers, relatively short in young animals, become longer during growth. Staining becomes negative in regenerating tissue after head removal, and possibly also in presumptive bud tissue. Fiber depolymerization (muscle process reorganization?) may occur in cells ready to start morphogenesis. (3) The antibody for clone-95 specifically stains muscle processes of both ectodermal and endodermal epithelial cells. Sequence analysis has not been completed for this clone.

(4) Another clone (clone-F2–1) was isolated using antisera raised against small-size cells separated by elutriation. Its DNA sequence shows homology to tropomyosin and myosin heavy chain. The antibody stains a fibrous structure throughout the entire cytoplasm of developing stenotele and desmoneme nematoblasts. In maturation, the structure gradually moves to and gathers at the base of the nematocyst capsule, suggesting that this molecule may play a role in the positioning and anchoring of the capsule within the nematocyte cytoplasm.

Screening is in progress to isolate more markers. Markers isolated will be used for fine structural examination of hydra morphogenesis. Epitope selection is a rapid and convenient approach for simultaneous isolation of specific antibodies and cDNA clones for marker molecules.

Disappearance and Reappearance of Mesoglea in Hydra Tissue During Regeneration after Head Removal

Hiroshi Shimizu and Michael J. Sarras, Jr*

The mesoglea is a thick layer of extracellular matrix that exists between the ectodermal and endodermal layers in hydra. Using monoclonal antibodies raised against hydra mesoglea, changes occurring in the mesoglea in regenerating tissue after head removal were examined.

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A monoclonal antibody, mAb52, specifically recognizes the laminin B1 chain in hydra mesoglea. mAb39 specifically recognizes the central fibrous zone of mesoglea. Indirect FITC-staining using these 2 preparations was employed to visualize the mesoglea in the regenerating tissue. Both antibodies gave similar results.

Soon after head removal, the mesoglea disappeared from the regenerating tissue. One hour after amputation, no staining was observed in the tissue near the amputation site using either of the two antibody preparations. Mesoglea was present in the adjacent tissue. The border of the remaining mesoglea was located 40–50 μm from the amputation site. The staining intensity was stronger at this site than in the rest of the tissue. Histological examination by electron microscopy showed that the thickness of the mesoglea was approximately 5 μm at the edge and 2–3 μm in the rest of the tissue. The mesoglea started to appear again in the regenerating tissue at about 24 hours after head removal. The staining intensity was weak initially, and became gradually stronger, recovering to almost the normal level in 4 days. During this period, however, the distinct edge of high staining intensity observed soon after amputation could still be recognized at the same position.

The mechanisms for the disappearance and reappearance of the mesoglea in regenerating tissue are unclear at present. The disappearance of the mesoglea can occur either through degradation at the site, or withdrawal of the mesoglea layer from the amputation site into a more basal part of the tissue. A strong staining intensity and thickening of the mesoglea at the edge suggests that withdrawal may be responsible for the disappearance. The reappearance of mesoglea could occur either through new synthesis or extension of the existing mesoglea. The former appears likely since strong staining intensity remains at the same site during the recovery process. Experiments are in progress to critically examine the mechanisms for disappearance and reappearance.

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

Yojiro MUNEOKA[1], Toshio TAKAHASHI[1], Toshitaka Fujisawa, Hiroshi SHIMIZU, Masayuki HATTA, Tsutomu SUGIYAMA, Osamu KOIZUMI[2], Thomas BOSCH[3], Maria deHaro[3], Jan LOHMANN[3], Charles DAVID[3] and Hans BODE[4]

Morphogens are thought to play important roles in regulating development in hydra. A few of them have been isolated and characterized in past studies. In order to systematically isolate new hydra morphogens, a project based on a novel approach was started. It aims to isolate peptides which function as signal molecules to regulate gene expression in hydra. The project consists of the following 5 parts: (1) Peptide isolation from hydra tissue, (2) selection of peptides capable of altering gene expression in hydra, using Differential Display PCR (Liang and Pardee, 1992), (3) structure determination of peptides active in DD-PCR, (4) chemical synthesis of peptides, and (5) examination of biological functions of synthetic peptides. Part (1), (3) and (4) are reported here, the rest are reported in the following abstracts.

(1) *Peptide isolation.* Two batches of crude peptide preparations were extracted from *Hydra magnipapillata* tissue, batch 1 (150 g tissue) using hot acetic acid method and batch 2 (500 g), the cold acetone method. Each extract was separated by large scale reverse-phase chromatography into 15 fractions. Fraction 8 of both batches were selected as pilot samples. From these samples, a total of 188 peptides were isolated by 5–8 alternating steps of reverse-phase and ion-exchange HPLC. (2) *Amino acid sequence.* Amino acid sequence analysis was successfully performed for 97 peptides using an automatic analyzer. It was, however, not successful for 39 samples (21%) because of N-terminal blocking (or non-peptide). A homology search suggested that at least 37% and 2% in batch 1 and 2, respectively, are fragments of known proteins in the data base. (3) *Chemical synthesis.* 14 peptides were chemically synthesized and confirmed to be identical to native peptides by HPLC. They include a family of 4 peptides having a common sequence of GLWamide at the C-terminus (as in Metamorphosin-A, Leitz *et al*, 1994).

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The results described so far suggest that the procedure developed in this study is a powerful approach for isolating biologically active peptides. They also show that batch 2 contains less protein fragments than batch 1. 500 or more peptides are estimated to be present in the remaining 14 fractions in batch 2. All these peptides will be processed by the procedures described above.

Hydra Peptide Project. II. Differential Display-PCR Assay For Signal Molecule Activity

Toshitaka FUJISAWA, Hiroshi SHIMIZU, Masayuki HATTA, Tsutomu SUGIYAMA, Thomas BOSCH[1], Maria DEHARO[1], Jan LOHMANN[1], Charles DAVID[1], Toshio TAKAHASHI[2], Yojiro MUNEOKA[2], Osamu KOIZUMI[3] and Hans BODE[4]

Differential Display-PCR (Liang and Pardee, 1992) was adopted as the critical step for detecting signal molecule activities of peptides isolated from hydra tissue. The procedure was originally developed to display small differences of gene expression present in two similar tissue samples. This procedure was used to detect alterations of gene expression in hydra tissue treated by isolated peptides.

A part of each isolated peptide was diluted in a small volume of hydra culture solution, producing a peptide solution ranging from 0.5 to 10×10^{-9} M in concentration. This solution was used to treat hydra in the presence or absence of 2% DMSO during the first hour. DMSO was used to make hydra "permeable" to peptides. RNA was extracted from the treated and untreated control hydra, and used to examine and compare gene expression patterns using DD-PCR. Peptides that alter gene expression in the treated animals were selected by this assay.

46 peptides were examined. Using 2–4 primer sets, 9 (20%) produced major changes and 4 (9%) produced minor changes in gene expression. The percentage values should become higher when using more primer sets. Some produced additional bands (up-regulation), some produced disappearing bands (down-regulation), and some produced both in the display. 8 out of

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the 9 peptides which produced strong effects required DMSO to be effective, indicating that "permealization" is required for the uptake of most peptides. Several cDNA clones were isolated for genes whose expression was affected by the peptides.

DD-PCR was found to be effective in selecting peptides capable of affecting gene expression in hydra. However, one unexpected feature was also found. Gene expression was affected by the peptide treatment as distinct additional or missing bands in DD-PCR. However, northern analysis carried out using the isolated cDNA clones has revealed that actual differences in expression of such genes were relatively small (2–3 X) in some cases. The reason for this is unclear at present. The display results, therefore, need to be interpreted with caution. The procedure, however, is highly powerful in examining signal molecule activities using limited quantities of isolated peptides.

Hydra Peptide Project. III. Biological Function Assay

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A functional test of the synthetic peptides is the final crucial step of the peptide project. Simple but reliable assay procedures are required to identify peptides which play roles in regulating developmental processes. Four synthetic peptides were selected as model peptides to establish such tests. They were (1) Hyd-1, KPPRRCYLNGYCSP-amide, (2) Hyd-23, KWVQGKPTGEVKQIKF, (3) Hym-33H, AALPW, and (4) Hym-54, GPMTGLW-amide. Hyd-23 and Hym-54 have identical amino acid residues to head-activator (Schaller and Bodenmüller, 1981) and Metamophosin-A (Leitz *et al.* 1994), respectively, in the positions indicated by underlines.

In *Hydractinia*, the peptide effect was tested on metamorphosis from planula to polyp. In hydra, it was tested on budding under a variety of conditions, head or foot regeneration and $TS19^+$ antigen expression after head or foot removal, expression patterns of Cnox-2 and other locally

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expressed genes, formation of RF^+ neuronal network, cell ratios (i/epi, nerve/epi, nerve/i, etc) in whole body column and different regions, and BrdU-labeling index for various cell types in pulse and continuous BrdU-labeling. The following results were obtained.

In *Hydractinia*, (1) Hym-54 (10^{-6}M) induced complete metamorphosis from planula to polyp. (2) Hym-33H (10^{-6}M) induced tissue contraction (first step of metamorphosis?), but not complete metamorphosis by itself. However, it enhanced metamorphosis with marginal concentrations (10^{-7} M) of Hym-54.

In hydra, (3) Hym-54 (10^{-6}M, +2% DMSO) enlarged the zone of RF^+ neuron distribution in the upper body column, and increased the nerve/epi ratio (about 1.5 X) in the same region. (4) Hym-33H (10^{-6}M) contracted the same zone of RF^+ neuron distribution.

The procedures developed in this project were found to be effective in isolating signal peptides involved in regulating various developmental processes. In the future, the project can be extended to isolate genes encoding the peptides. A new way, then, will be open to elucidate the complete "molecular circuitry" of a specific developmental process that starts from a master control gene, goes through signal pathways and early gene regulation, and finally ends in the expression and functioning of the realizator genes.

Intermittent Expression of BmFTZ–F1, a Member of the Nuclear Hormone Receptor Superfamily during Development of the Silkworm *Bombyx mori*

Guan-Cheng SUN, Susumu HIROSE and Hitoshi UEDA

BmFTZ–F1 is a sequence-specific DNA-binding factor in the silkworm *Bombyx mori* sharing similar biochemical characteristics with *Drosophila* FTZ–F1, a member of the nuclear hormone receptor superfamily. Using DNA sequence homology with FTZ–F1 and information on the tryptic peptide sequences of BmFTZ–F1, we isolated a cDNA encoding for BmFTZ–F1. Amino acid sequences in the zinc finger DNA-binding region and the putative ligand-binding domain of BmFTZ–F1 showed strong similarity not only to FTZ–F1 but also its mammalian homologues, LRH-1, ELP, and Ad4BP, suggesting the importance of each region for the function of these proteins. Northern blot analyses of RNA isolated from the middle and

posterior silk glands and fat bodies showed that a 6.1-kb BmFTZ–F1 mRNA is present in all tissues so far examined. Expression of BmFTZ–F1 mRNA is intermittent, being high during larval molting and both larval-pupal and pupal-adult transformations. Injection of 20-hydroxyecdysone on the third day of the 5th instar larvae induced BmFTZ–F1 mRNA in the posterior silk gland after 24 hr. When 5th instar silk glands were cultured *in vitro*, BmFTZ–F1 mRNA was induced by a 6-hr exposure to 20-hydroxyecdysone followed by 6 hr in hormone-free medium. These results suggest that BmFTZ–F1 is inducible through a decline in the ecdysteroid titer and may play an important role in the development of the silkworm as a transcription factor. For details, see *Dev. Biol.* **162**, 426–437, 1994.

Mediators of Activation of *fushi tarazu* Gene Transcription Activation by BmFTZ–F1

Feng-Qian LI, Hitoshi UEDA and Susumu HIROSE

Transcriptional activation by many eukaryotic sequence-specific regulators appears to be mediated through transcription factors which do not directly bind to DNA. BmFTZ–F1 is a silkworm counterpart of FTZ–F1, a sequence-specific activator of the *fushi tarazu* gene in *Drosophila melanogaster*. We report here the isolation of 18- and 22-kDa polypeptides termed MBF1 and MBF2, respectively, that form a heterodimer and mediate activation by BmFTZ–F1 of *in vitro* transcription from the *fushi tarazu* promoter. Neither MBF1, MBF2, nor a combination of them binds to DNA. MBF1 interacts with BmFTZ–F1 and stabilizes the BmFTZ–F1-DNA complex. MBF1 also makes direct contact with the TATA-binding protein (TBP). Both MBF1 and MBF2 are needed to form a complex between BmFTZ–F1 and TBP. We propose a model in which MBF1 and MBF2 form a bridge between BmFTZ–F1 and TBP and mediate transactivation by stabilizing the protein-DNA interactions. For details, see *Mol. Cell. Biol.* **14**, 3013–3021, 1994.

Sequences of Two cDNAs Encoding Silkworm Homologues of *Drosophila melanogaster squid* Gene

Feng-Qian LI, Guan-Cheng SUN, Hitoshi UEDA and Susumu HIROSE

The *squid* (*sqd*) gene of *Drosophila melanogaster* encodes a few isoforms of

a heterogeneous nuclear (hn) RNA-binding protein. We isolated two types of cDNAs coding for homologues of the Sqd protein from the silkworm *Bombyx mori*. The two predicted amino acid sequences are identical up to amino acid 280 and then diverge. The silkworm and fruit fly proteins share 80% homology in the RNA-binding motif region. These cDNAs detect 2.0-, 1.8- and 1-kb mRNAs in the middle and posterior silk glands. These transcripts were most abundant at the molting stage. This may respond to a dramatic change in RNA species synthesized in the silk glands during the molting stage. For details, see *Gene* **154**, 295–296, 1995.

The *Drosophila* Nuclear Receptors FTZ–F1α and FTZ–F1β Compete as Monomers for Binding to a Site in the *fushi tarazu* Gene

Carolyn K. OHNO*, Hitoshi UEDA and Martin PETKOVICH*

The striped pattern of *fushi tarazu* (*ftz*) expression found in the blastoderm of the *Drosophila melanogaster* embryo is generated largely through complex interactions between multiple transcription factors that bind to the zebra element of the *ftz* gene. A motif in the zebra element, the FTZ–F1 recognition element (F1RE), has been shown to bind a transcription factor, FTZ–F1, which is a member of the nuclear receptor family. A second, related member of this family, FTZ–F1β, which also binds to this motif has been identified recently. To investigate the possibility that FTZ–F1α and FTZ–F1β coregulate *ftz* transcription through the F1RE, we have studied the DNA binding properties of FTZ–F1α and FTZ–F1β. We found that recombinant FTZ–F1α and FTZ–F1β proteins produce similar *in vitro* DNase I footprint patterns on a 14-nucleotide region of the zebra element and bind to this site with similar affinities and sequence specificities. Using wild-type and N-terminally truncated receptors, we have determined that FTZ–F1α and FTZ–F1β both bind as monomers to the 9-bp F1RE in the zebra element, as well as to an imperfect inverted F1RE repeat present in the *Drosophila* alcohol dehydrogenase gene. A polyclonal antibody raised against FTZ–F1β identifies a predominant F1RE-binding component in embryonic nuclear extracts. Although FTZ–F1α is also present in these extracts, FTZ–F1α and FTZ–F1β do not appear to form heterodimers with each other.

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Cotransfection assays in mammalian cell culture indicate that both receptors contribute to the net transcriptional activity of a reporter gene through their direct interaction with the F1RE. These data suggest that FTZ–F1α and FTZ–F1β likely coregulate common target genes through competition for binding to a 9-bp recognition element. For details, see *Mol. Cell. Biol. 14*, 3166–3175, 1994.

The *Bombyx* silkworm Is a Semelparous Insect with a Long Life in the Most-reproductive Period

Akio MURAKAMI

There is a widely accepted view that semelparous organisms are quick aging and have a short lifespan in the post-reproductive term as seen in ocean salmon, trout and so on. The silkworm, *Bombyx mori* (L.), is a semelparous insect. It is debatable whether aging in the post-reproductive term for *Bombyx* follows the same pattern as in other semelparous animals. In *Bombyx* after a 2 week embryo period, newly hatched larvae start feeding on mulberry leaves. This period lasts about 3 weeks. However, female moths lay offspring within half a day after eclosion. Accordingly, the entire post-embryonic life of this insect, from egg hatching to moth eclosion, is calculated to be about 40 days in addition to several days of an adult period at the standard breeding temperature of 23–25°C. But the popular view of the adult lifespan of *Bombyx* has long been accepted as several days. Results of our observations indicate that the adult lifespan differs between the sexes: female moths generally live 1.5–1.9 times longer than males: freshly eclosed moths live *ca.* 5–6 days in the case of males and *ca.* 9–10 days in the case of females. It is worthwhile to note that some individuals, have had lifespan up to *ca.* 23 days in the post-reproductive period, regardless of sex. Accordingly, the lifespan of *Bombyx* is markedly longer than what is conventional view accepted: lasting about 45 days in males for the post-embryonic period and about 50 days in females.

