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GENERAL STATEMENT

Our institute was established 44 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. This is not to ignore the importance of more traditional approaches to genetics. Our institute is uniquely suitable for pursuing cooperative work by scientists of various disciplines. Through interactions between these lines of research, 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. However, as the manager responsible for these activities, I honestly feel frustration running service operations with inadequate staff numbers, salaries and funding. Nation-wide recognition of the importance of these activities and appropriate actions to amend these conditions are essential.

I hope that with guidance from people in and outside this institute and

further support from governmental and private sources, I could lead the institute into a more successful future.

In the past year, the Mammalian Development Laboratory was established in the Genetic Stock Research Center. Prof. N. Nakatsuji and Dr. Y. Shirayoshi were transferred to the new laboratory from the Mammalian Genetics Laboratory, where Dr. T. Shiroishi of the Division of Cytogenetics was transferred, being promoted to associate professor. Dr. Y. Ugawa was transferred to the Ministry of Agriculture and Fisheries and Dr. I. Nakamura to Iwate Biotechnology Research Center. The following three joined us as research members: Dr. T. Takano in the Division of Population Genetics, Dr. T. Kishi in the Division of Mutagenesis, and Dr. K. Kanamaru in the Microbial Genetics Laboratory of the Genetic Stock Research Center.

It is a pleasure to note that Dr. F. Tajima was honored by the Encouragement Award of the Genetics Society of Japan for his statistical studies on DNA polymorphism.

Junichi Tomigawa

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DNA Synthesis Laboratory

HAYASHI, Shigeo, D. Sc.

DNA Data analysis Laboratory

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IKEO, Kazuho, D. Sc.

IMANISHI,

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KOHARA, Yuji, D. Sc., Associate professor

ANDACHI, Yoshiki, D. Sc.

8. Radioisotope Center

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

9. Experimental Farm

SANO, Yoshio, D. Ag., Head of the Farm

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MITA, Akihiko, Chief of the Section

11. Department of Administration

EBIHARA, Akira, Head of the Department

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PROJECTS OF RESEARCH FOR 1993

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 and KISHI)

Division of Nucleic Acid Chemistry

Mechanism of mRNA capping (MIZUMOTO)

Transcription of the Sendai virus genome (MIZUMOTO)

Development of DNA Database management system and of unification tool of taxonomy Databases (KITAKAMI)

2. DEPARTMENT OF CELL GENETICS

Division of Cytogenetics

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

Theoretical and experimental bases for karyotype evolution (IMAI)

Study of adrenal function in mouse development by transgene (GOTOH,

* Genetic stock research center.

SHIROISHI* and MORIWAKI)

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, UEDA and HAYASHI)
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

Nerve net formation in *Hydra* (KOIZUMI)

4. DEPARTMENT OF POPULATION GENETICS

Division of Population Genetics

Theoretical studies of population genetics (OHTA and TAJIMA)
 Theoretical studies on the evolution of multigene family (OHTA)
 Theory of gene genealogy (TAJIMA)
 Statistics for DNA polymorphisms (TAJIMA)
 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 MATSUMOTO)
 Studies on genes in HLA locus (IKEMURA and MATSUMOTO)
 Studies on functions of extracellular matrix proteins (MATSUMOTO and IKEMURA)
 Molecular evolutionary analysis of nucleotide sequence data (SAITOU)
 Studies on the genetic affinity of human populations (SAITOU)
 Studies on molecular evolution of viruses (MORIYAMA)
 Studies on molecular evolution of *Drosophila* genes (MORIYAMA)

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 NAKA-

SHIMA)

Molecular genetics of human metabolic disorders (IMAMURA and NAKASHIMA)

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 and NAKAMURA)

Division of Applied Genetics

Molecular genetics of human immune mechanisms (WATANABE)

Theoretical studies on conservation biology (YONEZAWA)

6. GENETIC STOCK RESEARCH CENTER

Mammalian Genetics Laboratory

Recombinational hotspots in the mouse MHC (SHIROISHI and MORIWAKI)

Genetic Mechanisms for regulating tumor development in the laboratory and wild mice (MIYASHITA and MORIWAKI)

Invertebrate Genetics Laboratory

Molecular genetics of insect development (UEDA and HIROSE)

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)
Analysis of migration pattern of neurons in morphogenesis of the mouse central nervous system (NAKATSUJI)

7. DNA RESEARCH CENTER

DNA-Protein Interaction Laboratory

Interaction (SHIMAMOTO and NAGAI)

Recombinant DNA Laboratory

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

DNA synthesis Laboratory

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

DNA Data Analysis Laboratory

Computer (GOJOBORI and UGAWA)

Construction (GOJOBORI,)

Molecular (IKEO and GOJOBORI)

Gene Library Laboratory

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

Genome analysis of *Caenorhabditis elegans* (KOHARA)

8. RADIOISOTOPE CENTER

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

9. EXPERIMENTAL FARM

RESEARCH ACTIVITIES IN 1993

I. MOLECULAR GENETICS

Molecular Anatomy of *Escherichia coli* RNA Polymerase: Functional Map of the Amino-Terminal Assembly Domain of Alpha Subunit

Makoto KIMURA, Nobuyuki FUJITA and Akira ISHIHAMA

The α subunit of *Escherichia coli* RNA polymerase plays a major role in assembly of the core enzyme. Analysis of carboxyl-terminal deletion derivatives of α indicated that the amino terminal two-thirds of α subunit, down to amino acid 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). To define the site(s) for core enzyme assembly, we constructed a set of *rpoA* mutants with deletion or insertion mutations in this region. The over-expressed α derivatives were purified and examined for their abilities to assemble β and β' subunits *in vitro*. Analysis of a total of 22 α deletion mutants indicated that the minimal fragment with the full assembly activity is $\alpha(21-235)$, with deletions of 20 amino-terminal and 94 carboxy-terminal amino acids (Kimura, M. *et al.* (1994) *J. Mol. Biol.* **242**, 107–115). Most of the other assembly-defective deletion mutants appeared to lack the activity of formation of stable α dimers.

On the other hand, 11 species of insertion mutants, having two extra amino acids, Ala-Ser, at every 20 residues, showed defects in the assembly at various steps depending on the site of dipeptide insertion. Five mutants having the insertions between positions 40–41, 100–101, 140–141, 180–181 and 220–221 were defective in dimerization, nevertheless two of them, having the insertion at position 180–181 and 220–221, were able to assemble β subunit but unable to assemble β' subunit. The insertion at position 200–201 made α virtually inactive in β' assembly. The insertion at position 80–81 resulted in reduction of stability of $\alpha_2\beta$ complex, but the stability increased with formation of hybrid α dimer between the wild-type and the mutant α subunits. These

results altogether suggest that within the assembly domain of α , the N-terminal proximal region is involved in dimerization and contact with β while the C-terminal region is involved in β' assembly.

**Molecular Anatomy of *Escherichia coli* RNA Polymerase:
Functional Map of the Carboxy Terminal Contact
Domain with Class-I Factors**

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

The α subunit of RNA polymerase plays an essential role not only in subunit assembly but also in transcription activation by class-I transcription factors (reviewed in Ishihama, A. (1993) *J. Bacteriol.* **175**, 2483–2489). For detailed mapping the structural domains of α subunit, we analyzed the pattern of proteolytic cleavage by endoproteases. First we carried out limited proteolysis by protease V8 and trypsin under mild conditions and identified the sites of initial cleavage after sequencing of isolated proteolytic fragments. Results indicate that the proteolytic cleavage takes place at three regions, between amino acid residues 236–241 (full-length α is composed of 329 amino acid residues), implying that this region are exposed on protein surface, presumably forming interdomain linkers. Thus, we propose that two major structural domains exist within the α subunit, each being composed of amino acid residues 8–235 (domain 1) and 242–329 (domain 2).