In this insect species, eclosion of moths of both sexes takes place at sunrise and is completed by 8–9 a.m. at temperatures of 23–25°C throughout the year. Thirty minutes after eclosion newly emerged moths begin to mate and they continue copulation for 1–2 hrs. The separated female moth begins to lay eggs around sundown and continues for half the night. During this short

term, female moths deposit almost all the eggs from their bodies. Accordingly, it can be said that the reproductive term of *Bombyx* silkworms is less than half a day. The moth that develops seems useful only for reproductive purposes. The female moth that has completed her duty of laying offspring lives for a fairly long post-reproductive period. Generally, the male moth lives a markedly shorter period than the female. In any case, it is clear that *Bombyx* silkworms do no have a quick-aging or early death characteristic in the post-reproductive period. Their situation seems to be analogous to iteroparous animals including human beings.

Non-mated female moths in *Bombyx* slowly deposit unfertilized eggs in accordance with a clear-cut circadian rhythm for several days: they usually lay eggs from sundown until sunrise of the next day, but aged moths lose such the rhythm. It is of interest to note that there is no significant difference in the adult lifespan of mated moths from unmated ones so far as we have observed, suggesting that insemination stimulates only egg-laying. The behavior of egg-laying of a wild type silkworm, *B. mandarina* (M.), is to some extent different from the domesticated silkworm, *B. mori*. *B. mandarina*, a putative ancestor of *B. mori*, is a semelparous insect too, but has a peculiar habit of laying eggs on branches of the mulberry tree, bit by bit, extending over several days.

Thus, it can be inferred from the present observations and others that in *B. mori* slow aging (and a short lifespan) in the post-reproductive phase is a reflection of a genetic trait preserved in *mandarina* silkworms and semelparous animals can be grouped, at least, into two types with either a short or long lifespan in the post-reproductive term. Briefly, it appears there is no positive relationships between the lifespan of the post-reproductive phase and the number of reproductions that occur in the life of a semelparous organism.

Homoeostasis in the Process of Growth and Senility in the *Bombyx* Silkworm: the Relationship between Larval Growth Period and Temperature

Akio MURAKAMI

The domesticated mulberry silkworm, *Bombyx mori* (L.), has widely been bred throughout the Northern Hemisphere from the subtropics to the temperate zones and is a holometabolous insect passing through four different

stages: embryo (or egg), larva, pupa, and adult. Larvae principally consume mulberry leaves as a source of energy and during the other three stages use is made preserved nutrients: embryos use vitellins, but both pupae and moths utilize some nutrients remanufactured from several worn out tissues and organs from each of the previous developmental forms as well as preserved nutriments in adipose tissues. *Bombyx* larvae have a fairly narrow range of permissible breeding temperatures in contrast to the other three life forms: larvae are usually bred within a temperature ranging from 20 to 25°C in the temperate zones including Japan, while it is cultured in some measure in high temperatures in the subtropics. In general, the entire larval stage and first half of embryogenesis (prior to the completion of organogenesis), are affected negatively by low (below 15°C) and high (above 35°C) temperatures. On the other hand, the pupal, adult, and certain developed embryonic stages are fairly resistant to a wide range of temperatures from near the freezing point to around 35°C, except for certain developmental steps. This stage differential temperature sensitivity appears to be dependent upon the type of nutrients being used in each developmental stage. As a matter of course, embryos in organogenesis and pupae in the first half of metamorphosis as well as the entire larval stage are sensitive to extremely high and low temperatures above and below 19–25°C. Practically, this insect is bred around 20–25°C in the temperate zone, while in the tropics to some measure in temperatures higher than the standard rearing temperature of the temperate zones (but not exceeding 30°C).

In the *Bombyx* silkworm, a number of breeding records on the larval rearing period used with many tetramoulter stocks at various temperatures have accumulated. Using data from Morohoshi (1972), an analysis was carried out to obtain information on the relationship between larval growth terms and breeding temperatures at 19 and 24°C. As a result, it was found that there is no marked difference in the duration of the larval period between the two breeding temperatures: it takes *ca.* 700 hrs at the two temperatures on average among five pure stocks regardless of sex. However, for the pupal period a great difference was found between the two temperatures: its took about 300 hrs at 24°C for male pupae and 350 hrs for females, to go through the larval period, but *ca.* 550 hrs at 19°C for both sexes. This suggests that *Bombyx larvae* have the ability to stabilize the process of their growth in contrast to pupae. Such a large stage differential in temperature effect on growth-rate between the larval and pupal stages may be a reflection of

different physiological demands based on the nutrients utilized for each life form.

Homoeostasis in Growth and Senility in the *Bombyx* Silkworm: the Relationship between Adult Lifespan and Body-Weight

Akio MURAKAMI

Although the adult lifespan of *Bombyx* is known to be under the control of a complex genetic system, a peculiar phenomenon in which the duration of the moth stage is inversely proportional to the body-weight of adults (or pupae) has often been seen. As shown in Figure 1, the more body-weight gained above a population mean, the shorter the adult lifespan and covensely, the lower the body-weight, the more extended adult lifespan. The phenomenon is observable with both sexes. In addition, some corpulent individuals, whose weight is in excess of the mean population body-weight have a tendency to be susceptible to several diseases and also to be physically

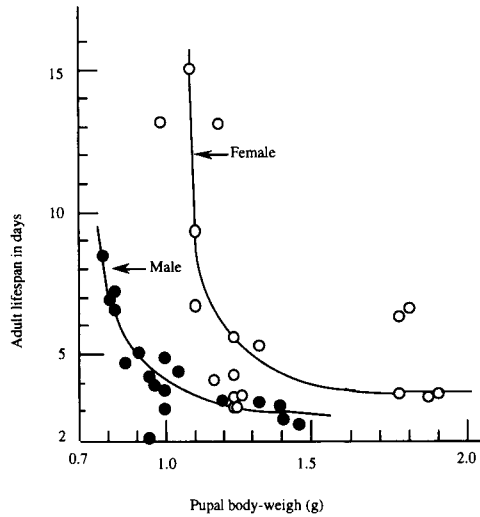

Fig. 1. Relationship between adult lifespan and body weight of a certain F_1 hybrid line in *Bombyx mori* (L). [A stock used is an F_1 hybrid between Kinshu and Showa.]

inactive as adults as well as pupae. Observations showed that within the same genetic population, the lifespan of adults is to some extent affected by the amount of food injested at the later larval phase, too. From the result of the present investigations, it is clear that lifespan of *Bombyx* adults depends on their body-weight, which goes up a larval feeding activity, and adults which have the average body-weight show their mean population lifespan for each stock. The average body-weight as well as lifespan of adults is appeared to be a reflection of physiologically more stable state for each genetic stock own. It can be said that this homeostatic state is peculiarly under the control of an inherently fixed genomic framework to each stock.

Growth Phenomena in *Bombyx mori* (L.) with a Special Reference to Genetic Factors Responsible for Growth Acceleration and Molutinism

Akio MURAKAMI

The tropical race has a certain growth accelerating factor as a novel biological trait, besides various peculiarities of multivoltinism, robustness (highly resistant to either high temperature or high humidity and some insect diseases, *etc.*) and a unique feel to the silk produced from this silkworm race.

The growth accelerating trait is recessive and seems to be comprised of the precocity compound gene loci located on the X chromosome of Cambodge stock. Each member is specially responsible for the growth acceleration for each developmental stage, in addition to the wide range over the growth phase. Considering such a genetic feature of the compound loci, it can be said that the growth phenomena in this insect species is under the control of the Mendelian genetic system rather than the so-called polygenic system. Phenotypic expression of the compound loci is independent of changes of photoperiod, light, temperature and other environmental variables unlike the moultinistic gene system.

For details, see Murakami (1994), *Indian J. Sericulture* **33**, 12–14.

Climatic Differential Phenotypic Expression of Voltine Genes in *Bombyx mori* L.

G. SUBRAMANYA* and A. MURAKAMI

Voltinism was considered to be under the control of autosomes and sex chromosomes in addition to autosomal modifiers. Earlier reports emphasized this phenomenon as a maternal trait. A detailed investigation on the genetic mechanism of voltinism resulted in the identification of a "*npnd*" stock, the genes for which are located on the 'X' chromosomes. As a result, it is now understood that a certain voltinism is a maternally inherited biological event under the control of sex linked genes. The photoperiod plays an important role in this phenomenon. This knowledge of voltinism and 'X' chromosomes is essential in silkworm breeding when studying combining ability or when evolving robust races. Polygenic involvement in silkworm breeding is questionable since it has been shown that 'X' chromosomes carry specific genes for various life history characters of *Bombyx mori* L. This new insight into the biology of voltinism of tropical races may open up new avenues for studying neurobiology, memory, learning, adaptation and evolution.

For details, see Subramanya and Murakami (1994), *Indian J. Sericult.* **33**, 103–109.

Isolation and Characterization of Enhancer Trap Lines expressed in Ovary in *Drosophila melanogaster*

Masa-Aki YAMADA

In *Drosophila*, development of the oocyte is controlled by various genes during oogenesis. In order to understand the mechanism of oogenesis, isolation of P-element mediated enhancer trap lines was carried out using histochemical detection of the β-galactosidase activity in the ovaries of transformants.

Of the 260 transformants carrying P[*lw*B] on the X chromosome, 26 lines expressed β-galactosidase specifically in the cells of the ovary. In 6 lines, the ooplasm and germarium were labeled and four lines were expressed within the germarium and all subsequent stages of the oogenesis. For three lines β-galactosidase activity was detected during stage 8–10 in nurse cell nuclei

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and ooplasm. One line was labeled in the germarium, nurse cell nuclei and ooplasm. In three lines, β-galactosidase activity was detected throughout the ovary and four lines were labeled in the columnar follicle cells and the border cells.

These enhancer trap lines capable of being stained in specific cells and at specific stages of the oogenesis provide useful markers for the recognition of cell types in ovaries. Further analyses on these lines are in progress.

The Retinoblastoma Binding Factor 1 (RBF–1) Site in *RB* Gene Promoter Binds Preferentially E4TF1, a Member of the Ets Transcription Factors Family

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The tumor suppresser retinoblastoma gene product, pRB, is a well known regulator of G1/S cell cycle progression. Moreover, mutational inactivations within the retinoblastoma gene (*RB*) are found in many human malignant tumors, and thus, believed to be an essential step in tumor formation. The human *RB* gene is considered a housekeeping gene with no characteristic TATA or CAAT elements in its promoter region, but the sequence between 206 and 185 bases upstream of the initiation codon, essential for *RB* promoter activity, contains putative Sp1 and ATF recognition sites. We previously reported that point mutations in this region, causing low penetrance retinoblastomas, completely reduced *RB* promoter activity, and that a nuclear factor, named RBF–1 (retinoblastoma binding factor 1), could specifically bind to this sequence, overlapping the SP1 recognition sequence. We show here, that RBF–1 can recognize a specific DNA sequence, 5′-GGCGGAAGT-3′, overlapping the Sp1 and ATF sites and corresponding to the consensus DNA binding site for members of the Ets transcription factors family. When the RBF–1 site was used for sequence specific DNA affinity purification from erythroleukemia cells, reconstitution assays, immunoblotting analysis and peptide mapping showed that the two major co-purified proteins are

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identical to human E4TF1–60 and –53 proteins. This reveals that E4TF1 can bind to the RBF–1 site of the *RB* gene promoter, which thus, constitutes a new target member of the Ets transcription factors family. For details, see *Oncogene.* **9**, 1839–1846, 1994.

Transcriptional Activation through the Tetrameric Complex Formation of E4TF1 Subunits

Jun-ichi SAWADA, Masahide GOTO, Chika SAWA, Hajime WATANABE and Hiroshi HANDA

Transcription factor E4FT1 is involved in the regulation of the adenovirus E4 gene. The factor is composed of two types of subunits, an ets-related DNA binding protein, E4TF1–60, and its associated proteins with four tandemly repeated Notch-ankyrin motifs, E4TF1–53 and E4TF1–47. To determine the functional domains, we constructed various mutants of the subunits. E4TF1–60 bound to DNA as a monomer. The ets domain and its N-terminal flanking region were necessary to recognize the specific DNA sequence. The 48 amino acids at the E4TF1–60 C-terminus were required for interaction with the other type of subunit. E4TF1–53 and E4TF1–47 share the N-terminal 332 amino acids but differ at the C-termini. They interacted with E4TF1–60 through the N-terminal flanking region to form a heterodimer. E4TF1–53 dimerized with itself, whereas E4TF1–47 did not. The C-terminal region specific for E4TF1–53 was required for the dimerization Therefore, heterodimers composed of E4TF1–53 and E4TF1–60 were further dimerized, resulting in the formation of a tetrameric complex, which stimulated transcription *in vitro.* Heterodimers of E4TF1–47 and E4TF1–60 weakly stimulated transcription *in vitro.* The results indicated that the tetrameric complex formation of E4TF1 subunits was necessary to activate transcription efficiently *in vitro.* For datails, see *EMBO J.*, **13**, 1396–1402, 1994.

Formation of Drosophila Wing and Haltare Imaginal Disc Depends on Two Related Zinc Finger Proteins Snail and Escargot

Naoyuki FUSE and Shigeo HAYASHI

Drosophila escargot (*esg*) and *snail* (*sna*) genes encode closely related C2-

H2 type zinc finger transcription factors. *esg* is expressed in all imaginal discs and histoblasts during embryogenesis and in the larva. In early embryo, *sna* is transiently expressed in mesodermal primordia and in the nervous system. During mid to late embryogenesis, expression of these two genes overlap in wing, haltare and genital imaginal discs. These imaginal discs do not develop in embryos deficient for both *sna* and *esg* activity. The presence of one copy of either one of the genes was sufficient for the formation of these discs. Furthermore, the ubiquitous expression of *esg* from a heat shock promoter partially rescued the mutant phenotype. In the rescued embryo, partial wing or haltare discs were observed in the position where they are normally formed, suggesting that *esg* and *sna* expression alone is not sufficient to induce wing and haltare discs in the ectopic position. These results suggest that local activation of *esg* and/or *sna* triggers differentiation of cells which were already made competent through the action of segmentation and homeotic genes.

Control of *esg* Expression

Yoshimasa YAGI* and Shigeo HAYASHI

esg is expressed in a spatially and temporally complex pattern during embryogenesis. Its expression in the imaginal cell primordia is thought to be responsible for the initial formation of some imaginal discs and for maintenance of diploidy. Expression in embryonic gonads and testes at later stages has a relevance for *esg's* role in spermatogenesis. In addition, *esg* is expressed in a subset of embryonic tracheal cells which play an important role in tracheal morphogenesis. To elucidate the role of *esg* in those tissues, and its relation to other known regulatory genes, we have been mapping cis-control elements in the upstream region of the *esg* gene. So far we have mapped the testis specific enhancer within a 1 kb fragment in the vicinity of the *esg* promoter. This fragment can direct expression on heterologous genes in the testis hub cell. Control elements for the nervous system, gut imaginal cells and tracheal tip cells are scattered within a region of more than 20 kb upstream from the esg promoter. These elements are candidates for the transcriptional target of regulatory genes such as, *wigless* and *dpp* which are involved in the morphogenesis of many tissues expressing *esg*.

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Use of Green Fluorescent Protein As a Live Cell Marker and Its Application to the Study of Tracheal Morphogenesis

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The morphogenesis of embryonic trachea involves initial determination of primordium from ectoderm, invagination, extension and final joining of precursors from each hemisegment to form a tubular network. During the final joining process, a set of marker genes are expressed in a single cell at the tip of each growing tracheal branch. These cells contact each other and joining occurs. *esg* is expressed in these tracheal tip cells and is required for fusion to occur. To elucidate the role of the tip cell, we are using two approaches. i) Investigate the role of *esg* in the tip cell. We are currently studying phenotypes of *esg* mutant tip cells using various markers. ii) Direct ablation of the tip cell and real time observation may be an effective way to access the role of the tip cell. We are using green fluorescent protein (GFP) to visualize tracheal cells in live embryo. A fusion gene, (nuc-GFP-lacZ) with the GFP coding sequence linked to the nuclear localization signal and bacterial β-galactosidase gene was expressed in embryo and in larva. The green fluorescence of nuc-GFP-lacZ was detected in both fixed and live embryo. This marker gene should allow us to follow the sequence of tracheal migration and to label a subset of tracheal cells for ablation experiments.

Developmental Mechanisms and Manipulation of Germ Cells in Mouse Embryos

Norio NAKATSUJI and Yasuaki SHIRAYOSHI

The postimplantation period of mammalian embryogenesis includes many important events, such as formation of the primitive streak, central nervous system (CNS) and primordial germ cells (PGCs). PGCs migrate to fetal gonads, become the gonia cells, and take different courses of differentiation in male and female embryos. We are studying the cellular and molecular mechanisms of such developmental processes in germ cells of mouse embryos.

There are several growth factors, such as the stem cell factor and leukemia inhibitory factor, which are known to affect proliferation of PGCs in culture. We found that another factor, tumor necrosis factor (TNF) -α, stimulates

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proliferation of PGCs at early migration stages when added to the culture medium (Kawase, E. *et al.* (1994) *Devel. Biol.* **161**, 91–95). For the PGCs at later stages, after arrival at fetal gonads, we found that retinoic acid acts as a potent mitogen (Koshimizu, U. *et al.* (1985) *Devel. Biol.* **168**, 683–685).

We are currently studying the differentiation of PGCs after they have settled in gonads. After having developed culture conditions to allow survival and differentiation of PGCs at these stages, we are now analyzing molecular and cellular changes of germ cells and their interactions with gonadal somatic cells. Our aim is to develop experimental methods to study developmental processes including meiosis and gamete formation in culture, and to enable the manipulation of germ cells, such as gene transfection, at various developmental stages to facilitate the molecular analysis of germ cell differentiation.

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, to clone 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 have constructed a cDNA library from 6.5–8.5 days 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 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 by ectopic expression or by altering the gene through the gene targeting method using ES cells and homologous recombination. Various ES cell lines we obtained (Kawase *et al.* (1994) int. *J. Devel. Biol.* **38**, 385–390) may be useful for such studies. 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.

Cell Differentiation and Morphogenesis of the Mouse Central Nervous System

Norio NAKATSUJI and Yasuaki SHIRAYOSHI

In collaboration with another laboratory (Dr. I. Nagata at Tokyo Metropolitan Institute for Neuroscience), 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 (Nagata, I. and Nakatsuji, N. (1994) *Devel. Growth Differ.* **36**, 19–27), and the ultrastructure of the neuroblasts showing perpendicular contact guidance (Ono, K. *et al.* (1994) *Devel. Growth Differ.* **36**, 29–38). We are now analyzing the role of perpendicular contact guidance in the actual morphogenesis of brain cortices using various histological and morphological methods.

We are also studying the cellular and molecular mechanisms of cell differentiation in CNS development. We have established several cell lines from early CNS neuroepithelium dissected from embryos of a transgenic mouse strain harboring a temperature-sensitive immortalizing gene. We are currently studying the differentiation potency of these cells *in vitro* and also *in vivo* after injecting them into embryonic brains.

Fluoride-resistant Mutants of the Nematode *Caenorhabditis elegans*

Minoru KAWAKAMI, Takeshi ISHIHARA, Misako URASAKI
and Isao KATSURA

Sodium fluoride (NaF) may interfere with a signal transduction system, because it depletes Ca^{++}, inhibits phosphatases, and activates trimeric G-proteins. We are trying to elucidate such a system through the isolation and characterization of *C. elegans* mutants resistant to NaF.

We have isolated 13 recessive fluoride-resistant mutants that map in 5 new genes, *flr-1 X*, *flr-2 V*, *flr-3 IV*, *flr-4 X* and *flr-5 V*. They are grouped into two categories, class 1 and class 2. Class 1 mutants (*flr-1*, *flr-3*, and *flr-4*) are resistant to 10 mM NaF, but they grow slowly and have a small brood size even in the absence of NaF. In contrast, class 2 mutants (*flr-2* and *flr-5*) are not completely resistant to 10 mM NaF, and they are almost normal in growth rate and brood size in the absence of NaF. Interestingly, class 2 mutations suppress the slow growth and small brood size but not strong fluoride-resistance of class 1 mutations. (Katsura, I. *et al.* (1994) *Genetics* **136**, 145–154)

To understand the biochemical reactions in fluoride-sensitivity we cloned *flr-1* and *flr-3* genes from transposon-insertion mutants, using the transposon Tc1 as the probe. Then we cloned cDNAs of these genes from a *C. elegans* cDNA library, using fragments of the genomic clones as the probes. So far only a partial-length cDNA of *flr-1* has been obtained. The predicted amino acid sequence shows weak homology to the MEC-4 and DEG-1 ion channels of *C. elegans* and amiloride-sensitive ion channels of mammals. Since the similarity is relatively high at the functionally important membrane-spanning region, we think *flr-1* also codes for an ion channel. For *flr-3* we obtained a full-length cDNA, which has the same length (2 kb) as the mRNA as determined by Northern analysis. The predicted FLR-3 protein consists of 525 (or 589) amino acid residues. It contains the subdomains II to XI of the protein kinase consensus sequences (Hanks, S. K. *et al.* (1988) *Science* **241**, 42–52) but lacks the consensus sequence of subdomain I, the ATP-binding site. Such examples have been found among protein kinases and kinase-like proteins.

During the course of the study it has become clear that the *flr* mutations affect neuronal functions. They cause worms to grow to dauer larvae, if the worms also have a certain neuronal mutation such as *unc-3*, *unc-31* or *osm-*

1 (See section 2b below). Furthermore, J. H. Thomas (University of Washington) and E. Jorgensen (University of Utah) found that the class 1 *flr* mutants have shorter defecation periods than wild type worms, which show a defecation every 45 seconds. We found also that some, if not all, *flr* mutants are abnormal in chemotaxis. We are trying to elucidate the function of the *flr* gene products by studying the time and site of gene expression as well as interactions between the gene products.

Caenorhabditis elegans Larval Lethal Mutations That Cause Detachment of the Outer Surface of the Intestine from the Inner Surface of the Body Wall

Ryuichi HISHIDA, Takeshi ISHIHARA and Isao KATSURA

We have been analyzing mutants in which the outer surface of the intestine is detached from the inner surface of the body wall and which die as larvae (Katsura, I. (1993) *Genetica* **88**, 137–146). We call them *clr-1*-like mutants, since the phenotype resembles that of *clr-1* among known mutations. Ten such mutants were isolated and mapped either to known genes that act in signal transduction (*let-23*, *let-341*, *lag-2* and *clr-1*) or to unknown genes. We think the latter genes may also act in signal transduction or in cellular functions regulated by signal transduction.

We cloned one of the unknown genes, *let(ut40, ut102)*, by the transposon-tagging method. Using a genomic fragment as the probe, we screened a *C. elegans* cDNA library and found three types of clones overlapping each other but differing in the positions of the 5′ and 3′ ends. The difference in the 3′ ends is probably caused by two closely located poly-A addition sites. The difference in the 5′ ends may be due to multiple promoters, or it may result from trans-splicing, since the shorter cDNA has the SL1 splice-leader sequence. The predicted amino acid sequence of *let(ut40, ut102)* has no clear homology to any known protein. To obtain further information we are determining the sites of gene expression in wild type worms and in signal transduction mutants.

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 are defective both in hatching and in migration of a neuroblast (Hedgecock, E. M. *et al.* (1987) *Development* **100**, 365–382). They cannot digest protein components of the eggshell, and a neuroblast called QL cell moves anteriorly instead of posteriorly during larval development. Since we isolated a transposon-insertion mutant in *hch-1* (unpublished results by K. Kondo (Soka University) and I.K.), we have cloned the gene by the transposon-tagging method and the cDNA by using the genomic fragment as the probe. The cloned cDNA has the same length (3 kb) as the mRNA as detected by Northern analysis. The predicted amino acid sequence contains a consensus of metal proteinases. It is intriguing to know the relation between this enzyme and the hatching enzymes of fish and sea urchin.

Construction of GFP Markers for Various Nerve Cells in *C. elegans* and Their Use for Mutant Isolation

Takeshi ISHIHARA, Manabi FUJIWARA and Isao KATSURA

To analyze formation and function of the neural circuit in *C. elegans*, we made 8 types of worms in which specific sets of neurons emit fluorescence. They were prepared by integration (into the worm genome) of the DNA constructs, in which the cDNA of GFP (jellyfish green fluorescent protein; Chalfie, M. *et al.* (1994) *Science* **263**, 802–805) was fused with various *C. elegans* neural promoters. The promoters were selected by the "promoter trapping" method (Hope I. (1991) *Development* **113**, 399–408) or by the DNA sequence as determined by the *C. elegans* genome project. The following are the neurons that express GFP (and results of a homology search of the genes that seem to be controlled *in vivo* by the promoters) for the 8 types of worms: (1) all or almost all neurons (no homology), (2) a pair of head neurons called AFD cells (no homology), (3) G2 cell and its descendants (no homology), (4) all or almost all neurons (*unc-31* gene itself), (5) 8 neurons in the head and PVC cells in the tail (AMPA-type glutamate receptor), (6) CEP, ADE and PDE neurons (dopamine transporter), (7) several head neurons including ASJ, ASI and URX as well as the

PQR neuron in the tail (cyclic nucleotide-gated channel), and (8) a small number of head and tail neurons including ASG, DVA, PDA and ALN as well as the excretory cell (*prospero* homolog). We confirmed that at least for the promoter (1), fluorescence is localized to cell nuclei and to processes and cell bodies, if the SV40 nuclear localization signal and a *C. elegans unc-76* cDNA fragment, respectively, are inserted into the DNA construct.

We plan to use these worms to isolate mutants with structural abnormalities in the nervous system. As a first step we mutagenized the worms (1) containing SV40 NLS using EMS (ethyl methanesulfonate) and screened 1000 independent F1 worms for those producing mutants abnormal in the number and/or location of neuronal nuclei in the tail. Mutants having different numbers of neurons in the tail were found and are being analyzed.

Analysis of the Head Neural System 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 limited food supply, they deviate from the normal life cycle and develop to enduring, non-feeding larvae called dauer larvae or dauers. The developmental decision to become dauers seems to be regulated by the head neural circuit, with the smell of bacteria and the pheromone as 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 those of chemotaxis, we are analyzing the head neural circuit by detecting dauer formation as the output.

To identify genes required for the neural circuit, we looked for combinations of known mutations that cause worms to grow to dauers irrespective of the environment, only if two of them are present in the worm genome as homozygous double mutations. We found more than 40 known mutations have such a synthetic dauer-constitutive phenotype. They include the following: (1) most sensory mutations that cause structural abnormalities in amphids (*che-2*, *che-3*, *che-11*, *che-13*, *daf-10*, *osm-1*, *osm-3*, *osm-5*, *osm-6*, *che-10*, *che-14*, *daf-6*, *mec-1*), (2) most chemotactic mutations that do not seem to affect amphid structures (*che-1*, *che-6*, *che-7*, *tax-2*, *tax-4*, *tax-6*, *odr-1*, *odr-2*, *odr-4*, *odr-5*), (3) some mutations giving rise to abnormality in

movement (*unc-3*, *unc-31*, *unc-64*, *unc-101*, *unc-104*), (4) a few mutations in the neuronal cell lineage (*lin-32*, *vab-3*), (4) some egg-laying mutations (*egl-4*, *egl-32*), (5) a mutation resulting in abnormality in defecation (*aex-3*), (6) fluoride-resistant mutations (*flr-1*, *flr-2*, *flr-3*, *flr-4*, *flr-5*), (7) some mutations affecting body-size (*sma-2*, *sma-4*), (8) a mutation causing decrease in the amount of serotonin and dopamine (*cat-1*). Since *cat-1* is included in the list, we tested dauer formation of some single mutants in the presence of various neurotransmitters, agonists and antagonists. Of 11 drugs that we tested, ketanserin, a serotonin antagonist, produced a dauer-constitutive phenotype in mutants of *unc-3*, *unc-31*, *osm-1* and *egl-4* but not in wild type worms.

In this study it become clear that many genes affect dauer formation in combination but not by themselves. The reason is probably that there are parallel pathways for the signals regulating dauer-formation. The pattern of combinations of mutations that have the dauer-constitutive phenotype show that there are more than three parallel pathways. Another peculiar aspect is that many mutations listed under (1) have a dauer-defective rather than dauer-constitutive phenotype by themselves. This means that one of the signals in the parallel pathway is unaffected by sensory inputs and always inhibits dauer formation. We are especially interested in the nature of this signal, which requires the function of *unc-3*, *unc-31*, *unc-64* and *unc-104* genes.

To look for new genes required for one of the parallel pathways and possibly for the formation or function of the neural system, we isolated 44 new mutations that have the dauer-constitutive phenotype in combination with *unc-31* mutation but not by themselves. Although we have mapped only about half of them, some of them are located evidently in new genes. We also tested all but two of the mutants for structural abnormality in amphid neurons by the dye-filling method (Hedgecock, E. M. *et al.* (1985) *Dev. Biol.* **111**, 158–170). Six of them were clearly abnormal, another six were weakly abnormal, and 30 seemed to be normal in this respect.

The *C. Elegans* cDNA Project: Towards an Expression Map of the Genome

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

Aiming to ultimately understand the network of gene expression in development of the worm, we are constructing an expression map of the 100 Mb genome by identifying and characterizing all of its cDNA species, whose number is estimated to be around 15,000. 36,000 cDNA clones were picked up randomly from 3 different cDNA libraries (from size-fractionated (>2 Kb) and unfractionated cDNA from a mixed-stage population, and from embryos) and were subjected to (1) tag-sequencing from both 5′- and 3′-ends, (2) mapping onto the genome, and (3) analysis of expression patterns. After analysis of every some 4,000 clones, cDNA species which were represented by more than 4 clones in the previous analysis were removed to minimize redundancy in analyses.

(1) Tag sequencing: The 3′-tag sequences were compared by FASTA to classify the cDNA clones into unique groups (genes). Thus far, 8,351 clean 3′-tag sequences have been obtained, and these were classified into 3,518 unique cDNA groups (assigned CELK00001 through CELK03521). Also detected were many pairs of clones which appeared to be generated by alternative splicing or differential poly-A addition. In a BLASTX search of the 5′-tags, 36% of the cDNA groups showed significant similarities (blastx score >100).

(2) Mapping onto the genome: Using YAC polytene filters, 1,416 cDNA groups were mapped onto the genome. Mapping *in silico* using the cosmid sequences available at the Sanger Centre was also effective.

(3) Analysis of expression patterns: We have developed a method of *in situ* hybridization on whole mount embryos of the multi-well format, and we are applying the method to the set of representative clones of the identified cDNA groups.

In Situ Hybridization of the Multi-Well Format on Whole Mount Embryos of *C. Elegans*

Hiroaki TABARA, Tomoko MOTOHASHI and Yuji KOHARA

In order to perform a large scale screening of the expression patterns of

worm genes during embryogenesis, we have adapted our method of *in situ* hybridization on whole mount embryos to the standard 96-well format using 96-well dot-blot apparatuses. The results of model experiments using stage-specific gene probes (*unc-54*, *myo-2*, *glp-1*, *clb-2*, pepsinogen gene, etc.) verified the effectiveness of this procedure. Currently, 32 different probes can be assayed on one apparatus at one time. We are applying the procedure to classified cDNA groups produced by the cDNA project in this lab. Thus far, an approximate estimation is that 1/20 of the cDNA groups shows specific patterns of expression during embryogenesis.

In the course of the in situ screening, we found that a cDNA clone, yk61h11, showed asymmetrical distribution of the mRNA in the 2-cell stage embryo. Closer examination showed that the mRNA (1) was detected first at the corner of the gonad, (2) was distributed evenly in oocytes, (3) was segregated posterially during psuedo-cleavage and first cleavage and (4) remained only in P4. This gene, named *pos-1* (posterior), was mapped on LG 5 using a YAC polytene filter. The cDNA sequence showed strong homology to the zinc finger region of the Tis11 family which are known to be transcription factors induced by stimulation of FGF and TPA etc. Another cDNA species having homology with Tis11 was obtained in our cDNA project, but the distribution of the mRNA turned out to be ubiquitous during embryogenesis. We are planning gene disruption experiments on the gene *pos-1*.

Efficient Isolation of Tc1-Insertion Mutants from a Mutant Bank Representing 20,000 Worms of *C. elegans*

Yoshiki ANDACHI and Yuji KOHARA

Aiming for efficient gene disruption, we have devised a system for isolating mutants of Tc1 insertion in desired genes from a bank of frozen mutator worms. The main point of the system is a pooling strategy. Empirically, at least one Tc1 insertion mutant in a given gene is expected in a population of 10^5 or fewer mutator worms. Thus, as a first step, 192 pools of about 100 worms (a total of about 20,000 worms) of a mutator strain, MT3126 (*mut-2*), were fed to F1 progeny and a part of the F1 worms of each pool was stored frozen to make a mutant bank. Genomic DNA were extracted from the remaining part of each pool. Parts of the genomic DNA of every 12 pools

were further pooled to make 16 larger pools. The larger pools were examined for Tc1 insertion in a target gene by PCR, using primers from the target gene and Tc1. When a positive one was detected, its 12 pools were assayed individually by the same PCR to detect a positive pool. Finally, the frozen worms (~200) corresponding to the positive pool were cultured separately and assayed by the PCR to identify a strain having the Tc1 insertion.