The N-terminal proximal fragment down to amino acid 7 is not essential for assembly and enzyme function (Kimura, M. *et al.* (1994) *J. Mol. Biol.* **242**, 107–115). Upon prolonged proteolysis, the domain 1 was deaved into two subdomain fragments (1a and 1b) between Arg45 and Glu68. The amino-terminal proximal 1a subdomain is rapidly degraded upon further incubation with trypsin [subdomain 1a contains four potential cleavage sites by trypsin]. The subdomain 1b of 168 amins acids in length is cleaved at Gly151 into two fragments. Domain 2 carrying the contact site I region for various class-I transcription factors is resistant to secondary cleavage by both V8 and trypsin, indicating that this domain is highly structured.

In order to analyze the higher-order structure of domain 2, we cloned a DNA fragment carrying the C-terminal region of *rpoA* into an expression

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vector and expressed in *E. coli* a C-terminal fragment consisting of amino acids 233–329. The purified C-terminal fragment [α (233–329)] was subjected to $^1\text{H-NMR}$ spectroscopic analysis, which is believed to be the best technique for analysis of static and dynamic structure of short polypeptides in solution. Preliminary results indicate the presence of regular secondary structure in this region. Sequential signal assignment by means of ^{15}N -single and $^{15}\text{N}/^{13}\text{C}$ -double labeling techniques is in progress.

**Molecular Anatomy of *Escherichia coli* RNA Polymerase:
Mapping of cAMP Receptor Protein Contact
Site on One Alpha Subunit**

Chao ZOU, Nobuyuki FUJITA and Akira ISHIHAMA

In transcription activation of positively controlled genes, transcription factors make contact with RNA polymerase at specific sites. Up to now, we identified clusters of the contact sites on narrow regions of both α (contact site I) and σ (contact site II) subunits (reviewed in Ishihama, A. (1993) *J. Bacteriol.* **173**, 2483–2389). cAMP receptor protein (CRP) contacts with the C-terminal region of α subunit (contact site I) in transcription activation of the *lacP1* promoter (Igarashi, K. and Ishihama, A. (1991) *Cell* **32**, 319–325; Igarashi, K. *et al.* (1993) *Proc. Natl. Acad. Sci. USA* **88**, 8958–8962). The fine mapping indicated that the contact site is clustered within a short segment between amino acid residues 265–270 (Zou, C. *et al.* (1992) *Mol. Microbiol.* **6**, 2599–2605). However, it remained unknown whether only one or both of the two α subunits is involved in the protein-protein contact with CRP.

This year we constructed a hybrid RNA polymerase consisting of one wild-type and one C-terminal truncated α subunits. Results of *in vitro* transcription assay indicates that this reconstituted hybrid RNA polymerase preparation has the activity of CRP-dependent *lacP1* transcription half the level of wild-type RNA polymerase, indicating that the reconstituted hybrid RNA polymerase is a mixture of two forms which differ in the arrangement of mutant α and that CRP interacts with only one form enzyme (Zou, C. *et al.* (1994) *J. Mol. Biol.* **236**, 1283–1288). Thus, we conclude that CRP makes contact with only one α subunit.

**Molecular Anatomy of *Escherichia coli* RNA Polymerase:
Interaction between Promoter -35 and -10
Recognition Domains of Sigma-70**

Ashok KUMAR, Richard S. HAYWARD* and Akira ISHIHAMA

RNA polymerase holoenzyme of *E. coli* is composed of core enzyme with the subunit structure $\alpha_2\beta\beta'$ and one of six different species of σ subunit. RNA synthesis is catalyzed by core enzyme but σ subunit is required for promoter recognition and transcription initiation. Each molecular species of σ subunit recognizes a specific and unique set of promoters. Prokaryotic σ family proteins carry four regions of conserved sequences, of which region 2 and 4 (from N-termini) are involved in recognition of promoter -10 and -35 sequence, respectively. Previously, we demonstrated that the region 4 (-35 recognition domain) is not needed provided that an "extended -10 sequence" with the sequence of TGxTATAAT exists in the test promoters (Kumar, A. *et al.* (1994) *J. Mol. Biol.* **232**, 406-418). These C-terminally truncated σ subunits often lose the response to a group of transcription factors including PhoB and CRP (Kumar, A. *et al.* (1984) *J. Mol. Biol.* **235**, 405-413) [we designated this group as "class-II transcription factors"].

The activity of C-terminally truncated σ subunits lacking the -35 recognition domain, as measured by promoter-dependent transcription by core enzyme, was, however, less than the intact σ subunit, but was enhanced by the addition of C-terminal fragments. Thus, it appears that the -10 recognition domain of region 2 and the -35 recognition domain of region 4 interact each other to enhance the sigma activity.

**Promoter Selectivity Control of *Escherichia coli* RNA Polymerase:
Competition between Different Sigma Subunits**

Shuichi KUSANO, Miki JISHAGE, Nobuyuki FUJITA and Akira ISHIHAMA

RNA polymerase core enzyme with the subunit structure $\alpha_2\beta\beta'$ is functionally differentiated into different forms of holoenzyme by interaction with one of multiple molecular species of the σ subunit. Thus, the σ subunit plays a major role in determination of the promoter recognition property of RNA polymerase (reviewed in Ishihama, A. (1979) *Trends Genet.* **4**, 282-286;

* University of Edinburgh, Institute of Cell and Molecular Biology, Edinburgh.

Ishihama, A. (1993) *J. Bacteriol.* **175**, 2483–2489). Up to now, six different species of σ subunit have been identified in *Escherichia coli*. Changes in global transcription pattern are believed to be mainly due to σ replacement on RNA polymerase. In order to get insight into the molecular mechanism(s) of σ subunit replacement, we first observed competition between different σ subunits in an *in vitro* transcription system in the presence of different σ subunits. For this purpose, we purified four molecular species of σ subunit, σ^D (σ^{70} ; the regular σ^{70} encoded by *rpoD*), σ^S (σ^{38} ; stationary-specific σ encoded by *rpoS*), σ^H (σ^{32} ; heat-shock gene-specific σ encoded by *rpoH*), and σ^F (σ^{28} ; flagella gene-specific σ encoded by *rpoF*). Using an *in vitro* single-round transcription assay system, we first measured the level of each σ subunit required for maximum level transcription in the presence of a fixed amount of core enzyme. Results indicate that σ^D forms a holoenzyme at an input molar ratio of 1 : 1 for σ^D :core, while 5- and 3-fold molar excess was required for σ^H and σ^F , respectively. In sharp contrast, about 100-fold molar excess of σ^S was required to saturate core enzyme. The affinity of each σ subunit to core enzyme is being measured directly by glycerol gradient centrifugation. Experiments are also in progress, for measurement of competition between different σ subunits and for analysis of replacement rate between free σ subunit and core-bound σ .

**Promoter Selectivity Control of *Escherichia coli* RNA Polymerase:
Differential Recognition of Osmoregulated Promoters
between $E\sigma^D$ and $E\sigma^S$**

Qingquan DING, Shuichi KUSANO, Nobuyuki FUJITA and Akira ISHIHAMA

Escherichia coli normally exists in vertebrate animals but can also survive in nature, including seawater, where nutrients are scarce and the osmolarity is high. In such hyperosmotic media, *E. coli* increases its internal solute concentration to avoid plasmolysis. A rapid increase in K^+ is the primary response of *E. coli* to osmotic stress. The intracellular glutamate concentration also increases as to balance the increase in K^+ concentration. Transcription regulation of three osmoregulated gene promoters, *osmB*, *osmY* and *proU*, was analyzed in an *in vitro* transcription system using two species of reconstituted RNA polymerase holoenzyme, $E\sigma^D$ and $E\sigma^S$. The *proU* gene is involved in transport of glycine betaine (the secondary response solute to

increase in intracellular osmolarity) while *osmB* and *osmY* encode osmotically inducible periplasmic protein and lipoprotein, respectively.

Results indicate that *proU* (P2) is recognized by Eo^D while both *osmB* and *osmY* are transcribed by both Eo^D and Eo^s . The maximum level transcription was observed above 300 mM K glutamate (but not KCl) for *osmB* and *osmY*, and above 200 mM K glutamate for *proU*. Our studies suggest that, in addition to the increase in glycine betaine concentration, the membrane composition changes, as a response to osmotic shock, by assembly of OsmB and OsmY proteins. Furthermore, the RNA polymerase itself seems to be a sensor of K glutamate concentration.