Thus far, we have obtained insertion mutants for 9 out of 10 genes for which the bank was screened. Although the insertions in many cases were within introns, deletion mutants have been successfully isolated from some of the insertion mutants by a sib-selection procedure. Therefore, we think that the bank is sufficient for producing disruption of any of most genes of this organism.

V. CELL GENETICS

Fine Analysis of DNA Replication Timing in the Human MHC Region by Fluorescence *in situ* Hybridization: Temporal Order of DNA Replication Closely Correlates with G+C% Gradient of Genome Sequences

Katsuzumi OKUMURA[1], Tatsuo FUKAGAWA[2], and Toshimichi IKEMURA[2]

We have used fluorescence *in situ* hybridization (FISH) on HL60 cells for structural analyses and determination of replication timing patterns of DNA segments over 2 Mb within the human MHC region. This region is composed of long-range G+C% (GC) mosaic structures related to chromosome bands, and a boundary of GC mosaic domains exists between MHC classes II and III. FISH with elongated chromatin fibers from the interphase nuclei of HL 60 cells showed that the flanking genome structures of the boundary are strictly consistent with previously reported mapping data. This confirmed that HL60 cells may not have large structural abnormalities in this region. Multi-color FISH with interphase nuclei of HL60 cells was useful in comparing the temporal replication timings of two DNA fragments which are located in adjacent genomic positions and which replicate at a similar time during the S phase in the cell cycle. Using this procedure it was possible to determine precisely the relative time of replication of each DNA clone which was randomly selected from the MHC region. The clones at the boundary region were found to replicate the latest among all the clones examined. 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 the genome mosaic structure at the level of genome sequences as well as in chromosome bands (manuscript in preparation).

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VI. POPULATION GENETICS

Interaction of Selection and Drift in Molecular Evolution

Tomoko OHTA

The nearly neutral theory proposes that interaction of selection and drift is important in molecular evolution. The theory predicts that evolutionary rate is negatively correlated with the species population size, for those genes whose function has been fixed long time ago. The prediction is tested through analysis of sequence data of 17 mammalian genes by estimating divergence among genes separately for synonymous substitutions and nonsynonymous substitutions. Star phylogenies composed of rodentia, artiodactyla and primates are examined. The generation-time effect is found to be more conspicuous for synonymous substitutions than for nonsynonymous substitutions. This result supports the nearly neutral theory. For details, see *Jpn. J. Genet.* **68**, 529–537.

On Hypervariability at the Reactive Center of Proteolytic Enzymes and Their Inhibitors

Tomoko OHTA

The pattern of synonymous and nonsynonymous substitutions at the reactive center of proteases (kallikrein) and their inhibitors (α_1-antitrypsin and serpin) was examined. In the case of α_1-antitrypsin, the proportion of different nonsynonymous sites exceeds that of different synonymous sites at the reactive center for sequence pairs of recent duplication. The result indicates that the positive selection has operated after duplication to increase functional diversity. In the cases of kallikrein, serpin, and remote sequence pairs of α_1-antitrypsin, the proportion of different synonymous sites at the reactive center exceeds that of different synonymous sites at the remaining region. The result indicates that gene conversion followed by natural selection is working. On the whole, it is concluded that hypervariability of amino acids at the reactive center is generated by an interaction among natural selection, random genetic drift, point mutation, and gene conversion.

Gene duplication may provide potential for them to interact. For details, see *J. Mol. Evol.* **39**, 614–619.

Further Examples of Evolution by Gene Duplication Revealed Through DNA Sequence Comparisons

Tomoko OHTA

To test the theory that evolution by gene duplication occurs as a result of positive Darwinian selection that accompanies the acceleration of mutant substitutions, DNA sequences of recent duplication were analyzed by estimating the numbers of synonymous and nonsynonymous substitutions. For the troponin C family, at the period of differentiation of the fast and slow isoforms, amino acid substitutions were shown to have been accelerated relative to synonymous substitutions. Comparison of the first exon of α-actin genes revealed that amino acid substitutions were accelerated when the smooth muscle, skeletal and cardiac isoforms differentiated. Analysis of members of the heat shock protein 70 gene family of mammals indicates that heat shock responsive genes including duplicated copies are evolving rapidly, contrary to the cognitive genes which have been evolutionarily conservative. For the α_1-antitrypsin reactive center, the acceleration of amino acid substitution has been found for gene pairs of recent duplication. For the details, see *Genetics* **138**, 1331–1337.

Statistical Methods for Estimating the Effective Number of Alleles, Expected Heterozygosity and Genetic Distance in Self-incompatibility Locus

Fumio TAJIMA, Tohru TOKUNAGA* and Naohiko T. MIYASHITA**

In order to understand the evolutionary process of self-incompatibility, we must know the genetic variability of the self-incompatibility genes within and between populations. Statistical methods for estimating the effective number of alleles, expected heterozygosity and genetic distance from pollination experiments were developed, which can be applied to both gametophytic and sporophytic self-incompatibility systems. In these methods, bud-pollination,

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which is necessary for obtaining homozygotes, is not required. Since bud-pollination, which is time-consuming, is not required in the present methods, they might be useful. For details, see *Jpn. J. Genet.* **69**, 287–295, 1994.

Comments on the Detection of Reciprocal Recombination or Gene Conversion

Naoyuki TAKAHATA

Reciprocal recombination and gene conversion (in short, exchanges) have undoubtedly been playing important roles in molecular evolutionary processes. Such exchanges may be revealed by comparing DNA sequences. However, it is not easy to obtain clear-cut evidence for the occurrence of exchanges between orthologous regions of DNA, because alleles at a functional locus generally differ very little within species. Demonstrable instances often involve exchanges between paralogous loci. Several statistical tests for detecting sequence exchanges are available, some of which examine whether or not nucleotide substitutions are randomly distributed along the DNA sequences compared. For instance, Satta (1992) considers two different regions separately, measures their relative sizes p and q ($p+q=1$), and counts the total number of substitutions (M). If both regions had evolved without exchanges, the number of substitutions in each region is expected to follow the binomial distribution with parameters p and M. Other tests rely on phylogenetic trees constructed by the maximum parsimony or maximum likelihood methods. Discordant phylogenetic trees between different regions, if found, are taken as evidence for exchanges. In this communication, I consider the statistical strength of the tests developed by Stephens (1985) and Sawyer (1989) in comparison with that of the Runs Test. All these tests are applied to *Mhc* DNA sequences. For details, see *Immunogenetics* **39**, 146–149, 1994.

The Neutral Theory of Molecular Evolution

Naoyuki TAKAHATA and Motoo KIMURA*

There are a variety of molecular mechanisms that are responsible for changing the genome. However, molecular evolution has not been driven

* deceased.

solely by these mechanisms. Mechanisms acting at the individual and population levels have played an equally important role in determining the process of molecular evolution. Of particular importance are natural selection and random genetic drift. The neutral mutation and random genetic drift theory of molecular evolution, the neutral theory in short, claims that the overwhelming majority of evolutionary changes at the molecular level are caused by random fixation of selectively neutral (i.e., selectively equivalent) mutants. The theory also asserts that most of the protein or DNA variation within species is maintained by the balance between mutational input and random extinction. In this Chapter, we overview molecular evolutionary mechanisms, and describe the neutral theory and its predictions in the light of recent observations. For details, see pp. 205–234 in *Principles of Medical Biology* (E. E. Bittar and N. Bittar eds.), JAI PRESS London 1994.

Structure, Function, and Evolution of Mouse *TL* Genes, Nonclassical Class I Genes of the Major Histocompatibility Complex

Yuichi OBATA, Yoko SATTA, Kazuo MORIWAKI, Toshihiko SHIROISHI, Hitomi HASEGAWA, Toshitada TAKAHASHI and Naoyuki TAKAHATA

In contrast to well-studied "classical" class I genes of the major histocompatibility complex (*MHC*), the biology of nonclassical class I genes remains largely unexamined. The mouse *TL* genes constitute one of the best defined systems among nonclassical class I genes in the *T* region of the *MHC*. To elucidate the function and the evolution of *TL* genes and their relationship to classical class I genes, seven *TL* DNA sequences, including one from a Japanese wild mouse, were examined and compared with those of several mouse and human classical class I genes. The *TL* genes differ from either classical class I genes or pseudogenes in the extent and pattern of nucleotide substitutions. Natural selection appears to have operated so as to preserve the function of *TL*, which might have been acquired in an early stage of its evolution. In a putative peptide-binding region encoded by *TL* genes, the rate of nonsynonymous (amino acid replacing) substitution is considerably lower than that of synonymous substitution. This conservation is completely opposite that in classical class I genes, in which the peptide-binding region has evolved to diversify amino acid sequences so as to recognize a variety of antigens. Thus, it is suggested that the function of *TL* antigens is distinct

from that of classical class I antigens and is related to the recognition of a relatively restricted repertoire of antigens and their presentation to T-cell receptors. For details, see *Proc. Natl. Acad. Sci. USA* **91**, 6589–6593, 1994.

Polymorphism at *Mhc* Loci and Isolation by the Immune System in Vertebrates

Naoyuki TAKAHATA

The major histocompatibility complex (*Mhc*) contains a family of genes whose products play crucial roles in self and nonself discrimination. Self is the mature T cell repertoire that is restricted by Mhc molecules, whereas nonself is processed peptides of foreign antigens that can be presented in the context of Mhc. Because the T cell repertoire and the complex of Mhc-peptides provide molecular basis of self defense mechanism and both of them depend critically on *Mhc* genotypes, the genes must be subjected to natural selection. A model of individual fitness is formulated in terms of the number of functional *Mhc* loci, the number of heterozygous loci, the size of T cell repertoire, and the size of Mhc peptide repertoire. Available data delimit the range of values of these parameters in the model. The mode of natural selection on *Mhc* loci depends primarily on the T cell repertoire shaped by different gene products. If each *Mhc* gene product deletes a substantial portion of T cells, balancing selection cannot operate simultaneously on a large number of loci. Only when the number of functional *Mhc* loci is limited, natural selection acts so as to produce the unusual polymorphism. If individuals are heterozygous at all these loci or possess haplotypes being different in the constellation of *Mhc* loci, their fitness may be reduced. The reduction may happen in hybrids between antigenically diverged populations, in which case the immune system acts as a reproductive isolation mechanism. For details, see pp. 233–246 in *Non-Neutral Evolution* (B. Golding ed.), Chapman & Hall, New York, 1994.

Intensity of Natural Selection at the Major Histocompatibility Complex Loci

Yoko SATTA, Colm O'HUIGIN, Naoyuki TAKAHATA and Jan KLEIN

Long persistence of allelic lineages, prevalence of nonsynonymous over synonymous substitution in the peptide-binding region (PBR), and deviation

from neutrality of the expected gene identity parameter F all indicate indirectly that balancing selection is operating at functional major histocompatibility complex (*MHC*) loci. Direct demonstrations of the existence of balancing selection at *MHC* loci are, however, either lacking or not fully convincing. To define the conditions under which balancing selection could be demonstrated, we estimated its intensity, from the mean number of nonsynonymous substitutions, K_B, at the PBR and the mutation rate μ. We compared the five available methods for estimating K_B by computer simulation and chose the most reliable ones for estimation of selection intensity. For the human *MHC*, the selection coefficients of the *HLA-A*, *-B*, *-C*, *-DRB1*, *-DQB1*, *-DQA1*, and *-DPB1* loci are 0.015, 0.042, 0.0026. 0.019, 0.0085, 0.0028, and 0.0007, respectively. This low selection intensity places severe restrictions on the possibility of measuring selection directly in vertebrate populations. For details, see *Proc. Natl. Acad. Sci. USA* **91**, 7184–7188, 1994.

Repeated Failures That Led to the Eventual Success in Human Evolution

Naoyuki TAKAHATA

The effective population number estimated in humans is about 10,000. This rather small number may contradict the fact that humans had been occupying vast areas in Africa and Eurasia for a long time since *Homo erectus* first migrated out of Africa 1–2 Mya. One possible cause is a recent bottleneck, or a sudden reduction in population size, but a severe bottleneck is incompatible with the unusual polymorphism at the major histocompatibility complex (*Mhc*) loci. In this letter, I argue that a more likely cause is frequent extinction and recolonization (turnover) of subpopulations in the lineage leading to modern humans. For details, see *Mol. Biol. Evol.* **11**, 803–805, 1994.

Population Genetic Studies on the Evolution of SINE II

Hidenori TACHIDA

In eukaryote genomes, there are many dispersed repetitive elements called retroposon which amplify themselves via RNA intermediates. In order to investigate how these amplifications occur, three models of retroposons were

developed and analyzed. In the transposon model, all elements can duplicate themselves. In the master copy model, only one element per haploid genome has the ability of duplication. Intermediate between the two extremes is the third model in which some elements have ability of duplication but others do not have such ability. Variances of consensus gene frequencies in the three models were computed. The variances were shown to be different among the three models if the amplification period is not short. One subfamily of the human Alu family of retroposon was analyzed by computing the variances of the consensus frequencies and it was found that the data is compatible with any of the three model if we assume that the amplification period is short. However, mutations were found to have occurred on duplicative elements in several sites and the probability of obtaining this pattern would be small if the duplication period is short and the number of duplicative elements is small. From these considerations, it was inferred that this subfamily of the Alu element amplified in a short period and there were multiple duplicative elements.

Genetic and Geographical Differentiation of Subspecies Groups of *Mus musculus* in East Asia

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Since 1983, we have surveyed genetic variations among natural populations of house mouse, *Mus musculus*, in vavious areas of China, the area west of Lanzhou, the southern area including Tibet, the central area, the northern area including Mongolia and the northeast area of China. Molecular

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analyses of genetic polymorphisms in mitochondrial DNAs, ribosomal DNAs, SRY DNAs on the Y-chromosome, hemoglobin-beta (Hbb) and others, have revealed the presence of two major subspecies groups of the house mouse in these areas which are the castaneus group and the *musculus* group. The former inhabits the area south of the Yangtze river and the latter inhabits the area north of the river including the northeast, Korean peninsula and Japan. More detailed analyses demonstrated a further differentiation of the subspecies groups. In the *musculus* subspecies group, the western China populations which were classified as the gansuensis subspecies based on morphology could also be characterized by a new Hbb-w1. The central populations, the homourus subspecies, were characterized by Hbb-p and the northern populations including the northeast, Korea and Japan, the *molossinus* subspecies, were separated by their characteristic SRYmo. In the castaneus subspecies group, the southern China population and southeast Asian population could be clearly discriminated by D-loop analysis in mitochondrial DNAs. It should be noted that the northern Japanese populations show the latter type mt-DNA. This is probably related to the histoucal human movement from southeast Asia to Japan.

VII. EVOLUTIONARY GENETICS

Early Evolution of Genes and Genomes

Tomoko OHTA

In the early evolution of gens and genomes, ribozymes and reverse tarnscriptase probably played important roles. In such a system, reverse flow of genetic information may have been more frequent than in the present DNA world. The interaction between natural selection and reverse flow of genetic information might have provided an efficient environmental feedback to genes and genomes. For details, see In *Early Life on Earth* (Bengtson, S. ed.) pp. 135–142, Columbia Univ. Press.

Estimation of Evolutionary Distance for Reconstructing Molecular Phylogenetic Trees

Fumio TAJIMA and Naoko TAKEZAKI*

The most commonly used measure of evolutionary distance in molecular phylogenetics is the number of nucleotide substitutions per site. However, this number is not necessarily most efficient for reconstructing a phylogenetic tree. In order to evaluate the accuracy of evolutionary distance, $D(t)$, for obtaining the correct tree topology, an accuracy index, $A(t)$, was proposed. This index is defined as $D'(t)/\sqrt{V[D(t)]}$, where $D'(t)$ is the first derivative of $D(t)$ with respect to evolutionary time and $V[D(t)]$ is the sampling variance of evolutionary distance. Using $A(t)$, namely, finding the condition under which $A(t)$ gives the maximum value, we can obtain an evolutionary distance that is efficient for obtaining the correct topology. Under the assumption that the transversional changes do not occur as frequently as the transitional changes, we obtained the evolutionary distances which are expected to give the correct topology more often than are the other distances. For details, see *Mol. Biol. Evol.* **11**, 278–286, 1994.