Structure and Function of Yeast RNA Polymerase II: Attempts for *in vitro* Reconstitution

Yoshinao AZUMA, Makoto KIMURA, Makiko KAWAGISHI,
Masahiro YAMAGISHI and Akira ISHIHAMA

Three forms of nuclear RNA polymerase exist in eukaryotes, each being composed of more than ten polypeptide chains. Structural and functional complexity of eukaryotic RNA polymerases must therefore be higher than that of prokaryotic enzymes. Recent progress in molecular cloning of the genes coding for eukaryotic RNA polymerase proteins from various organisms has revealed that the three large components are structurally related to the β' , β and α subunits of prokaryotic RNA polymerase core enzyme. Since the core enzyme carries the catalytic activity of RNA synthesis, it is reasonable to consider that these three components of eukaryotic RNA polymerases form an assembly with the basic function of RNA polymerization.

To address this question and furthermore to get insight into the function of each polypeptide component of RNA polymerase II, we tried to express in *E. coli* the genes for the putative subunits of RNA polymerase II from the budding yeast *Saccharomyces cerevisiae* [a collaborative study with Dr. R. Young, Whitehead Institute, MIT]. Although we succeeded the expression of small subunit genes, it seems extremely difficult to express two large subunits in *E. coli* because of their instability. In parallel, we cloned the genes encoding three large subunits of RNA polymerase II from the fission yeast *Schizosaccharomyces pombe* (Azuma, Y. *et al.* (1991) *Nucleic Acids Res.* **19**, 461-468; Kawagishi, M. *et al.* (1992) *Nucleic Acids Res.* **21**, 469-473;

Azuma, Y. *et al.* (1993) **21**, 3749–3754), and tried to express the genes in *E. coli*. Again we have so far failed to get the stable expression of two large subunits, which is therefore the major difficulty to be overcome to proceed this line of studies.

Attempts are also being made to set up a reversible dissociation system of purified *S. pombe* RNA polymerase II. Since the reassembly of completely dissociated RNA polymerase may require large amounts of purified proteins judging from the efficiency of well-established assembly system of *E. coli* RNA polymerase, we started to find out reconstitution conditions of partially dissociated RNA polymerase II with low concentrations of urea.

Molecular Dissection of Influenza Virus RNA Polymerase: RNA Synthesis by PB1 Subunit

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

RNA polymerase of influenza virus consists of three P proteins, PB1, PB2 and PA, and catalyzes multiple step reactions in transcription and replication of the genome RNA. Systematic studies have been carried out in this laboratory for identification of the function(s) of each subunit P protein. For this purpose, we established an *in vitro* reconstitution system of RNA polymerase from individual P proteins expressed using recombinant baculoviruses carrying cDNA for each P protein (Kobayashi, M. *et al.* (1992) *Virus Res.* **22**, 235–245). As an extension of this line studies, we examined the activity of viral RNA synthesis in nuclear extracts prepared from insect cells infected with each of the recombinant baculoviruses or combinations of these viruses. RNA synthesis activity was measured using short model RNA templates, each carrying 5'- and 3'-terminal sequences of the genome RNA segment 8.

Results indicate that nuclear extracts expressing only PB1 subunit are able to catalyze viral RNA synthesis in the presence of ApG primer. In contrast, nuclear extracts of cells expressing all three P proteins displayed complete replication cycle of model RNA template in the absence of primer. Thus, we conclude that PB1 is the catalytic unit of influenza virus RNA polymerase

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and it alone is able to catalyze RNA synthesis (Kobayashi, M. *et al.*, submitted for publication). Essentially the same results were obtained using nuclear extracts infected with recombinant vaccinia viruses (Toyoda, T. *et al.*, submitted for publication).

Molecular Dissection of Influenza Virus RNA Polymerase: Photoaffinity Labeling of PB1 with 8-Azido GTP

Yukiyasu ASANO, Tokumi MARUYAMA*, Kiyohisa MIZUMOTO**
and Akira ISHIHAMA

8-Azidopurines are used for monitoring nucleotide-binding sites on proteins at the primary sequence. Upon exposure to a low dose of UV light, the azido moiety of these nucleotide analogues is converted into a highly reactive nitrene, which forms a covalent bond with most amino acids within the cross-linking distance. In this study, we used 8-azido guanosine 5'-triphosphate (8-N₃ GTP) for identification of nucleotide-binding sites on influenza virus RNA polymerase because GTP is the first nucleotide substrate incorporated into 3' termini of primers by virion-associated RNA polymerase (Ishihama, A. *et al.* (1986) *J. Biol. Chem.* **261**, 10417-10421). We found that 8-N₃ GTP is recognized as a substrate by influenza virus RNA polymerase and can be polymerized into RNA chains. Upon exposure of mixtures of radioactive 8-N₃ GTP and RNP cores to UV light, PB1 was selectively photolabeled and this PB1 labeling was competitively inhibited by not only GTP but also GDP, GMP and dGTP. In the presence of ApG primer, the cross-linking of GTP analogue decreased almost half the level of its absence. These observations altogether suggest that the substrate-binding site (S site) is located on PB1 but the GTP analogue can also binds to the primer- and product-binding site (P site) on the same PB1 subunit.

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Molecular Dissection of Influenza Virus Nucleoprotein (NP): Identification of RNA-binding Domain

Makoto KOBAYASHI*, Tetsuya TOYADA, Djanybek ADYSHEV
and Akira ISHIHAMA

Influenza virus nucleoprotein (NP) is associated with the RNA genome at a constant interval of 15–20 nucleotides (Yamanaka, K. *et al.* (1990) *J. Biol. Chem.* **265**, 11151–11155), together forming ribonucleoprotein (RNP) cores. Naked RNA is active as a template for viral RNA-dependent RNA polymerase (Parvin, J. *et al.* (1989) *J. Virol.* **63**, 5142–5152; Honda, A. *et al.* (1990) *J. Biochem.* **107**, 624–628) but NP is required for elongation of RNA chains (Honda, A. *et al.* (1988) *J. Biochem.* **104**, 1021–1026). In order to identify the amino acid sequence involved in RNA binding, we performed north-western blot analysis using a set of N- and C-terminal deletion mutants of NP produced in *Escherichia coli*. The RNA-binding region was mapped between amino acid residues 91 and 188. This region contains a sequence highly conserved among not only NP from A-, B- and C-type influenza viruses, but also a plant virus movement protein (Kobayashi, M. *et al.* (1994) *J. Virol.* **68**, 8433–8436).

Molecular Dissection of Influenza Virus M Proteins: Identification of Growth Control Activity for M1

Jiro YASUDA** and Akira ISHIHAMA

The genome of influenza A virus is composed of 8 RNA segments of negative-polarity. Segment 7 encodes two matrix proteins. In virions, M1 protein forms a membrane matrix between ribonucleoprotein (RNP) cores and lipid envelope, while M2 protein, produced after splicing of M1 mRNA, forms an ion channel in infected cells, thereby controlling transport and assembly of hemagglutinin (HA). Previously, we found that the difference in virus growth rate between fast-growing WSN and slow-growing Aichi is attributed to the M gene (Yasuda, J. *et al.* (1994) *Arch. Virol.* **133**, 283–294). In this study, we tried to determine which of the two M proteins, M1 and M2, encoded by the M gene is responsible for the rapid virus growth. For this

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purpose, we produced recombinant viruses, each carrying a chimeric M gene between Aichi and WSN after transfection of chimeric RNAs, which were produced *in vitro* by transcribing hybrid cDNA (Yasuda, J. *et al.* (1994) *J. Virol.* **68**, 8141–8146). Analysis of the growth rate for these transfectant viruses indicated that the WSN M1 protein is responsible for rapid virus growth. Since M1 binding to RNP interferes with RNA polymerase functions (Hankins, R.W. *et al.* (1989) *Virus Genes* **3**, 111–126; Hankins, R.W. *et al.* (1990) *Res. Virol.* **141**, 305–314), the rate of M1 association to and/or M1 dissociation from RNP is different between WSN and Aichi M1 molecules and this difference may ultimately lead to changes in virus growth rate.