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Human Pseudoautosomal Boundary-Like Sequences (PABLs): Core and Consensus Sequence, Expression, and Involvement in Evolutionary Formation of the Present Day Pseudoautosomal Boundary of Human Sex Chromosomes

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The human genome, like those of warm-blooded vertebrates in general, has long-range mosaic structures of G+C% (GC%), which are thought to be related to chromosome bands. We previously disclosed a boundary for Mbp-level domains of G+C% mosaic structures in the human MHC and found in the domain boundary, a sequence highly homologous with the pseudoautosomal boundary (PAB) of human sex chromosomes. We designated the sequence "PABL" and found many PABL-type sequences in the human genome. Expression of the PABL sequences was also disclosed. In analyzing a total of twelve PABLs (six genomic and six cDNA sequences), a ca. 650 nt of the core and consensus sequence could be defined; a strict conservation of the 3′ and 5′ edges of the PABLs including sex-chromosome PABs was found. Northern blot analysis showed the sizes of PABL transcripts to be 5–10 kb.

The divergence time of PABLs was estimated to be 60–120 million years ago after analyzing five human PABLs and sex-chromosome PABs of seven primates. The evolutionary rates deduced showed PABLs to have been under a selective constraint. A model of evolutionary formation of the present-day pseudoautosomal boundary was proposed by postulating an illegitimate recombination between two PABLs. For details, see *Genomics* **25**, 184–191 (1995) & *Human Molecular Genetics* (in press).

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A Notch-family Gene Found in the Human MHC Class III Region Near the Junction with the Class II: Human Counterpart of Mouse Mammary Tumor Gene *int-3*

Kimihiko SUGAYA[1], Asako ANDO[2], Hidetoshi INOKO[2] and Toshimichi IKEMURA[1]

The genomes of higher vertebrates are composed of long-range mosaic structures of G+C content (GC%), which are related to chromosome bands. During the processes to characterize the border of the Mb-level GC% mosaic domains previously located in the human MHC, we found the human counterpart of the mouse mammary tumor gene *int-3* near the junction of the MHC class III with the class II. Integration of the mouse mammary tumor virus (MMTV) into the *int-3* locus promotes the transcription of the flanking mouse cellular *int-3* sequence which shares significant homology with the intracellular domain of the *Drosophila* neurogenic *Notch* gene. The human gene found in the present work contained not only the intracellular domain part present in the *int-3* sequence but also the extracellular part present in typical Notch-family genes, showing it to correspond to the human counterpart of an uninterrupted form of the transmembrane protein gene predicted at the mouse *int-3* locus. Human cDNA clones of this NOTCH-related gene were isolated and sequenced, clarifying its gene organization. In the 5′ upstream region, there was a trinucleotide-repeat cluster, which shows evident polymorphism among human populations and thus is a useful marker in studying the correlation of this gene with the diseases suspected in the respective region, as well as in searching for other disease-related genes. By constructing phylogenetic trees based on all available sequences of Notch-family genes including the present gene, four subfamilies for mammalian Notch were found (manuscript in preparation).

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Estimation of Protein-Production Levels for ORFs Registered in *E. coli* Genome Projects, Based on Levels of "Optimal Codon" Usage, in Connection with the Feasibility of Protein Coding Ability and with Assignment to Foreign-Type Genes

Yasukazu NAKAMURA[1] and Toshimichi IKEMURA[1]

The choice among synonymous codons is clearly non-random, although it does not affect the nature of proteins synthesized. Among the genes of each unicellular organism, there is a clear similarity of codon choice patterns, regardless of gene function; i.e., the "codon dialect" of the organism. We previously found the extent of codon bias (accent of the codon dialect) is related to the protein production level. Codon usage in genes encoding abundant proteins is always much more dependent on tRNA content (strong accent) than that in moderately or poorly expressed genes (moderate accent). It is also known that foreign-type genes (e.g., transposons, plasmids and virus genes) often have quite different codon patterns from the host dialect. To examine these features quantitatively, the frequency of use of "optimal codons" was previously defined; the "optimal codon" is the codon translated by the most abundant isoacceptor. The cellular content of many *E. coli* proteins was measured by Neidhardt and his colleagues allowing us to study the correlation between frequency of optimal codon use (Fop) and gene expressivity. A definite correlation between Fop and protein content was revealed; Fop for the highly expressed genes was almost always high, but low for weakly expressed genes. The Fop of foreign-type genes was significantly lower than that of intrinsic genes. We calculated the Fop of 603 ORFs registered in *E. coli* genome projects of Japan and USA. Based on the Fop levels, we could classify these ORFs into highly-, moderately- or weakly-expressed genes, as well as into foreign-type genes. Among those registered by the USA group, they often included ORFs that are shorter than those in the Japanese project. The feasibility of individual short ORFs as protein-coding regions was proposed. For details, see Genome Informatics Workshop Series No. 5, pp. 188–189 (1994) & *Nucleic Acids Research* (in press).

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Evolutionary Rate of Insertions and Deletions in Non-Coding Nucleotide Sequences of Primates

N. SAITOU and S. UEDA*

Insertions and deletions are responsible for gaps in aligned nucleotide sequences, but they have been usually ignored when the number of nucleotide substitutions was estimated. We compared six sets of nuclear and mitochondrial non-coding DNA sequences of primates, and obtained the estimates of the evolutionary rate of insertions and deletions. The maximum parsimony principle was applied to locate insertions and deletions on a given phylogenetic tree. Deletions were about twice as frequent as insertions for nuclear DNA, and single nucleotide insertions/deletions were the most frequent in all events. The rate of insertions/deletions was found to be rather constant among branches of the phylogenetic tree, and the rate (ca. 2.0 per kb per million years) for mitochondrial DNA was found to be much higher than that (ca. 0.2 per kb per million years) for nuclear DNA. The rates of nucleotide substitutions were about ten times higher than those of insertions/deletions both for nuclear and mitochondrial DNAs. For details, see *Mol. Biol. Evol.* **11**, 504–512 (Saitou and Ueda, 1994).

Genetic Analysis and Molecular Phylogeny of Simian T-cell Lymphotropic Virus Type I: Evidence for Independent Virus Evolution in Asia and Africa

K.-J. SONG**, N. SAITOU and R. YANAGIHARA**

Type C retroviruses, designated simian T-cell lymphotropic virus type I (STLV-I), have been isolated from several genera of Old World monkeys and apes, but not from New World monkeys and prosimians. To determine the genomic diversity and molecular evolution of STLV-I and to clarify their genetic relationship to human T-cell lymphotoropic virus type I (HTLV-I), we enzymatically amplified, then directly sequenced selected regions of the *gag*, *pol*, *env*, and *pX* genes of STLV-I strains from Asia and Africa. STLV-I strains Si-2, Matsu, and JM86 from Japanese macaques, which exhibited sequence similarities ranging from 98.5 to 99.8% among themselves, diverged

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by 12.9 to 13.3% from STLV-I strain MM39-83 from a naturally infected rhesus macaque, by 9.7 to 11.2% from STLV-I strains from Africa, and by 8.8 to 11.2% from HTLV-I strains originating in Japan, India, Africa, the Caribbean, the Americas, Polynesia, and Melanesia. By contrast, the interspecies nucleotide sequence similarity among African STLV-I strains from green monkey, yellow baboon, sooty mangabey, and common chimpanzee was remarkably high, ranging from 96.9 to 97.4%, and these STLV-I strains diverged by only 2.2 to 2.8% from HTLV-I strain EL from equatorial Zaire. Phylogenetic trees constructed by using the neighbor-joining and maximum parsimony methods indicated that the Asian STLV-I strains diverged from the common ancestral virus prior to African STLV-I and cosmopolitan and Melanesian HTLV-I strains. Thus, our data are consistent with an archaic presence of STLV-I in Asia, probably predating macaque speciation, with subsequent independent virus evolution in Asia and Africa. For details, see *Virology* **199**, 56–66 (Song *et al.* 1994).

Population Genetic Study in Hainan Island, China. II. Genetic Affinity Analysis

N. SAITOU, K. OMOTO*, C. DU** and R. DU***

A population genetic study was carried out on six populations (two Li populations, two Miao populations, one Hui population, and one Han population) in Hainan Island, Southern China. Allele frequency data for these six populations and those for some other Asian populations were used to estimate genetic distances between populations, and genetic affinity dendrograms and networks were constructed by using UPGMA and the neighbor-joining method, respectively. The two Li populations showed a close relationship, while the two Miao populations were relatively distant from each other. These six populations of Hainan Island showed a close affinity with Zhuang of Southern China, Thailanders, and Javanese, but they are distant from populations around northern China (Japanese, Mongolian, and Korean). Divergence time between the Li-Miao group and Japanese was estimated to be roughly 19,000–26,000 years. For details, see Anthropological *Science* **102**, 129–147 (Saitou *et al.* 1994).

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Molecular Evolution of Serine Protease and Its Inhibitor with Special Reference to Domain Evolution

Takashi GOJOBORI and Kazuho IKEO

The evolution of serine protease and its inhibitor are 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 are made up 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 b precursor protein in Alzheimer's disease patients were examined by 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 evolutionarily movable. Thus, it was concluded that various 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 those functional domains must have been minigenes possibly at the time of primordial life or the origin of life. We call these minigenes 'ancestral minigenes'. Every effort should be made to answer questions about the minimum set of ancestral minigenes that must have existed and must have been needed for maintaining life forms. The DNA sequence database is useful for making attempts to answer such difficult but significant questions. For details, see *Phil. Trans. Royal Soc. Lond.* **B344**, 411–415.

NRSub: A Non-redundant Data Base for the *Bacillus subtilis* Genome

Guy PERRIERE, Manolo GOUY and Takashi GOJOBORI

We have organized the DNA sequences of *Bacillus subtilis* from the EMBL collection to build the NRSub data base. This data base is free from duplications and all detected overlapping sequences were merged into contigs. Data on gene mapping and codon usage are also included. NRSub is publically available through anonymous FTP in flat file format or structured on the form of an ACNUC data base. Under this format, it is possible to use NRSub with the retrieval program Query win. This program integrates a

graphical interface and may be installed on any kind of UNIX computer under X Window and on which the Vibrant and Motif libraries are available. For details, see *Nucl. Acids Res.* **25**, 5525–5529

Confirmation by Molecular Evolutionary Analysis of Hepatitis C Virus Transmission through Needlestick Accidents

Kaoru SUZUKI, Masashi MIZOKAMI, Johnson Y. N. LAU, Naoto MIZOGUCHI, Koji KATO, Yoshiki MIZUNO, Takeshi SODEYAMA, Kendo KIYOSAWA and Takashi GOJOBORI

To document the transmission of hepatitis C virus (HCV) through needlestick accidents, 3 health workers who acquired HCV through such accidents and their HCV donor patients were studied using molecular evolutionary analysis based on the HCV E2 region. At least six clones were sequenced from each subject. Nucleotide substitutions were estimated by the six-parameter method, and a phylogenetic tree was constructed by the neighbor-joining method. HCV isolates from the donor patient and the recipient were nested in one monophyletic cluster; this clustering was confirmed to be statistically significant by bootstrap analysis. The nucleotide divergence among the isolates from the recipient was always smaller than that from the donor, supporting the notion that the direction of transmission was from the donor to the recipient. These findings provide evidence, at a molecular evolutionary level, that HCV was transmitted through needle-stick accidents. For details, see *J. Infectious Diseases* **170**, 1575–1578.

Evolution of Pathogenic Viruses with Special Reference to the Rates of Synonymous and Nonsynonymous Substitutions

Takashi GOJOBORI, Yumi YAMAGUCHI, Kazuho IKEO and Masashi MIZOKAMI

For pathogenic viruses such as human immunodeficiency virus (HIV), hepatitis B virus (HBV), hepatitis C virus (HCV), human influenza A virus, and human T-cell leukemia virus type I (HTLV-I), evolutionary features were briefly reviewed with special reference to the rates of synonymous and nonsynonymous substitutions. In particular, these rates were discussed in connection with the neutral theory of molecular evolution. Common to all

five pathogenic viruses was the fact that the rate of synonymous substitution was higher than that of nonsynonymous substitution particularly when entire gene regions were compared between different isolates. This suggests that viral proteins are quite conservative in regards to functional and structural changes even though most of these viral genomes are evolving at a speed much higher than that of their host genomes. Thus, this feature is consistent with the neutral theory. However, it can also be pointed out that positive selection may be operating on some specific sites such as antigenic sites in order for the pathogenic viruses to escape from the host immune system. For details, see *Jpn. J. Genet.* **69**, 481–488.

Reduction of Synonymous Substitutions in the Core Protein Gene of Hepatitis C Virus

Yasuo INA, Masashi MIZOKAMI, Kenichi OHBA and Takashi GOJOBORI

Molecular evolutionary analyses were carried out to elucidate the phylogenetic relationships, evolutionary rate, and divergence times of hepatitis C viruses. Using the nucleotide sequences of viruses isolated from various locations in the world, we constructed phylogenetic trees. The trees showed that strains isolated from a single location were not necessarily clustered as a group. This suggests that the viruses may be transferred with blood on a worldwide scale. We estimated the evolutionary rates at synonymous and nonsynonymous sites for all genes in the viral genome. We then found that the rate (1.35×10^{-3} per site per year) at synonymous sites for the C gene was much smaller than those for the other genes (e.g., 6.29×10^{-3} per site per year for the E gene). This indicates that a special type of functional constraint on synonymous substitutions may exist in the C gene. Because we found an open reading frame (ORF) in the C gene region, the possibility exists that synonymous substitutions for the C gene are constrained by the overlapping ORF whose reading frame is different from that of the C gene. Applying the evolutionary rates to the trees, we also suggest that major groups of hepatitis C viruses diverged from their common ancestor several hundred years ago. For details, see *J. Mol. Evol.* **38**, 50–56.

Phylogenetic Subtypes of Human T-lymphotropic Virus Type I and Their Relations to the Anthropological Background

Tomoyuki MIURA, Yasuo INA, Takashi GOJOBORI and Masanori HAYAMI

Isolates of human T-lymphotropic virus type I (HTLV-I) from native inhabitants in India and South America (Colombia and Chile) and from the Ainu were phylogenetically analyzed. Their genomes were partially sequenced together with isolates from Gabon in central Africa and from Ghana in West Africa. A phylogenetic tree was constructed from the sequence data obtained and those of previously reported HTLV-I isolates and simian T-lymphotropic virus type I (STLV-I) isolates. The heterogeneity of HTLV-I was recently recognized, and one major type, generally called the "cosmopolitan" type, contained Japanese, Caribbean, and West Africa isolates. The phylogenetic tree constructed in the present study showed that this cosmopolitan type can be further separated into three lineages (subtypes A, B, and C). Subtype A consists of some Caribbean, two South America, and some Japanese isolates, including that from the Ainu, in addition to an India isolate. Subtype B consists of other Japanese isolates in addition to another Indian isolate, suggesting that there might be at least two ancestral lineages of the Japanese HTLV-I. Subtype C consists of the West African and other Caribbean isolates, indicating that not all parts of the Caribbean strains directly originated from West Africa. The tree also has showed that the HTLV-I isolate from Gabon in central Africa forms a cluster with STLV-I from a chimpanzee, suggesting a possible interspecies transmission between man and the chimpanzee in the past. For details, see Proc. *Natl. Acad. Sci. USA* **91**, 1124–1127.

Toward Unification of Taxonomy Databases in a Distributed Computer Environment

Hajime KITAKAMI, Yoshio TATENO and Takashi GOJOBORI

All the taxonomy databases constructed with the DNA databases of the international DNA data banks are powerful electronic dictionaries which aid in biological research by computer. The taxonomy databases are, however, not consistently unified with a relational format. If we can achieve consistent unification of the taxonomy databases, it will be useful in comparing many

research results, and investigating future research directions from existent research results. In particular, it will be useful in comparing relationships between phylogenetic trees inferred from molecular data and those constructed from morphological data. The goal of the present study is to unify the existent taxonomy databases and eliminate inconsistencies (errors) that are present in them. Inconsistencies occur particularly in the restructuring of the existent taxonomy databases, since classification rules for constructing the taxonomy have rapidly changed with biological advancements. A repair system is needed to remove inconsistencies in each data bank and mismatches among data banks. This paper describes a new methodology for removing both inconsistencies and mismatches from the databases on a distributed computer environment. The methodology is implemented in a relational database management system, SYBASE. For details, see ISMB94, pp. 227–235, Stanford Univ. Press.

Molecular Evolutionary Virology: Its Application to Hepatitis C Virus

Masashi MIZOKAMI, Takashi GOJOBORI and Johnson Y. N. LAU

We described the importance and usefulness of molecular evolutionary analysis in the study of pathogenic viruses. In particular, HCV (hepatitis C virus) and HIV (human immunodeficiency virus) are known to have a rate of nucleotide substitution million times greater than hosts such as humans. The extremely high rate of nucleotide substitutions for these viruses enables us to predict future evolution and to test it a few years later. For example, the patterns of nucleotide substitutions can be estimated by constructing phylogenetic trees. In particular, the direction from a particular nucleotide to other nucleotides can be identified if we can successfully infer the ancestral nucleotide at each branching point from the phylogenetic tree. This will be very useful for developing effective vaccines because the future direction of substitutions can be predicted with certain probabilities. For details, see *Gastroenterology* **107**, 1181–1182.

Molecular Evolution of the Major Epitopes of HIV within a Single Host

Yumi YAMAGUCHI and Takashi GOJOBORI

The third variable envelope region (V3) is one of the major epitopes of

HIV (human immunodeficiency virus). We intended to elucidate the evolutionary mechanisms of the V3 region of HIV within a single host. We collected nucleotide sequence data of HIV clones isolated from a single host at several time points after infection. We estimated the rates of synonymous and nonsynonymous substitutions for the V3 region of HIV within a single host during each period of time. In some periods of time after infection, the rate of nonsynonymous substitution for the V3 region was higher than that of synonymous substitution. We also estimated the number and type of amino acid substitutions that occurred at each amino acid site in the V3 region. It was found that amino acid substitutions dominantly occurred at several particular amino acid sites where the substitutions are known to be responsible for the production of antigenic variation and determination of viral phenotypes. These observations indicate a strong possibility that positive selection is operating in the V3 region of HIV within a single host.

A Large-Scale Search for Genes on which Positive Selection May Operate

Toshinori ENDO, Kazuho IKEO and Takashi GOJOBORI

The neutral theory of molecular evolution predicts that most genes evolve by accumulation and fixation of neutral mutations. The theory, however, does not deny the possibility that positive selection operates on some genes. The proportion of such genes among all the genes available has not yet been estimated. Thus, we have searched for such genes from the three DNA sequence databases of DDBJ, EMBL, and GenBank. By making a large number of alignments for homologous DNA sequences, we then compared the estimated numbers of synonymous substitutions with those of nonsynonymous substitutions. As a result, we found that in 3,595 homologous gene groups, positive selection may operate on sixteen gene groups. Eight out of sixteen gene groups were surface proteins of parasites and viruses. We then performed the newly developed window analysis method to find the within-gene region on which positive selection may operate. The window analysis revealed that positive selection may operate on a within-gene region where an antigenic epitope is located. We further searched the genes that contained in within-gene regions where positive selection may operate. We found that these 186 gene groups were regarded as candidate genes on which positive selection may operate.

Gene Conversion in the Major Histocompatibility Complex Genes

Tadashi IMANISHI

Human major histocompatibility complex (MHC) genes comprise a multigene family, and many of them show high degrees of genetic polymorphisms. One of the hypothesized mechanisms that generate polymorphism is gene conversion, by which allelic segments of dozens of nucleotides are copied to other alleles or alleles of other loci. In this study, I examined the possibility of gene conversion in the MHC genes and its evolutionary significance through extensive comparisons of nucleotide sequence data. In particular, distribution of locus-specific substitutions, nucleotides observed exclusively in a particular locus and not in other loci, and shared DNA polymorphism between different loci, were examined. Results showed shared DNA polymorphism was significantly abundant in the hyper-variable exons of both class I (HLA-A, B, and C) and class II (HLA-DRB1, DQB1, and DPB1) MHC loci. This observation can be accounted for, either by gene conversion between different, duplicated loci or by natural selection favoring the same sets of amino acid sequences.

Studies of Genetic Variability in Human MHC Genes

Tadashi IMANISHI

The genetic variability in human MHC genes (HLA) was investigated at the DNA level and at the population level. New alleles of class I HLA were found and their nucleotide sequences were determined. The molecular evolution of the new alleles was examined by constructing a phylogenetic tree (Ishikawa, Y. *et al.* (1994) *Human Immunology* **39**, 220–224). Moreover, the frequencies of DNA-typed HLA alleles and haplotypes were estimated for the Japanese population (Hashimoto, M. *et al.* (1994) *Tissue Antigens* **44**, 166–173; Akaza, T. *et al.* (1994) Transplantation Now **7**: s87–s99 (in Japanese)). Furthermore, the gene frequencies and haplotype frequencies in HLA were estimated for the Buryat population in northeast Asia and Jamaican populations in South America. The phylogenetic relationships of these populations and the surrounding populations were analyzed by using dendrograms (Tokunaga, K. *et al.* (1995) *Tissue Antigens* **45**, 98–102; Blank, M. *et al.* (1995) *Tissue Antigens* **45**, 111–116). These studies revealed not

only the distribution of genetic variability in human MHC genes at the population level and at the molecular level, but also the evolution of human populations and MHC genes themselves.

Evolution of Glutamine Synthetase Gene Is in Accordance with the Neutral Theory of Molecular Evolution

Yoshio TATENO

The evolution of the glutamine synthetase gene was studied by analyzing 30 homologous DNA sequences of the gene. The sequences of various organisms spanning from prokaryotes to eukaryotes were collected from the DDBJ database. They were translated first, aligned next, then evolutionary distances were computed, and the molecular phylogeny was finally obtained.

The results of the alignment revealed that functionary important regions of this enzyme have been evolutionary more conserved than the remaining regions which are not directory related to its function. The evolutionary distances computed show that the rate of synonymous substitutions is higher than that of nonsynonymous substitutions. These results are in accordance with the neutral theory of molecular evolution.

Moreover, the molecular phylogeny estimated indicates that the origin of glutamine synthetase gene is much older than the divergence between eukaryotes and prokaryotes, suggesting that the gene is one of the oldest genes functioning at present. (published in *Jpn. J. Genet* **69**, 489–502, 1994)

Yamato and Asuka: DNA Database Management System

H. KITAKAMI, T. SHIN-I, K. IKEO, Y. UGAWA, N. SAITOU, T. GOJOBORI and Y. TATENO

We recently developed a relational schema for effectively building, integrating, and searching the DNA database on the relational database management system, Sybase at DDBJ. The schema is called the DDBJ schema. The schema allows us to implement a DNA database management system with two types of window interfaces, Yamato and Asuka, to easily build the DNA database. The first type is used by the data reviewers for processing a single entry of the data sent by a researcher. The second one is used by genome project groups for processing multiple entries. Both types

have a window interface similar to that for the flat file format.

We have also developed two subsidiary systems for Yamato and Asuka. One is a restructuring tool for converting the data in the GenBank schema into those in the DDBJ schema. The other is a tree search tool needed for correcting errors in the taxonomy database. A structured SQL programming method is proposed for implementing these subsidiary systems. The method was developed by making use of the SQL expansion and the control flow language of Sybase. (published in The Proceedings of the 28 Hawaii International Conference on System Sciences, pp. 72–80, IEEE Computer Society, 1994)

Building and Search System for a Large-Scale DNA Database

H. KITAKAMI, Y. YAMAZAKI, K. IKEO, Y. UGAWA, T. SHIN-I,
N. SAITOU, T. GOJOBORI and Y. TATENO

We at DDBJ had used a flat-file system to operate our DNA database system until two years ago. But the flat-file system was inadequate for integrating and searching a large-scale database in which the number of entries was increasing at an explosive rate. Then we adopted a relational database management system, the GenBank Schema, from GenBank in the United States. This system worked on Sybase. We also installed the annotator's work bench (AWB), which was a tool for building the DNA database with the schema. AWB, however, did not have a simultaneous processing function for large amounts of DNA data such as EST data. Moreover, the integrating function was not operated, nor was the searching function at DDBJ.

Thus we recently developed a hierarchical relational schema for effectively building, integrating, and searching the DNA database on Sybase. The schema is called the DDBJ schema. The schema allows us to implement a window interface to easily build the DNA database. The database in the GenBank schema can be converted to that in the DDBJ schema by use of a restructuring tool we also developed. We have proposed a structured SQL programming method to implement the restructuring tool. The method was developed by using the control flow language of Sybase. We have also proposed two more methods by which to execute those tools on the UNIX based workstation connected to the computer network. (published in Advances in Molecular Bioinformatics, pp. 123–138, IOS press, 1994)

VIII. HUMAN GENETICS

Molecular Definition of the Prader-Willi Syndrome Chromosome Region and Isolation of Transcribed Sequences by Positional Cloning Method

Takashi IMAMURA, Masako SAKAI, Rie INABA, Kenji IZUHARA and Tomoko HASEGAWA

The prader-Willi syndrome (PWS) is characterized by psychomoter and growth retardation, infantile hypotonia, characteristic dysfunction causing hypogonadism and hyperphagia with obesity. 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 q11.1–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) that is clinically quite distinct and characterized by more severe mental retardation, absent speech, microcephaly, seizures, a movement disorder, inappropriate laughter and typical facies. AS can also be caused by paternal disomy or maternally inherited as yet unidentified submicroscopic mutations. Thus, PWS and AS are distinct neurogenic disorders, which are 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 deletions 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 another 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. To identify the locus from which these cDNAs were derived and to characterize the genomic structure of loci, we have carryed out quantitative polymerase chain reaction amplification of human genomic DNA using primers derived from the cDNA sequences. Since the patient with PWS are identified to be hemizygous for the genes, the results indicate that these transcribed sequences appear to be deleted on the paternally derived chromosome 15, and that genes on the maternally derived chromosome 15 are imprinted and completely suppressed.

Isolation of Candidate Transcribed Sequences in Cloned Genomic DNA with Known Lacation on Human Chromosome 18

Takashi IMAMURA, Masako SAKAI, Rie INABA and Hitoshi NAKASHIMA

Identification and recovery of transcribed sequences from cloned mammlian 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 exons from random pieces of cloned genomic DNA. We have 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 have 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.

The approaches are designed to detect transcribed sequences from genomic

DNA of known location. A regional assignment of cosmids could be made by fluorescence *in situ* hybridization to metaphase chromosomes. The purpose of this work was to get a molecular approach for identifying genes within the human genome. 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 candidtate 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. This screen of uncharacterized cosmids will determine the utility of transcribed sequence fragments of known chromosomal location, as identifiers of candidate coding sequences.

Chromosome 18 is involved in tetrasomy 18p syndrome as well as in trisomy 18. 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.

Abnormality of JAK-STAT Pathway Induced by Interleukin-4 in Patients with X-linked Severe Combined Immunodeficiency

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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 (γc) with IL-2, IL-7 and IL-9 receptor systems. Mutations of γc are known to result in human X-linked severe combined immunodeficiency (XSCID). Since JAK3 has been shown to associate with γc, we analysed activation of

JAK-STAT pathway in EBV-transformed B cell lines established from two XSCID patients (XSCID-B), in order to investigate the involvement of γc 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 γc plays a pivotal role in activation of JAK–STAT pathway by IL-4.

A New Point Mutation at Nucleotide Pair 3291 of the Mitochondrial $tRNA^{leu(UUR)}$ Gene in a Patient with Mitochondrial Myopathy, Encephalopathy, Lactic Acidosis, and Stroke-Like Episodes (MELAS)

Yu-ichi GOTO*, Kazuo TSUGANE, Yuzo TANABE**,
Ikuya NONAKA* and Satoshi HORAI

Examples of the association between mitochondrial DNA (mtDNA) mutations and mitochondrial encephalomyopathies have followed in succession since the first report by Holt and his colleagues. This genotype-phenotype relationship includes, for instance, mtDNA deletion or duplication in chronic progressive external ophthalmoplegia (CPEO), a point mutation at nucleotide pair 8344 in myoclonus epilepsy with ragged-red fibers (MERRF) and a point mutation at 3243 in mitochondrial myopathy, encephalopathy, lactic acidosis and stroke like episodes (MELAS). In MELAS, we have found a second common mutation at nucleotide pair 3271 in Japanese patients. Both 3243 and 3271 mutations are not limited to the Japanese but have been found in other racial groups. We have also shown that a mutation at nucleotide pair 11084 which has been proposed as a third one could be an mtDNA polymorphism prevalent in Japanese. A new point mutation at nucleotide pair 3291 in the mitochondrial $tRNA^{Leu(UUR)}$ gene was found in a Japanese MELAS patient. The nucleotides at the mutated site were evolutionarily invariant from humans through sea urchins. The mutant genomes were detected in a

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heteroplasmic fashion in muscle and blood cells of the proband by means of PCR–RFLP. Among 46 MELAS, 5 MERRF, 23 CPEO and 55 normal controls examined, this is the only patient with the mutation. This is the third mutation associated with MELAS in addition to nucleotides at 3243 and 3271. All three mutations occurred within the tRNA-Leu(UUR) region indicating that the tRNA alteration is responsible for the MELAS phenotype. For details, see *Biochem. Biophys. Res. Comm.* **202**(3), 1624–1630 (1994).

Point Mutations in Mitochondrial tRNA Genes: Sequence Analysis of Chronic Progressive External Ophthalmoplegia (CPEO)

Yuko HATTORI*, Yu-ichi GOTO**, Ryoichi SAKUTA**, Ikuya NONAKA**, Yoshikuni MIZUNO* and Satoshi HORAI

We have sequenced all mitochondrial tRNA genes from 9 Japanese patients with chronic progressive external ophthalmoplegia (CPEO) who had no detectable large mtDNA deletions nor mutations previously reported, and identified 6 different base substitutions in 6 patients. Since 5 of the 6 substitutions were homoplasmic in distribution and recognizable in some normal controls, they were thought to be polymorphisms in normal individuals. One mutation at nucleotide (nt) 12311 in the $tRNA^{Leu(CUN)}$ gene was not present in 90 normal controls nor in 103 patients with other mitochondrial myopathies. This mutation was in a heteroplasmic state, and the mutated site was conserved among other species during evolution, suggesting a disease-related mutation. However, the significance of this mutation has to be studied further. In Japanese CPEO patients without large deletions, a point mutation in the mitochondrial tRNA gene is not likely to be a frequent cause. For details, see *J. Neurol. Sci.* **125**, 50–55 (1994)

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Mitochondrial Myopathy with Progressive Decrease in Mitochondrial tRNALeu(UUR) Mutant Genomes

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Ikuya NONAKA** and Satoshi HORAI

Since the first description of mitochondrial myopathy by Luft and colleagues in 1962, many mitochondrial disorders have been described in which almost 40 of patients have the following three major clinical forms: chronic progressive external ophthalmoplegia (CPEO) including Kearns-Sayre syndrome (KSS); mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke like episodes (MELAS); and myoclonus epilepsy associated with ragged-red fibers (MERRF). Each disorder has disease-specific mtDNA abnormalities including large-scale deletions in CPEO and KSS, point mutations at nucleotide pairs (nt) 3243 and 3271 in MELAS and 8344 in MERRF. We present a female patient who had an A-to-G substitution at nt 3243 in mtDNA (3243 mutation), commonly seen in patients with MELAS but with the unusual feature of improvement in her muscle weakness. While the 3243 mutation is thought to induce various clinical expressions from none to typical MELAS phenotype, this is the first report describing a patient who had reversible muscle weakness and pathology associated with a change in the mutant genome population. A female patient with mitochondrial myopathy had a mitochondrial DNA mutation at nucleotide pair 3243, commonly seen in patients with MELAS but unlike MELAS patients, she had no central nervous system symptoms. Muscle weakness, which was most severe when she was 7 years old, improved gradually with age. Comparison of two muscle biopsies obtained at an interval of 12.5 years (7 and 20 years of age, respectively), revealed that the number of ragged-red fibers was markedly decreased and histochemical cytochrome c oxidase activity increased in parallel with the decrease in population of mutant genomes. For details, see *Ann. Neurol.* **35**, 370–373 (1994).

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A Caucasian Family with the 3271 Mutation in Mitochondrial DNA

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MELAS (mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes) is a distinct clinical entity characterized by episodic headache and vomiting, convulsions, transient hemiplegia, hemianopsia, and high lactate levels in serum and cerebrospinal fluid (CSF). Approximately 80% of the patients have an A-to-G transition mutation at nucleotide position 3243 in the mitochondrial (mt) tRNALeu(UUR) gene (3243 mutation), while about 10% have a T-to-C transition in the same gene at nucleotide position 3271 (3271 mutation). Patients with the 3243 mutation have been found in several races, and biochemical and genetic studies have shown that the mutation plays a substantial role in the pathological process in MELAS. Since all the patients with the 3271 mutation reported so far are of Japanese descent, it has been debated whether the mutation is pathognomonic of mitochondrial diseases. Here we describe a Brazilian family with the 3271 mutation, offering further evidence that the 3271 mutation is closely related to mitochondrial diseases. This mutation was found in a Brazilian family of Portuguese and Italian descent, indicating that this mutation also exists in a race other than Japanese. The propositus had mild clinical manifestations atypical of MELAS, suggesting that patients with the 3271 mutation exhibit heterogeneous phenotypic expression as seen in the 3243 mutation. For details, see *Biochem. Med. Metabol. Biol.* **52**, 136–139 (1994).