Host Factors for Influenza Virus Growth Control: Cellular Factor(s) for Influenza Virus RNA Replication

Tetsuya TOYODA and Akira ISHIHAMA

Replication of the influenza virus genome involves two discrete step reactions: vRNA-directed primer-independent (unprimed) synthesis of cRNA; and cRNA-directed unprimed synthesis of vRNA. Nuclear extracts from both MDCK and HeLa cells infected with influenza virus A/PR/8/34 exhibited model vRNA template-directed unprimed synthesis of not only complementary model RNA (model cRNA) but also model vRNA (Toyoda, T. *et al.* (1994) *Arch. Virol.* **136**, 269–286). Ribonucleoprotein (RNP) complexes with the replication activity were isolated from these nuclear extracts by glycerol gradient centrifugation in the presence of 0.1 M KCl. At 0.5 M KCl, however, these complexes were dissociated into stripped RNP and soluble protein fractions. The soluble protein fraction contained the activity of exogenous template-dependent unprimed RNA synthesis, indicating that the RNA replicase is dissociated from RNP upon exposure to high salt concentrations. On the other hand, the high salt-treated RNP catalyzed only primer-dependent RNA synthesis, but regained a low level activity of exogenous template-dependent unprimed RNA synthesis by adding nuclear extracts from uninfected cells, suggesting that host factor(s) are involved in the functional conversion of influenza virus RNA polymerase from transcriptase to replicase.

Host Factors for Influenza Virus Growth Control: Antiviral Activity of Mouse Mx1 Protein

Tetsuya TOYODA, Yukiyasu ASANO and Akira ISHIHAMA

The Mx1 protein is an interferon-inducible nuclear protein of mice, which confers resistance to influenza virus infection. Using Mx1 purified from both the Mx1 gene-expressing *E. coli* and interferon-treated mouse liver, we demonstrated that mouse Mx1 is a GTP-binding protein with GTPase activity (Nakayama, M. *et al.* (1991) *J. Biol. Chem.* **266**, 21404–21408; Nakayama, M. *et al.* (1992) *Virus Res.* **22**, 227–234) and forms self-assemblies (Nakayama, M. *et al.* (1993) *J. Biol. Chem.* **268**, 15033–15038). In order to determine whether the GTPase and self-assembly activities are required for antiviral function of Mx1, we constructed mutant Mx1 with mutations in the regions required for GTPase and self-assembly and examined their antiviral activity.

For this purpose, we established cell lines expressing wild-type and mutant Mx1 proteins each carrying single amino acid substitution. A mutant Mx1 (S50I) with Ser-to-Ile substitution at amino acid residue 50 within the GTP-binding motif has reduced GTPase activity (Nakayama, M. *et al.* (1993) *J. Biol. Chem.* **268**, 15033–15038). Wild-type Mx1 forms big dots of self-assemblies in induced nuclei, while the S50I formed linear threads of Mx1 in cell nuclei. The growth of influenza virus was not affected so much even in the presence of S50I Mx1. Another mutant (C71S) with an amino acid substitution of Cys-to-Ser at position 71 within the self-assembly domain showed diffuse distribution in the nuclei. The virus growth inhibition was not affected by the C71S mutation, suggesting that the self-assembly domain is not involved in antiviral activity (Toyoda, T. *et al.*, submitted for publication).

Abnormal Integrity of Nucleolus Associated with Cell Cycle Arrest in A Mouse Cell Mutant of The Temperature-Sensitive Ubiquitin-Activating Enzyme E1

T. SUDHA*, Sumiko KANEDA, Yukiko NAGAI, Fumiaki YAMAO,
Hideo TSUJI**, Masazumi SAMESHIMA***,
Yoichi MATSUDA** and Takeshi SENO

The ubiquitin-activating enzyme, E1, catalyzes the first step of the sequential transfer of ubiquitin to target proteins. The ubiquitin system is essential for a wide spectrum of cellular phenomena, including cell cycle control, induced error prone DNA repair, and the stress response.

Temperature-sensitive (ts) mutants of the ubiquitin-activating enzyme, E1, isolated from a mouse cell line FM3A including the authentic ts-E1 mutant, ts85, give rise to different arrest points in the cell-cycle, although the mutants fall into the same complementation group. The mutation site in the E1 coding region was mapped to show that each mutant had a different single amino acid change but the mutation sites were clustered somewhat at the C-terminal half of the E1 protein (Kaneda *et al.*, submitted). However, the molecular consequences leading to different cell cycle arrests have not yet been explained.

The long term goal is to identify the target proteins, whose ubiquitination via a specific E2(s) is impaired due to the mutation of E1, which leads to a specific terminal mutant phenotype. We attempted to identify genetic traits of a more qualitative nature other than the ritual flow cytometric profile. Through cytological analysis, we observed with Azure C staining, a morphological change in the nucleolus in G2-arrested mutant ts85 cells after exponentially growing cells were incubated for 10h at the nonpermissive temperature (39°C). The altered nucleolar structure consisted of a U-shaped or ring-shaped arrangement of nucleolar lobes with an unstained region in the center. Silver staining of the nucleolar organizer region (NOR) and fluorescence *in situ* hybridization analysis with rDNA, both gave signals in Azure C-positive regions as expected. Electron microscopic examination revealed that a cloud of unidentified electron-dense particles of homogeneous size (diameter, approx. 70 nm) was present in the Azure C-negative region

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surrounded by the nucleolar lobes (Sudha *et al.*, Chromosome Res., in press). Immunochemical and biochemical characterizations of the electron-dense particles are in progress.

When the arrested ts85 cells were released into metaphase, we observed a peculiar pulverization-like figure consisting of several chromosomes arrayed together. NOR-silver staining, fluorescence *in situ* hybridization studies with rDNA, and centromeric satellite DNA, consistently demonstrated that the pulverized regions coincided with those of NORs. Judging from the results, this pulverization may reflect under-condensation of the NORs associated with delayed dissolution of the nucleolus during G2-M transition (Sudha *et al.*, Chromosome Res., in press). Attempts were made to substantiate this possibility, by chasing the abnormal nucleolar structure following release from the arrest. It appears that the ubiquitin system plays a role in the dissociation processes of the nucleolus, possibly involving the NOR.

**Cell cycle control by the ubiquitin system in mammalian cells;
the ubiquitin-activating enzyme, E1, is targeted
by cdc2 kinase**

Yukiko NAGAI, Sumiko KANEDA, Takeshi SENO
and Fumiaki YAMAO

The ubiquitin system is essential for cell growth since the mutational block of one of the ubiquitin-conjugation steps is lethal to cells, arresting them at specific stages of the mitotic cell cycle. Historically, ts85, a temperature-sensitive (ts) mutant of the mouse mammary carcinoma cell line FM3A, has been shown to be defective in the ubiquitin-activating enzyme and arrested at the late S to G2 period of the cell cycle under restrictive temperatures. Recently, other types of E1 mutants from the same cell line were also reported; some are arrested predominantly at S phase while others are arrested at all phases of G1, S and G2/M, implying that multiple steps during the cell cycle are differentially regulated by the ubiquitin system. In addition, a series of key proteins regulating the cell cycle were shown to be ubiquitinated. Cyclin B, a regulatory subunit of the mitosis promoting factor, has been shown to be degraded by the ubiquitin-dependent proteolytic pathway. p53 and c-Mos, have also been reported to be ubiquitinated at least *in vivo*. Thus, the conjugation of ubiquitin to target molecules is associated with

various functions of cell cycle progression, presumably through the proteolytic breakdown of the proteins involved in the essential function of the processes.