A Subtype of Diabetes Mellitus Associated with a Mutation of Mitochondrial DNA

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Several families have been described in which a mutation of mitochondrial DNA, the substitution of guanine for adenine (A G) at position 3243 of Lucite transfer RNA, is associated with diabetes mellitus and deafness. The

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prevalence, clinical features, and pathophysiology of diabetes with this mutation are largely undefined. We studied 55 patients with insulin-dependent diabetes mellitus (IDDM) and a family history of diabetes (group 1), 85 patients with IDDM and no family history of diabetes (group 2), 100 patients with non-insulin-dependent diabetes mellitus (NIDDM) and a family history of diabetes (group 3), and 5 patients with diabetes and deafness (group 4) for the mutation. We also studied the prevalence and characteristics of diabetes in 39 patients with a syndrome consisting of mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes who were known to have the mutation and 127 of their relatives (group 5). We identified 16 unrelated patients with diabetes associated with the A→G mutation: 3 patients from group 1 (6 percent), 2 patients from group 3 (2 percent), 3 patients from group 4 (60 percent), and 8 patients from group 5 (21 percent). We also identified 16 additional subjects who had diabetes and the mutation among 42 relatives of the patients with diabetes and the mutation in groups 1, 2, 3, and 4 and 20 affected subjects among the 127 relatives of the patients in group 5. Diabetes cosegregated with the mutation in a fashion consistent with maternal transmission, was frequently (in 61 percent of cases) associated with sensory hearing loss, and was generally accompanied by impaired insulin secretion. Diabetes mellitus associated with the A→G mutation at position 3243 of mitochondrial leucine transfer RNA represents a subtype of diabetes found in both patients with IDDM and patients with NIDDM in Japan. For details, see *N. Engl. J. Med.* **330**, 962–968 (1994).

Enzymatic and Genetic Adaptation of Soleus Muscle Mitochondria to Physical Training in Rats

Taro MURAKAMI*, Yoshiharu SHIMOMURA*, Takayuki OZAWA**
and Satoshi HORAI

To evaluate the effects of physical training on mitochondrial gene expression and mitochondrial biogenesis in slow-twitch muscle, adult female Sprague-Dawley rats were trained for 3, 6, and 12 wk by running on a motor-driven treadmill (speed of 25 m/min and duration of 90 min/day, 5 days/wk), and the activities of citrate synthase, ubiquinolcytochrome-c ox-

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idoreductase, cytochrome oxidase, mitochondrial cytochrome b mRNA (by Northern blot analysis), and mitochondrial DNA (by slot-blot and Southern blot analyses) were measured in rat soleus muscle. A DNA probe for detection of mitochondrial mRNA and DNA was prepared from a 1,500-bp fragment of human mitochondrial DNA that included the coding region of the cytochrome b gene. Training for 3, 6, and 12 wk significantly increased the activities of citrate synthase (31, 28, and 47%, respectively), ubiquinolcytochrome-c oxidoreductase (61, 63, and 77%, respectively), and cytochrome oxidase (25, 26, and 32%, respectively) in muscle. The concentration of cytochrome b mRNA in the muscle was proportionally elevated with the enzyme activities. On the other hand, the mitochondrial DNA concentration in the muscle was not altered by training for 3 or 6 wk but increased significantly after training for 12 wk (35% in the slot-blot analysis and 31% in the Southern blot analysis). These results suggest that an increase in the oxidative capacity of slow-twitch muscle by the relatively short-term training is regulated at the pretranslational step in mitochondrial protein synthesis but that the increase by the long-term training involves mitochondrial replication. For details, see *Am. J. Physiol.* **267** (*Endocrinol. Metab.* **30**), 388–395 (1994).

Polymorphisms of Complement Component I and C1R Subcomponent of C1 in Nine Aboriginal Taiwanese Populations

Kazuo UMETSU*, Isao YUASA**, Tsuneo SUZUKI*, Cheih-Shan SUN***, I-Hung PAN****, Takafumi ISHIDA*****, Naruya SAITOU and Satoshi HORAI

Complement component I (IF) and C1R subcomponent of C1(C1R) types were determined by isoelectric focusing and subsequent immunoblotting techniques for 658 individuals from nine aboriginal Taiwanese populations. The frequency of the *IF*A* allele ranges from 0.075 (Bunun) to 0.430 (Saisiat), and a new variant allele *IF*B2* was found to have polymorphic frequency in the Atayal. The frequency of the *C1R*1* allele ranges from 0.410 (Yami) to 0.650 (Atayal), and the frequency of the *C1R*2* allele

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ranges from 0.265 (Atayal) to 0.586 (Saisiat). The *C1R* ∗*5* allele was found in five populations (Atayal, Bunun, Ami, Puyuma, Yami), and the *C1R* ∗*9* allele was found in two populations (Tsou, Puyuma). The results indicate a remarkable degree of genetic variability among these populations. The variability may reflect long-term genetic and geographic isolation of each population. For details, see *Human Biology* **66**(2), 339–348 (1994).

Construction and Expression of Chimeric Antibodies by a Simple Replacement of Heavy and Light Chain V Genes into a Single Cassette Vector

Hidetoshi KANDA, Keiko MORI, Katsumi TANIGUCHI and Takeshi WATANABE

A novel vector pMH-gpt, which is proved to be useful for cloning mouse immunoglobulin heavy and light chain V genes and for expression of mouse-human chimeric antibody, was constructed. The vector contains human genomic Cγ and Cκ genes, cloning sites for immunoglobulin V region genes, murine Ig promoters, a human Ig heavy chain enhancer, and the selection marker gene Eco-gpt. Because VH and Vκ genes can be cloned into a single vector, a chimeric antibody gene is easily constructed by this simple insertion procedure.

The usefulness of the vector was confirmed by construction of two mouse-human chimeric antibodies. Mouse monoclonal antibody (MAb) 196–14 recognizes the ovarian cancer-associated antigen (CA125), and MoAb 2–18 reacts with carcinoembryonic antigen (CEA). Mouse-human chimeric 196–14 and 2–18 antibodies were readily constructed and efficiently produced in a mouse myeloma cell line by utilizing the vector. Both chimeric antibodies retained binding activity to their respective antigens. In biodistiribution and immunoscintigraphy studies, specificity of radiolabeled chimeric 196–14 antibody was identical to that of its murine counterpart and significant accumulation at the tumor site was observed. The pMH-gpt vector is useful for construction and production of mouse-human chimeric antibodies.

IX. PLANT GENETICS

Genetic Diversity of Chinese Wild-Rice Populations

Hong-Wei CAI* and Hiroko MORISHIMA

The Asian common wild rice (*Oryza rufipogon*), progenitor of cultivated rice, is widely distributed in China. The genetic diversity of Chinese wild rice and phylogenetic relationships with other strains belonging to the same species in tropical Asia have not been fully investigated. In the present study, three Chinese population samples collected in their natural habitats, 1) Dongxiang, Jiangxi Province (the northernmost among the known sites of this species), 2) Yuanjiang, Yunnan Province, and 3) Guigang, Guangxi province, were examined regarding various phenotypic characters, 29 isozymes and nuclear genome RFLP. As controls, three perennial populations collected in Thailand, India and Indonesia and one Thai annual population were selected. Perennial and annual types represent two major ecotypes of this wild-rice species. A total of 292 plants were examined.

(1) Chinese wild rice showed a perennial trait, but differed from other perennial types collected from tropical countries in plant type, photoperiodic response, cold tolerance, etc.

(2) The three Chinese populations studied were found to share some common molecular markers which are rarely found in the strains found in other countries.

(3) The Dongxiang and Yuanjiang populations which are completely isolated from cultivated rice fields showed a lower degree of intra-populational polymorphism than Guigang populations and other control perennial populations. The Guigang population which proved to be highly polymorphic most probably received repeated gene flow from neighboring cultivated rice fields.

(4) Chinese wild rice populations contained "Japonica-specific alleles" at higher frequencies than the populations of other countries. At the same time, they carried "Indica-specific alleles" as well as alleles which are not found either in Japonica or in Indica cultivars.

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The above results suggest that Chinese wild rice has played an important role in the differentiation of Indica and Japonica types of Asian cultivated rice.

Variation Study of Weedy Rice by Morpho-Physiological Characters, Isozyme and RAPD Markers

Hak Soo SUH*, Yo-Ichiro SATO** and Hiroko MORISHIMA

There are various weedy types of rice which are not intentionally cultivated but which naturally grow in farmers' rice fields. Such weedy types is an important genetic resource for evolutionary study and breeding in rice. The variation patterns of 152 weedy rice strains collected from various regions in the world were investigated to elucidate the genetic background of weedy rice, based on morpho-physiological characters, isozymes and RAPD markers.

The weedy rice strains examined could be clearly classified into Indica and Japonica types, the two major varietal groups in Asian cultivated rice, by the first vector of principal component analysis (PCA) based on allelic variation at 14 isozyme loci. In the Indica group, strains from tropical regions were separated from those of temperate regions by the second vector of PCA. The Japonica group, however, showed little variation. In the scatter diagram from the first and second vectors of factor analysis based on six characters (glume hair length, $KClO_3$ resistance, score of the first vector of PCA based on isozymes, 100-grain weight, seed dormancy and seed shattering degree), the weedy rice strains tested were classified into four groups. These groups were designated as groups I, II, III and IV. Group I was Indica type with cultivar characters, group II was Indica type with wild-rice characters, group III was Japonica type with cultivar characters and group IV was Japonica type with wild-rice characters. Fifty-four strains were identified as belonging to group I, 24 to group II, 63 to group III and 21 to group IV, respectively.

Variation in seven RAPD markers (six for nuclear and one for chloroplast DNA) and one isozyme locus, *Est-10*, whose alleles were effectively used for identification of Indica and Japonica cultivars, were examined in 28 weedy rice strains. Six Japonica-type weedy strains showed no variation in the eight

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molecular markers tested. Among twenty weedy strains of Indica type, however, two from Korea and two from Japan showed Japonica-specific RAPD markers at one or two loci, and two from Brazil and two from the USA showed both Indica- and Japonica-specific markers in one or two loci. Two strains from Brazil, one from the USA, one from China and one from Nepal among the Indica type showed non-deletion type cpDNA which was frequent in Japonica cultivars. One weedy strain from Bangladesh had allele 4 at *Est-10* which was specific to wild rice. These results suggest that some weedy rices might have originated from the progenies of natural hybridization between Indica and Japonica types of cultivated rice or hybridization between wild and cultivated rice. This is additional support for the hypothesis proposed by Oka (1988) and others concerning the origin of weedy forms in crops.

Allozyme Variation and Genetic Structure of *Oryza glumaepatula* Populations Distributed in the Amazon Basin

Masahiro AKIMOTO*, Yoshiya SHIMAMOTO* and Hiroko MORISHIMA

Oryza glumaepatula found in tropical America is a diploid wild rice species, closely related to Asian cultivated rice (*O. sativa*) and its wild progenitor (*O. rufipogon*) sharing the AA genome. The plants of this species were found in the flood plain of the Amazon, where the annual oscillation of the water level is 10 to 20 m. Based on our field study and information from Brasilian scientists, the following unique life history was revealed. Growth is initiated on the parched riverbed in the late dry season mostly from seedlings and their internodes elongate in response to the rapid increase of water level to a certain extent. Then, the culms are easily broken at the lower internodes and their bodies are released from the ground to the surface of the water. Forming floating meadows, they develop many shoots and roots from each node and continue reproduction. These plants drift in the river according to the wind and current, mostly downstream. This habit enables the plants of *O. glumaepatula* to disperse their seeds over a long distance.

To study genetic variability and the inter- and intra-populational structure of Amazonian *O. glumaepatula*, we assessed allozyme variability at 30 isozyme loci of 16 enzymes using about 700 plants of 62 populations collected

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in 1992 and 1993. Genetic structure was quantified by several parameters such as F-statistics and Nei's gene diversity. The main results obtained are as follows.

1) Allozymes were not so variable in this Amazonian wild rice as in Asian wild rice. Overall gene diversity had low values, though our collection sites covered more than 30 myriad km^2. However, population genotypes tended to be differentiated in tributaries isolated geographically.

2) Populations in the lower basin showed higher gene diversity than those in the upper basin, suggesting that the gene flow is directed from upper to lower basin in the Amazon.

3) The observed frequency of heterozygotes and estimates of the inbreeding coefficient had about the same values as found in the annual populations of *O. rufipogon* which are predominantly autogamous. This suggests that *O. glumaepatula* has developed a selfing system to assure safe pollination against the threat of decrease in population size.

4) Inter-populational gene diversity is relatively higher than intra-populational gene diversity, indicating that gene flow among populations is restricted.

Various wild rice species are distributed pantropically, and adapted to the diverse environments of their habitats. *O. glumaepatula* also has developed its own adaptive strategy in the Amazon basin. The results of this study demonstrate a pattern of gene flow and inter- and intra-population genetic structure which reflects its unique life history.

Genetic Mechanism of Unidirectional Cross-Incompatibility in Rice

Yoshio SANO[1)]

Information on genetic bases for reduced crossability is limited in rice. A dominant gene, *Lcr* (low crossability), has been detected on chromosome 6 of a wild strain (W593, *O. rufipogon*) and has been transferred into the genetic background of Taichung 65 (T65, Japonica type). When the plant carrying *Lcr* was crossed with pollen of T65, the seed setting was markedly reduced due to an occurrence of shrunken and ungerminable seeds while the reciprocal crosses produced only viable seeds, resulting in unidirectional cross-incompatibility. Cyto-histological examinations revealed that no abnormal-

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ities were observed in pollen tube growth and fertilization, and that the endosperm began to deteriorate about 2–5 days after fertilization. When T65 was crossed as the pollen parent, the hybrid embryo continued to develop without endosperm, and the retardation of the endosperm caused defective seeds, while shriveled hybrid seeds were not obtained because of abortion at the early stage of development when crossed with Koshihikari. These observations suggested that the reduced crossability is not due to cross-incompatibility before fertilization but due to zygotic lethality. Genetic experiments showed that the role of cytoplasm as well as gene dosage effect of *Lcr* in the triploid endosperm was ruled out for explanations of the phenomenon.

When the plant carrying *Lcr* was crossed as the maternal parent, Japonica type tended to show reduced seed setting while *O. rufipogon* and Indica type tended to show a normal seed setting. A similar unidirectional cross incompatibility was recently reported in hybrids between IR and Japanese varieties. IR36 and others showed a low rate of crossability when Japanese varieties were used as the pollen parent. Genes which rescue zygotic lethality were detected in W593 and an Indica type of *O. sativa.* The modifiers were also transferred into the genetic background of T65 and one from W593 was proved to be located near Se_1 (photoperiod sensitivity) on Chromosome 6. A modifier detected in W593 rescued defective seeds only when transmitted through pollen. Thus, crossability observed in rice seemed to be associated with an epigenetic, gamete-of-origin dependent modification of the genome.

The Expression of the Rice *Wx* Gene in Response to Sugars

Motoji KUNI-EDA[1], Yoshio SANO and Hiro-Yuki HIRANO

The *wx* locus encodes an enzyme, ADPglucose starch glycosyl transferase, which catalyzes amylose synthesis in the rice endosperm. Carbon fixed during photosynthesis is transported as sucrose through phloem to seeds during their maturation. Since rice seeds contain a large amount of starch, such as amylose, the gene for starch synthesis is expected to be controlled by sucrose, which is converted to the substrate for starch synthesis. In this study, we analyzed the effect of sugars on the gene expression of the *Wx* gene. The results indicated that the rice *Wx* gene is activated in seeds by supplying

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sugars endogeneously, such as sucrose, glucose and fructose, as compared with water. However, activation was not observed when mannitol or sorbitol, which is not metabolized in plant cells, were supplied. The extent of the activation depended on the concentration of sucrose; the most effective activation was observed at a concentration of 100 mM, which corresponds to the concentration of sucrose in the phloem of rice. These results suggest that the *Wx* gene is regulated by the sugars metabolized in the cell.

We developed a transient assay system using rice protoplast to analyze the promoter function in response to sugars. The promoter of the rice *Wx* gene was activated in the protoplast when the culture medium contained sugars to be metabolized, as the gene was activated in the seed. Using this transient assay system, we are trying to identify the *cis*-acting element responsive to sucrose.