It has been well established that the transitions of the critical points in the eukaryotic cell cycle is controlled by a series of cyclin dependent protein kinases. A variety of mammalian cyclins and kinases associated with them have been identified. Their functions, however, are largely unknown except for cyclin B/p34cdc2. Which cyclin associates with which kinases, the timing of the associations, and the *in vivo* substrates of the kinases are a matter of great interest. To investigate the coupling of the ubiquitin system and the cyclin dependent kinases during cell cycle control, we examined the cdc2 kinase mediated phosphorylation of the ubiquitin activating enzyme E1 in mouse cells. In the amino acid sequence of mouse E1 enzyme deduced from its cDNA, at least five possible target sites of phosphorylation by cdc2 kinase were found. The E1 enzyme from mouse cells proved to be phosphorylated *in vivo*. This phosphorylation was shown to be mediated by cdc2 kinase since FT210, a temperature-sensitive mutant of cdc2 kinase, failed to achieve phosphorylation. *In vitro* phosphorylation of cellular and recombinant E1 proteins by immuno-purified cdc2 kinase, and their peptide mapping analysis indicated that the serine residue at the fourth position of the E1 amino acid sequence was the phosphorylation site both *in vivo* and *in vitro*. These results strongly suggest that the E1 enzyme is a substrate of cdc2 kinase in the cell. Such a post-translational modulation of E1 may afford enzyme specific functions towards the recognition of cell cycle-dependent ubiquitin pathways.

Cell cycle control by the ubiquitin system in mammalian cells; Ubiquitin pathways specific for DNA synthesis

Sumiko KANEDA, Yukiko NAGAI, Takeshi SENO,
Tatsuo YAGURA* and Fumiaki YAMAOKA

Ubiquitin is activated by the ubiquitin-activating enzyme, E1, and ATP is transferred from the E1 enzyme to one of a family of isoenzymes called ubiquitin conjugating enzymes (E2's) which catalyze the conjugation of ubiquitin to specific target proteins. There is increasing evidence that each E2

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enzyme has a preferred target protein(s). Detailed analysis of pleiotropic phenotypes of mutations of the ubiquitin-activating enzyme (E1) showed that the inhibition of the ubiquitin system caused cell cycle arrest not only at phase G2/M but also at G1 and S in mammalian cells. To identify the ubiquitin pathway associated with each phase of the cell cycle, we examined *in vitro* ubiquitin transfer from E1 enzyme to E2 conjugating enzymes in the extracts of various ts E1 mutants of the mouse cell line, FM3A. Ubiquitin transfer to an E2 protein of approximately 20 kDa was found to be reduced specifically in the lysates of mutant cell lines which were predominantly blocked in the S phase at restrictive temperature (39°C). From such S phase-specific E1 mutants, we isolated partial revertants by selecting clones that could grow at the semi-restrictive temperature, 37°C, but not at 39°C. The arresting points during the mitotic cell cycle of these revertants at restrictive temperatures were G1 and G2/M phases, specifically suppressing the defect in S-phase progression of the parental mutant cell lines. In the extract of the partial revertants of E1 mutants, the reduced ubiquitin transfer to the particular E2 was restored. Thus, E2 (20 kDa) appears to be involved specifically in DNA synthesis. The E2 proteins including that of 20 kDa, were purified and subjected to partial amino acid sequences for cloning of their genes. A 2.6 kb mouse E1 cDNA encoding the E1 protein lacking the first 255 amino acid residues, was transfected to various E1 mutant cells, and found to complement their defect in S-phase progression, but not in G2-phase progression. This suggested a domain for the E1 enzyme, which is necessary for recognition of the S phase-specific ubiquitin pathway, in the C-terminal two thirds of the enzyme. The S phase-specific ubiquitin pathway is possibly mediated by the E2 enzyme described above. On the contrary, the N-terminal fragment of the E1 enzyme seems to be essential to the G2 phase-specific ubiquitin pathway, which supports the fact that the cdc2 kinase-mediated phosphorylation of the E1 enzyme is at its fourth serine residue, if the phosphorylation has any functional role at all.

Cell Cycle Control by the Ubiquitin System in Yeast *Saccharomyces cerevisiae*

Tsutomu KISHI, Fumiaki YAMAO and Takeshi SENO

Yeast cells harboring a temperature-sensitive mutation in the CDC34 are

arrested at the G1/S phase of the cell cycle. CDC34 has already been shown to code for the ubiquitin-conjugating enzyme E2 (UBC3). Therefore, degradation of some specific target proteins has been suggested to be essential for cell cycle progression.

The aim of this project is to isolate and characterize the proteins to be ubiquitinated by the Cdc34. In addition, we are trying to isolate the gene(s) of the protein(s) that interacts with Cdc34.

This is a new project in this laboratory. The approaches and present results are as follows.

(1) Isolation of extragenic suppressor of *cdc34*^{ts}: We have isolated four suppressor strains and one of the four genes was cloned and is being analyzed.

(2) Cloning of the genes whose products interact with the Cdc34: With the use of the yeast two-hybrid system, several candidates were screened, and their characterizations are under way.

(3) Detection of target proteins of the Cdc34 by using *in vitro* system: Cdc34 was expressed and purified from *E. coli*. First, this was mixed with labeled ubiquitin and yeast crude extracts prepared from the G1 phase of the cell cycle. However, the protein by CDC34 was not detected. Now, we are trying to detect the protein using yeast nuclear extracts.

Reference

F. YAMAO, Y. NAGAI, S. KANEDA, S. YOSHIDA, and T. SENO. Conditional resistance to thymineless death predominantly selects DNA synthesis- deficient mutants of mammalian cells. *Mutat. Res.*, **289**: 83-89 (1993).

Mechanism of mRNA Capping Reaction

Kiyohisa MIZUMOTO

The yeast mRNA capping enzyme is composed of two polypeptide chains of 52 kDa (α) and 80 kDa (β), responsible for the activities of mRNA guanylyltransferase and RNA 5'-triphosphatase, respectively. Recently, we have isolated the gene encoding the α subunit (*CEG1*) (Shibagaki, Y. *et al.* (1992) *J. Biol. Chem.* **267**, 9521-9528). In this study, the *CEG1* gene was expressed in *Escherichia coli* under the control of a bacteriophage T7 promoter and the α subunit protein was purified to near homogeneity. The enzyme-³²P]GMP covalent reaction intermediate of the recombinant α subunit was digested with lysylendopeptidase and trypsin, and the resulting [³²P]GMP-

peptide was purified. After the [^{32}P]peptide was converted to [^{32}P]phosphoryl-peptide through periodate oxidation followed by β -elimination, it was hydrolyzed with alkali to give a [^{32}P]phosphoamino acid. [^{32}P](N- ϵ)phospholysine was detected as the only phosphoamino acid, indicating that GMP in the enzyme-GMP intermediate is bound to a lysine residue via a phosphoamide linkage, as previously observed with rat liver and vaccinia virus capping enzymes (Mizumoto, K. and Kaziro, Y. (1987) *Prog. Nucl. Acid Res. Mol. Biol.* **34**, 1-28). Microsequencing of the [^{32}P]GMP-peptide revealed that the GMP binding site was localized to a region between amino acids 60 and 75, which contained an internal trypsin-resistant lysine residue at position 70. The *CEG1* gene was subjected to site-directed mutagenesis *in vitro* and mutant polypeptides were expressed in *E. coli*. Substitution of Lys 70 to His or Ile abolished ability to form the enzyme-GMP complex. These results indicate that the GMP binding site in the α subunit of yeast capping enzyme is localized to a lysine residue at position 70.

Transcription of Sendai Virus (HVJ) Genome

Kiyohisa MIZUMOTO

Sendai virus (HVJ), a member of paramyxovirus group, contains a single non-segmented negative-strand RNA genome of approximately 15 kb-long. The genetic information of this RNA genome is expressed through at least 6 monocistronic mRNA species that are transcribed by a virion associated RNA-dependent RNA polymerase. We are studying the mechanism of transcription of the HVJ genome using an *in vitro* transcription system with purified virus particles, in which mRNA synthesis is almost entirely dependent on host factors. In HVJ-infected cells, an abundant, short and uncapped plus-strand leader RNA encoded by the first ~ 50 nucleotide sequence of the 3'-genomic terminus is synthesized. However the significance of leader RNA synthesis has not been understood yet. In the present study, to investigate the correlation of plus-strand leader RNA synthesis with mRNA production, we characterized the synthesis of short RNA species in the *in vitro* transcription system.