Genetic Diversity of Wild Soybean Populations in Japan

Yoshiya SHIMAMOTO

The wild progenitor of crop species is a primary gene pool for the improvement of respective crops and also gives valuable information about the domestication process. Wild soybean (*Glycine soja*) is distributed throughout Japan. Seeds from 447 collection sites were used to elucidate the degree and characteristics of genetic diversity in Japanese wild soybean populations. Electrophoretic assays of the germinated seeds were carried out to determine the genotype of each plant at 17 isozyme loci. The results showed that the number of alleles per polymorphic locus, frequency of polymorphic populations and the expected heterozygosity were 2.83, 82.4% and 0.267, respectively. This level of genetic diversity was higher than that found in the landraces and economic cultivars of Japanese soybean (Hirata *et al.*, in preparation).

The Hokkaido district showed remarkably low genetic diversity and especially the Hidaka area was completely monomorphic. Genetic diversity in the Kanto district was the highest in Honshu and also higher than the Shikoku and the Kyushu districts. One of the predominant alleles in cultivated soybean, *Ap–b*, was not found in Hokkaido, but found at a low frequency in Tohoku and increased proportionately with distance traveled south. Wild soybeans were found isolated from soybean fields in the Hidaka

area, while adjacent to soybean fields in southern Japan. The variation patterns mentioned above suggests that the alleles of cultivars have invaded wild soybean populations. The Hokkaido populations were considered to be genetically differentiated from other Japanese populations judging from their genetic distances and other information. Thus, the distribution pattern of genetic variation in wild soybean populations in Japan seemed to reflect the effect of gene flow from the cultivated soybean.

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ABSTRACTS OF DIARY FOR 1994

Biological Symposium

408th–Jan. 13	1. The transition from host to phage metabolism after T4 infection (Elizabeth Kutter) 2. Cellular and population responses to starvation in *E. coli* (Roberto Kolter)
409th–Jan. 13	DNA domains: The role of transcriptional control, replication and recombination (G. P. Georgoev)
410th–Mar. 14	1. Ribosomal phenotype and bacterial growth (Ritta Mikkola) 2. Structure and regulation of eukaryotic translation initiation factors (John Hershey)
411th–Mar. 16	Ribosomal pausing; some reasons and consequences (Leif Isaksson)
412th–Feb. 28	Recognizing distant and evolutionary relationships amongst protein sequences with special application to viral capsids (Patrick Argos)
413th–Mar. 1	Prediction and recognition in signal transduction pathways (Geoff Barton)
414th–Mar. 14	HTLV-I sequence variability is associated with diseases (Charles R. M. Bangham)
415th–Mar. 17	Sporadic distribution of Fn3 domains in animal and bacterial proteins (Russel Doolittle)
416th–Mar. 28	Hydra: how does it maintain its nervous system as it constantly produces and loses neurons? (Hans R. Bode)
417th–Apr. 13	Structure-Function relationships of *Escherichia coli* RNA polymerase (Dipankar Chatterji)
418th–June 6	Mx proteins: antiviral activity against tick-borne Thogoto and Dhori viruses (Otto Haller)
419th–July 26	1. Micro- and macroevolutionary analysis of breeding system diversity in the Pontederiaceae (Joshua R. Kohn) 2. Evolution of mating incompatibilities (Marcy K. Uenoyama)

420th–Aug. 5	A telomeric-like sequence as a regulatory element at the origin of replication of trypanosomal kinetoplast DNA (Joseph Shlomai)
421st–Aug. 31	Negative phototaxis by *Escherichia coli* bacteria and 100-fold increased phtotaxis by a mutant (Julium Adler)
422nd–Oct. 5	Combination of data in phylogenetic analysis (Jans-Hurgen Bandelt)
423rd–Oct. 17	Mechanism of HIV DNA integration (Robert Craigie)
424th–Oct. 21	Biochemical and biophysical changes in the human eye lens with ageing and in cataract (D. Balasubramanian)
425th–Nov. 15	How spirochetes swim? (Nyles W. Charon)
426th–Dec. 7	The signal transduction mechanism of interleukin-4 (Nobuyuki Harada)
427th–Dec. 9	"The genomic TAG hypothesis; modern viruses as molecular fossils ancient strategies for genomic replication, and clues regarding the origin of protein synthesis" and "evolution of U2 RNA genes"
428th–Dec. 9	*In vitro* control of SV40 DNA replication (Jerard Hurwitz)
429th–Dec. 12	Early segmentation in flies, beetles and butterflies (Lisa Nagy)
430th–Dec. 21	Transcriptional regulation of the N-ras oncogene: *In vitro* analysis of a TATA-less promoter (Fujiko Watt)
431st–Dec. 19	1. Dual errors hypothesis of variation and heterogeneity of DNA base composition (Noboru Sueoka) 2. Isochore-Genome structure of higher organisms (Giorgio Bernardi)

Mishima Geneticists' Club

415th–Jan. 10	Phosphorylation of RNA polymerase IICTD: Its role in the initiation of transcription and its regulation (Hiroaki Serizawa)
416th–Jan. 7	Progress in predicting DNA-binding specificity of proteins from amino-acid sequence (Masashi Suzuki)
417th–Jan. 31	Regulation of secondary metabolism by protein-phosphorylation in *Streptomyces* (Kouji Ishizuka)

418th–Feb. 1 The role of traM gene in initiation of DNA transfer in bacterial conjugation (Tatsuhiko Abo)

419th–Feb. 2 Regulation of heat-shock gene expression in *Pseudomonas aeruginosa* (Masaya Fujita)

420th–Feb. 15 Site-specific transposition of IS630 and Tnr1 to 5′-TA-3′ (Toyoaki Tenzen)

421st–Feb. 15 Molecular orgnization of centromeric alphoid repeats on the human chromosome 21 (Masashi Ikeno)

422nd–Feb. 16 Initiation factors of homologous recombination during meiosis of budding yeat (Katsuki Johzuka)

423rd–Mar. 1 Function and developmental expression of a steroidgenic cell-specific transcription factor, Ad4BP (Kenichirou Morohashi)

424th–Feb. 21 Evolution of human populations based on polymorphism in the major histocompatibility complex genes (Tadashi Imanishi)

425th–Mar. 23 Identification of genes regulating neurogenesis of Drosophila and its application to study mammalian nervous system (Hideyuki Okano)

426th–Apr. 19 Flightless mutations of Drosophila (Kaname Mogami)

427th–Apr. 20 Movile genetic elements and natural mutations of Drosophila (Kou Harada)

428th–Apr. 27 Trials for genetic analysis of relationship between genome structure and chromosome function (Nori Kurata)

429th–Apr. 28 Molecule species and their differential function of phytochrome (photoreceptor protein in plants) (Akira Nagatani)

430th–Apr. 28 Dynamic analysis of genome in higher plants (Yasunori Ogiwara)

431st–May 6 The signal transduction mechanisms of interleukin-4 (Kenji Izuhara)

432nd–May 24 Three-dimensional structure and function of RNase H (Kousuke Morikawa)

433rd–Mar. 30 Analysis of the functional domains of regulator of chromosome condensation, Rcc1 protein (Hiroaki Seino)

434th–June 7 Limb pattern formation and retinoic acid (Kouji

	Tamura)
435th–June 27	Transmembrane signaling of the aspartate receptor (Ichiro Maruyama)
436th–July 22	Estimation of the numbers of synonymous and non-synonymous substitutions (Yasuo Ina)
437th–July 27	Figuration and function in genetic research of plants (Ki-ichi Fukui)
438th–Nov. 2	Analysis of suppressors of programmed cell death, using the nematode *Caenorhabditis elegans* as a model system (Asako Sugimoto)
439th–Nov. 24	Three-dimensional structure and function of the *E. coli* RuvC protein (Holliday junction resolvase) (Kosuke Morikawa)
440th–Dec. 20	Role of dpp, a member of TGF-β superfamily, in Drosophila pattern formation (Shigeru Morimura)
441st–Dec. 21	Structure and function of nucleosomes (Kiyoe Ura)

FOREIGN VISITORS IN 1994

Dec. 1, 1991–	Thangirala Sudha, Indian Association for Down's Syndrome, India
Aug. 27, 1993–July 5, 1994	Ashok Kumar, University of Edinburgh, U.K.
Oct. 5, 1993–	Dzhanybek M. Adyshev, Institute of Biochemistry and Physiology, Kyrgys Academy of Sciences, Kyrgyz
Dec. 1, 1993–Mar. 10, 1994	Ritta H. Mikkola, Uppsala University, Sweden
Dec. 1, 1993	Stefanie A. Margarson, University of Nottingham Medical School, U.K
Feb. 20, 1994	
Dec. 3, 1993–	Dipankar Chatterji, Centre for Cellular and Molecular
Apr. 15, 1994	Biology, India
Jan. 13, 1994	Elizabeth Kutter, Harvard Medical School, U.S.A.
Jan. 13	Roberto Kolter, Harvard Medical School, U.S.A.
Jan. 13	G. P. Georgiev, Institute of Gene Biology, Russian Academy of Sciences, Russia
Feb. 28	Patrick Argos, European Molecular Biology Laboratory, Germany
Mar. 1	Geoff Barton, University of Oxford, U.K.
Mar. 10–	Hak-Soo Suh, Yeungnam University, Korea
Mar. 14	John Hershey, University of California at Davis, U.S.A.
Mar. 14	Charles R. M. Bnagham, John Radcliffe Hospital, U.K.
Mar. 16	Leif Isaksson, University of Stockholm, Sweden
Mar. 17	Russel Doolittle, University of California, U.S.A.
Mar. 25–30	Hans Bode, University of California at Irvine, U.S.A.
Apr. 1–28	Guy B. Perriere, Centre National de la Recherche Scientifique, France
Apr. 3–7	Ling Hua Tang, Institute of Food Crops, Jiangsu Academy of Agricultural Sciences, China
May 2–4	V. Purushothaman, India
May 2–15	William B. Provine, Cornell University, U.S.A.
June 5–15	Otto Haller, University of Freiburg, Germany
June 10–	Hong Wei Cai, Beijing Agricultural University, China

June 27–Sept. 30	Joshua R. Kohn, University of California at San Diego, U.S.A.
June 29	M. K. Tadjudin, University of Indonesia, Indonesia
June 30–July 30	Marcy K. Uyenoyama, Duke Univercity, U.S.A.
July 5–27	K. W. Jair, Maryland University, U.S.A.
July 8–Aug. 24	S. Wedgewood, University of Edinburgh, U.K.
July 8–Aug. 29	Konstanze Beck, University of Munich, Germany
July 8–Aug. 29	Seielstad, Mark T., Harvard University, U.S.A.
Aug. 5–6	Joseph Shlomai, Hebrew University-Hadassah Medical School, Israel
Aug. 31	Julius Adler, University of Wisconsin, U.S.A.
Sept. 1–	John R. Wakeley, University of California at Berkeley, U.S.A.
Sept. 21–Oct. 13	Hans-Juergen Bandelt, University of Hamburg, Germany
Sept. 23–Oct. 3	Hans Bode, University of California at Irvine, U.S.A.
Sept. 24–Oct. 5	Charles David, University of Munich, Germany
Sept. 24–Oct. 5	Maria de Haro, University of Munich, Germany
Sept. 25–Oct. 1	Tomas Bosch, University of Munich, Germany
Sept. 25–Oct. 1	Jan Lohmann, University of Munich, Germany
Oct. 3–29	Chitrakon Songkran, Pathumthani Rice Research Center, Thailand
Oct. 3–Nov. 29	Goonnapa Fucharoen, Khon Kaen University, Thailand
Oct. 13–23	Hans Bode, University of California at Irvine, U.S.A.
Oct. 17–18	Robert Craigie, NIDDK, National Institutes of Health, U.S.A.
Oct. 20–21	D. Balasubramanian, Centre for Cellular and Molecular Biology, India
Nov. 8–Dec. 24	L. W. Chuang, Singapore University of Technology, Singapore
Nov. 8–Dec. 9	Y.E.L. Elgin, Singapore University of Technology, Singapore
Nov. 9–11	Masashi Suzuki, MRC Laboratory of Molecular Biology, U.K.
Nov. 15	Nyles W. Charon, West Virginia University, U.S.A.
Nov. 22–Dec. 24	Yap E. Lin, Singapore University of Technology, Singapore

Dec. 7	Nobuyuki Harada, DNAX Research Institute of Molecular and Cellular Biology, U.S.A.
Dec. 8–9	Alan M. Weiner, Yale University School of Medicine, U.S.A.
Dec. 9	Jerard Hurwitz, Memorial Sloan-Kettering Cancer Center, U.S.A.
Dec. 12	Lisa Nagy, University of Wisconsin, U.S.A.
Dec. 18–19	Noboru Sueoka and Tamiko Sueoka, University of Colorado at Boulder, U.S.A.
Dec. 18–	K. Sharif, New York City University, U.S.A.
Dec. 19	Giorgio Bernardi, Institut Jacques Monod, France
Dec. 20–21	Fujiko Watt, CSIRO, Australia

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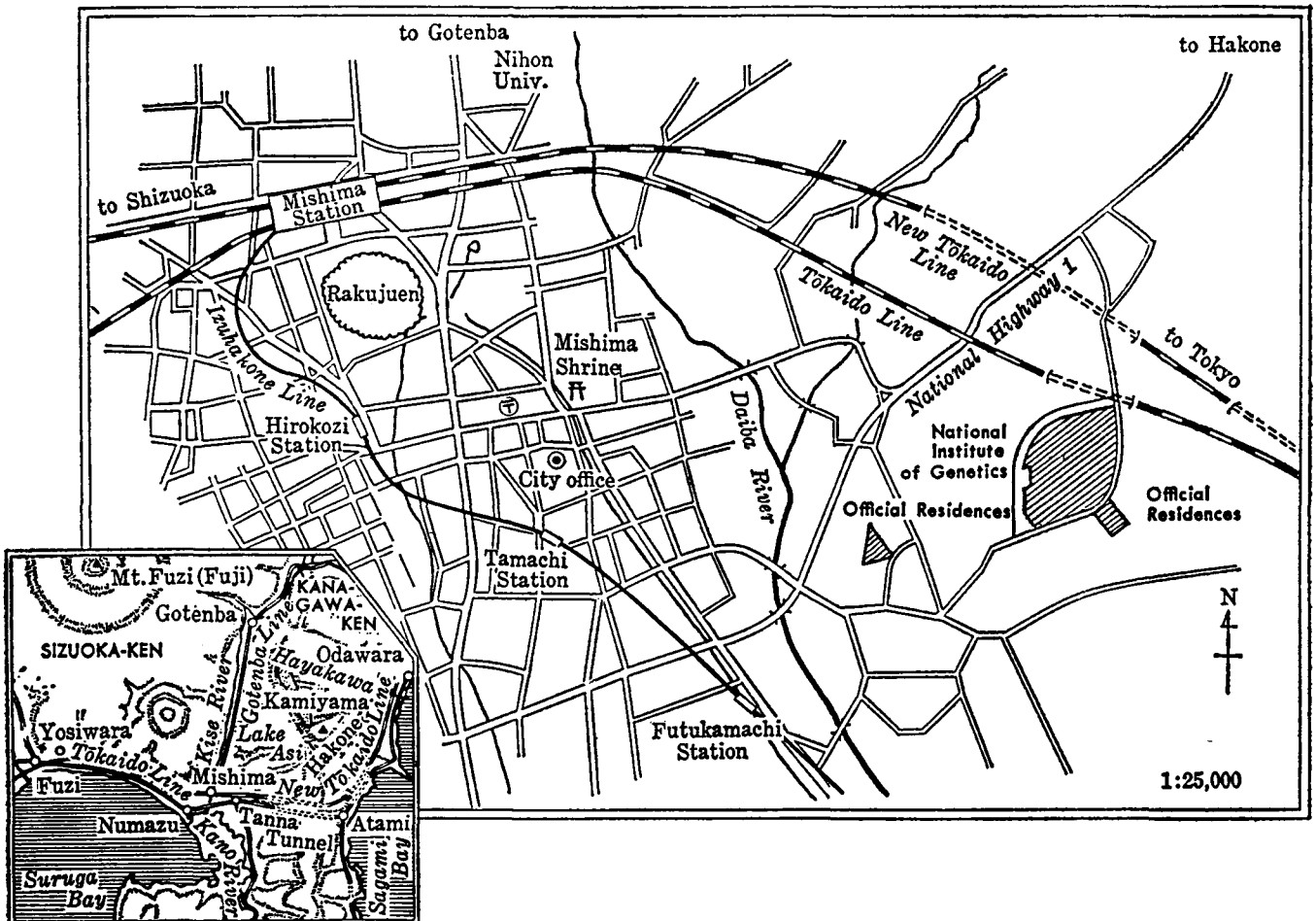
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