Transcription reaction was carried out in the absence of UTP to discriminate the leader transcripts from nascent short mRNA chains. Several discrete short RNAs ranging 20-25 nucleotides in length, which cor-

responded to the sizes expected from premature termination points at U residues, were synthesized. The 5'-termini of major RNA species were found to be (p)ppA- by post-labeling procedure with mRNA capping enzyme. They formed duplex molecules when annealed with the minus-strand genome RNA (vRNA). These results support that the short RNAs synthesized *in vitro* are the products transcribed from the 3'-end leader regions of the genome and have the positive polarity. The synthesis of these short RNAs was almost completely dependent on the addition of host cell extracts. An activity that stimulates the leader RNA synthesis was partially purified from HeLa cells and was shown to be separated from the host factor activity required for mRNA synthesis.

***Wx* gene Expression is Enhanced in Response to Cool Temperatures**

Hiro-Yuki HIRANO, Motoji KUNI-EDA* and Yoshio SANO

The *wx* locus controls amylose synthesis in the endosperm and pollen. We found that *Wx* gene expression was enhanced by exposing plants to cool temperatures during seed development and the amylose content increased in mature seeds causing a lower quality of rice.

Rice plants were grown during seed maturation at two different temperatures, 28°C (normal) and 18°C (cool), and the amount of *Wx* protein and amylose content in mature seeds were estimated. The results indicated that not only amylose content but also the amount of *Wx* protein in seeds matured at 18°C were higher than those matured at 28°C.

Northern blot analysis showed that the steady state level of the *Wx* transcript increased about 2- or 3-fold by exposing the plants to a temperature of 18°C and it decreased to the initial level after the plants were grown at 28°C for 2 days. These results suggest that the response to cool temperatures is regulated at the transcriptional level of the *Wx* gene and this regulation is reversible.

Next, plants were grown at 18°C for different lengths of time, 1, 2, 4, 8, or 12 days from the 7th day after anthesis, and then grown at normal temperatures (in the field) until seed maturation. The longer plants were exposed to a temperature of 18°C, the higher the levels of amylose and *Wx* protein accumulated in mature seeds. The extent of increase in amylose content was positively correlated with that of the *Wx* protein level.

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These results indicated that the *Wx* gene was activated by cool temperatures, causing the elevation of the *Wx* protein level. Thus, the elevated levels of *Wx* protein, the enzyme for amylose synthesis, resulted in a higher content of amylose in matured seeds.

The *Wx* gene transcript increased at 10 and 18°C but not at 4 or 35°C. These results indicated that cold or heat shocks did not affect the expression levels of the *Wx* gene. Thus, the *Wx* gene may be activated only by cool temperatures.

Gene regulation responding to slight differences of temperatures may be very important in the adaptation of plants for normal growth. The *Wx* gene may be one of the best clues for investigating the regulation in response to slight differences of temperatures.

Kinetic Study on Transcription Using Immobilized Operons: Early events in transcription elongation

Tomoko KUBORI and Nobuo SHIMAMOTO

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

In the *E. coli* transcription system, we found the 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 32nt from a promoter, a misincorporation at 6nt 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 holoenzyme and packed in a 5 μ l column. Reactions were started by eluting the column with a buffer

containing substrates. Kinetics of releases of abortive transcripts and and 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 was proved by direct visualization of single molecules of the enzyme. To address the question whether sliding motion is common to other DNA binding proteins, we applied a similar single molecule dynamics to a bacterial repressor protein, *P. putida* CamR. 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 three positions on a λ DNA, one was its cognate operator cloned on λ DNA, and the other two were likely to be regions in λ homologous to the operator. The DNA fragments containing the homologues regions competed with the cognate sequence in a gel-shift assay when they were added 400 and 4,000 times excess to the cognate fragments. They are, therefore, very weak binding site of CamR by gel-shift assay, but CamR stayed on them for a long time in the direct visualization, showing the visual detection is a very sensitive method.

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.

Accumulation of single stranded DNA in cells induces SOS response, one of the most intensively studied stress responses in bacteria. The *ssb* gene has SOS box (LexA binding site) around -35 position of minor promoter (P1). In contrast it has not been clear whether the synthesis of SSB protein is SOS inducible. We directly examined both the synthesis rate of SSB protein and the level of SSB mRNA upon exposure to SOS inducing agents. The protein synthesis rate increased by 2 fold upon SOS induction. Amounts of transcripts from P1 promoter increased by 10 fold but that from major constitutive P2 promoter was hardly affected: total amounts of mRNA increased by 2 fold. These results indicated that SSB protein is SOS inducible and the induction occurs primarily at transcription level likely as the other SOS inducible genes.

Diploidy of *Drosophila* Imaginal Cells is Maintained by a Transcriptional Repressor Encoded by *Escargot*

Naoyuki FUSE, Susumu HIROSE and Shigeo HAYASHI

The *Drosophila escargot* (*esg*) gene encodes a C2-H2 type zinc finger protein. *esg* is expressed in imaginal discs and histoblasts in the embryo and in the larva. In some *esg* mutants, abdominal histoblasts become polyploid. In our previous work, we suggested that the role of *esg* is to maintain the diploidy of the imaginal cells. We have found that *esg* encodes a DNA binding protein with high affinity to G/ACAGGTG, the consensus sequence for the bHLH family transcription factor. DNA binding activity is essential for *esg* function *in vivo* since one of the strong embryonic lethal alleles is caused by an amino acid change within the zinc finger which reduces affinity to DNA. In cultured cells, *esg* protein strongly inhibits transcriptional

activation by a heterodimer of bHLH proteins SCUTE and DAUGHTER-LESS, suggesting that *esg* may regulate processes dependent on bHLH proteins in development. In embryos, *esg* protein expressed by the heat shock promoter can rescue the polyploid phenotype of abdominal histoblasts, demonstrating that the phenotype is due to a loss of *esg* function. *esg* expression is required continuously during the larval period for efficient rescue. Ectopic expression of *esg* in the salivary gland inhibited endoreplication. These results suggest that *esg* is involved in transcriptional inhibition of endoreplication.

Control of *esg* Expression During Development

Yoshimasa YAGI*, Susumu HIROSE and Shigeo HAYASHI

esg is expressed in a complex pattern during embryogenesis. In that pattern, expression in the imaginal cell primordia is thought to be responsible 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 understand the role of *esg* in those system, it is important to know how such expression patterns are established. To analyze the regulatory mechanism of *esg* expression, we have started to dissect the cis-control region for enhancers responsible for expression in imaginal tissues, testes, and trachea. A lacZ fusion construct containing 3.5 kb of *esg* promoter was introduced into the germ line to establish transformants and lacZ expression was examined in embryo, larva and adult. This construct did not reproduce the expression pattern in imaginal and tracheal cells, but we found that lacZ was expressed in a subset of adult testis, similar in pattern to several *esg* enhancer trap lines. This expression in testes is probably responsible for the function of *esg* in fertility. We are currently making more constructs to search further upstream for other enhancers. Once we find such enhancers, we are planning to analyze interaction with genes such as *wingless*.

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Analysis of the Role of the Tip Cells in Tracheal Morphogenesis

Yasuhiro SHIGA* and Shigeo HAYASHI

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 is expressed in a single cell at the tip of each growing tracheal branch. These cells will contact each other and the joining occurs. These cells are called the tip cells and are thought to play important roles in pathfinding, target recognition and the fusion process. To elucidate the role of the tip cell, we started the following experiments. i) Some *esg* mutants are defective in tracheal morphogenesis. We found that *esg* is expressed in the tip cell and its expression is down regulated during fusion suggesting *esg* plays an important role in that process. The exact role of *esg* is being examined using various genetic and molecular techniques. ii) Direct ablation of the tip cell should be an effective way to assess the role of the tip cell. The laser microscope system recently set up in NIG will be used for this experiment. To identify targets for ablation, we are making constructs with various promoters fused to green fluorescent protein (GFP). GFP has been used successfully as a marker in live *C. elegans*. The constructs are now being injected into flies to establish transformants. From these projects we hope to solve the problem of pattern formation in this relatively simple organ.

The Function of *argos* in Regulating Cell Fate Decisions during *Drosophila* Eye and Wing Vein Development

Kazunobu SAWAMOTO**, Hideyuki OKANO**, Yoshitaka KOBAYAKAWA***,
Shigeo HAYASHI, Katsuhiko MIKOSHIBA**
and Teichi TANIMURA***

The *Drosophila argos* gene, which was previously called *strawberry*, encodes a secreted protein with an EGF motif. *argos* is involved in several developmental processes regulating cell-cell interactions such as eye morphogenesis. Loss-of-function mutations in the *argos* gene cause an increase in the number of photoreceptor cells and cone cells, impaired retinal projections to the optic

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lobe, and the formation of extra veins. We found the ubiquitously expressed *argos* product restored all these loss-of-function phenotypes. Overexpression of *argos* in the wild-type background resulted in a reduced number of photoreceptor cells, cone cells, and pigment cells, which are phenotypes opposite to those of the loss-of-function mutants. The *argos* gene is expressed in developing wing veins. Ubiquitous *argos* expression caused loss of veins in a dose dependent manner. This phenotype was enhanced by the loss-of-function *rhomboid* mutation, implying the possibility that *argos* and *rhomboid* play key roles in a common pathway for normal wing vein formation. We propose that *argos* acts as an inhibitory signal for cellular differentiation in the developing eye and wing. This work will be published in *Developmental Biology* (Sawamoto *et al.*, (1994) *Dev. Biol.* in press).

II. MICROBIAL GENETICS

The Nicking Reaction in Rolling Circle Replication Requires a Single-Stranded Nicking Region and a Double-Stranded Binding Region

Atsushi HIGASHITANI and Kensuke HORIUCHI

The core origin for plus strand DNA replication of filamentous bacteriophage f1 binds the initiator protein (gpII), which subsequently introduces a specific nick in the plus strand. The origin consists of a nicking region and a binding region. We previously proposed that the nicking reaction with gpII occurs via the following steps: 1) binding of gpII forms bent DNA structures in the origin (no requirement for either Mg^{2+} or negative superhelicity for this step); 2) the DNA bending induces melting of the duplex structure around the nicking site if the DNA is negatively superhelical; and 3) a specific nick is introduced within the melted region in the presence of Mg^{2+} (Higashitani, A., *et al.*, (1993) *Ann. Rep. of NIG*, **43**, 36–38).

In this study, the ability of gpII to cleave DNA when the nicking site is in a single-stranded state was tested using synthetic oligodeoxyribonucleotides as substrates. One substrate used was a 5'-terminally labeled 39-mer that carries the f1 plus strand sequence ranging from position -9 to +30 (relative to the nicking site), which includes the entire gpII binding sequences β , γ and δ . Upon cleavage at the nicking site, this substrate should yield a 9-mer fragment from its 5' end. The results showed that an extremely weak cleavage reaction took place yielding an 8-mer and a 9-mer in approximately equal amounts. The reaction was extremely slow, continuing for more than 120 min, and only about 1 per cent of the substrate was cleaved in 120 min. When RFI was the substrate under the same conditions, the reaction was completed within 1 min, with 60 percent of the substrate nicked. To make the gpII binding domain double-stranded, while keeping the nicking domain single-stranded, the 39-mer was annealed to a 25-mer oligonucleotide of the f1 minus strand sequence, ranging from position +29 to +5, and was subsequently used as a substrate for the nicking reaction. This annealed product was completely cleaved by gpII in less than 5 min to yield the 9-mer. The results indicate that efficient nicking requires the presence of the gpII

binding domain in a double-stranded form. Since restriction fragments (linear duplexes) carrying the complete f1 origin are very poor substrates for gpII nicking (Higashitani, A., *et al.*, (1992) *Nucl. Acids Res.* **20**, 2685–2691), the results also indicate that the region immediately flanking the nicking site needs to be single-stranded for the cleavage reaction to occur. In fact, when the entire region of the 39-mer was made double-stranded by annealing with a complementary oligonucleotide, the cleavage reaction was strongly inhibited. These results indicate that efficient nicking reaction with gpII takes place only when the substrate DNA carries a single-stranded nicking region and a double-stranded binding region. It is thus likely that the duplex structure around the nicking site must melt first for the nicking to occur in the natural substrate, f1 RFI.

**SOS Induction in *Escherichia coli* by Single-stranded DNA of
Filamentous Phages: Monitoring Through Cleavage of
LexA Repressor**

Nahoko HIGASHITANI, Atsushi HIGASHITANI
and Kensuke HORIUCHI

We previously reported that the SOS response is induced in *Escherichia coli* through infection with mutant filamentous phages that are defective in initiation of the complementary (minus)-strand synthesis (Higashitani, N., *et al.*, (1992) *J. Bacteriol.* **174**, 1612–1618). In these mutants, the rate of conversion of parental single-stranded DNA (ssDNA) to the double-stranded replicative form (RF) was extremely slow. Conversion to RF of the ssDNA molecules that were produced by rolling-circle replication also appeared very slow. These observations strongly suggested that it was phage ssDNA that induced the SOS response. However, since the mutants were still able to synthesize RF to some extent, and since we monitored the SOS induction through the expression of SOS genes, it was not possible to exclusively conclude that the phage ssDNA alone induced the SOS response. In the present study, we monitored the SOS induction through cleavage of the LexA repressor, which represents the first step of SOS response, after phage infection in the presence of chloramphenicol. The LexA levels in the infected cells were measured by Western blotting using the anti-LexA antibody. The results indicated that infection with the mutant phage reduced the cellular

level of LexA within a few minutes. Infection with the wild-type phage did not affect the level of LexA. These results unambiguously show that the parental ssDNA by itself can induce the SOS response in infected cells.

Stabilization of DnaA Protein with Chaperone Proteins

Seiichi YASUDA

DnaK is a major heat shock protein of *Escherichia coli* and participates in a variety of cellular processes including cell division and DNA replication. We have previously shown that DnaK protects the initiator protein DnaA from heat inactivation by forming a complex with it. DnaA which is normally inactivated completely by heating at 40°C for 10 min, becomes resistant to heating at 60°C for 10 min in the presence of DnaK. In these experiments, the activity of heated DnaA was assayed through *in vitro* replication of *oriC* DNA using a crude enzyme fraction. When DnaA was assayed by *oriC* DNA-binding without using the crude enzyme, the resistance of DnaA against heating at 60°C in the presence of DnaK was not observed. This implies that the crude enzyme fraction contained activity which reactivates DnaA that had been heated at 60°C in the presence of DnaK. An assay system for this reactivation activity was established. None of the *E. coli* heat shock proteins, DnaK, DnaJ, GrpE, GroES or GroEL could substitute for the crude enzyme fraction. The reactivating activity is being isolated from the crude enzyme fraction of an *E. coli* strain carrying a temperature-sensitive *dnaA* mutation.

A Mutation that Suppresses Thermosensitive Growth Defect at Low Osmolarity of the *Escherichia coli* Δ *prc* Mutation Is in the Gene Coding for Penicillin-Binding Protein 7

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

The *prc* gene of *Escherichia coli* codes for a periplasmic processing enzyme that removes the C-terminal 11 residues from the precursor form of penicillin-binding protein 3 (PBP 3), a murein-synthesizing enzyme essential for septum formation. A *prc* deletion mutant shows thermosensitive (ts) growth

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at low osmolarity. However, it does not form long filaments, a typical cellular morphology found whenever the function of PBP 3 is inhibited (Hara *et al.* (1991) *J. Bacteriol.* **173**, 4799–4813). The processing defect of PBP 3 seems unlikely to be directly related to the growth defect of the Δprc mutant.

We expected that the gene(s) of the target(s) of the Prc vital function other than PBP3 might be found as suppressor mutation(s) of Δprc , and isolated 22 independent, spontaneous suppressor mutants that restored growth on salt-free L agar plates at 42°C. None of them restored PBP 3 processing. As for the leakage of periplasmic proteins into the medium by the Δprc mutant, only partial suppression was observed. When a prc^+ -carrying mini-F plasmid was introduced, they all showed ts growth at low osmolarity again. All the isolates looked very much alike, and one of them was further studied.

First Δprc was displaced with the wild-type allele that was co-transduced with *eda* by P1 phage. The resultant strain was ts on salt-free L agar. Chromosomal Tn10 insertions co-transducible with this ts mutation were selected from a random Tn10 insertion pool. Transductions of these Tn10 insertions from strains that also carried the ts mutation to a Δprc strain gave thermoresistant transductants at expected co-transduction frequencies, showing that this ts mutation was identical to the suppressor. It seems that this mutation and Δprc similarly affect the protection of cells against thermal and osmotic stresses and are mutually suppressors of each other. The most tightly linked Tn10 insertion (90% co-transducible) was mapped to the region a little beyond *his* at 44 min in a clockwise manner by Hfr matings, and then by P1 transduction, to the vicinity of *fruA* at 47 min (75% co-transducible). This Tn10 insertion was co-transducible with other 21 suppressor mutations at high frequencies. All these 22 suppressors isolated independently are most probably allelic to one another.

Among cosmids carrying chromosomal fragments around the 47 min region in the ordered cosmid library (Tabata *et al.* (1991) *J. Bacteriol.* **171**, 1214–1218) some were found to complement the ts mutation, and a 3.5-kilobase *EcoRI-HindIII* fragment carrying the complementing ability was subcloned. The nucleotide sequence of a part of this fragment has already been reported together with the adjacent *dld* gene (Campbell *et al.* (1984) *Eur. J. Biochem.* **144**, 367–373). It included a 3'-terminal region of an open reading frame (ORF). Removal of this region abolished the complementing

ability, indicating the ORF is the wild-type allele of the suppressor, which we named *sprA* (suppressor of *prc*). Its precise map position was determined as 47.8 min by comparison with the revised *E. coli* genomic restriction map (Rudd (1992) in *A Short Course in Bacterial Genetics*, pp. 2.3–2.43, Cold Spring Harbor Laboratory Press). The product of the *sprA* gene was identified as a protein of about 31 kilodalton (kDa) by a maxicell experiment.

Examination of the deduced amino acid sequence of the C-terminal region revealed high homology to the C-terminal region of *E. coli* PBPs 5 and 6. Through PBP assay using radioactive penicillin we found that a *sprA*-carrying plasmid led to an overproduction of PBP 7 of about 31 kDa in an *ompT* mutant, and also of PBP 8 of about 28 kDa in a wild-type strain. The latter was reported to be a degradation product by OmpT protease of the former during envelope fraction preparation for PBP assay (Henderson *et al.* (1994) *J. Bacteriol.* 176, 256–259). PBP 7 was not reproducibly detected unless overproduced, and has not been studied in detail. Sequencing of the whole *sprA* gene, cloning and sequencing of the suppressor allele, and the study of the effect of the Δprc mutation on PBP 7 is needed to elucidate the possible function of PBP 7 against thermal and osmotic stresses and the mechanism of the suppression.

A General and Fast Method for Mapping Mutations on the *Escherichia coli* Chromosome

Atsushi HIGASHITANI, Nahoko HIGASHITANI, Seiichi YASUDA
and Kensuke HORIUCHI

One of the first steps in characterizing newly isolated mutations is to determine their positions on the chromosome map. In *Escherichia coli*, the mapping is often carried out using genetic methods such as Hfr crosses or transduction with P1 phage, which usually require construction of strains harboring a suitable set of genetic markers. With Kohara's ordered library of DNA clones (Kohara, Y., *et al.*, (1987) *Cell*, 50, 495–508) in hand, any piece of chromosomal DNA or its transcript can now be mapped easily through

Fig. 1. Schematic illustration of Tn10 and the mapping method. Horizontal arrows, #ah1 and #ah2, indicate 31-mer (5'-GAT TTT TAC CAA AAT CAT TAG GGG ATT CAT C-3') and 28-mer (5'-CAT TAA GTT AAG GTG GAT ACA CAT CTT G-3') oligonucleotides, respectively, used as PCR primers.

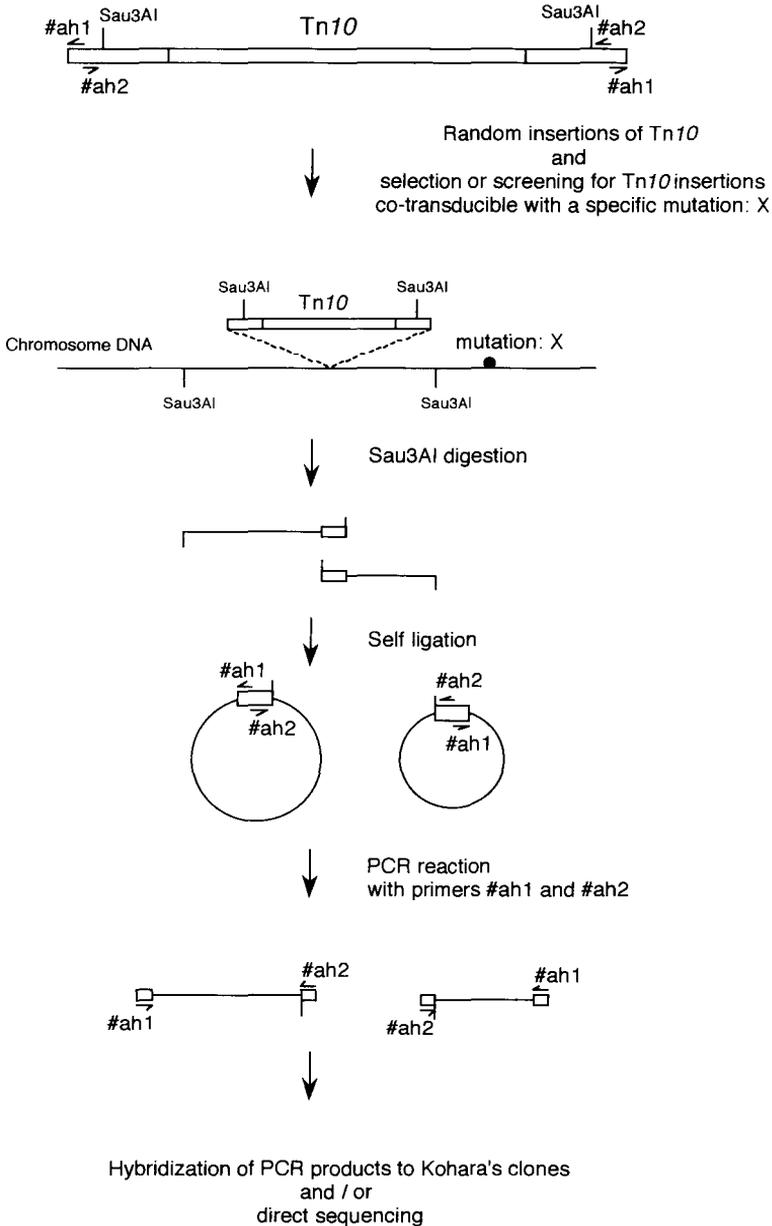


Fig. 1.

hybridization to membranes that retain immobilized DNA of Kohara's clones ("the *E. coli* gene mapping membrane" commercially available from Takara). Therefore, if a general method to obtain DNA fragments from the region of mutation were available, mapping of mutations would be greatly facilitated. We have developed a general and fast method for determining approximate positions of mutations on the *E. coli* chromosome. The method consists of the following steps: (i) random insertion of transposon Tn10 into the mutant chromosome by infection with $\lambda 496(b221, cI171 :: Tn10, O_{am29})$, (ii) selection or screening for the Tn10 insertion which is co-transducible with the given mutation, (iii) inverse PCR amplification of the chromosomal region flanking the Tn10 insertion site, and (iv) hybridization of the PCR products to the ordered set of Kohara's clones (Fig. 1). Tn10 carries 1400 bp inverted repeats, IS10, on its ends, and each repeat carries a *Sau3AI* site at a distance of about 70bp from the end (Kleckner, N. (1983) in *Mobile Genetic Elements*. Academic Press, pp 261–298). Hence, the border sequences between chromosomal DNA and Tn10 can be cut out by *Sau3AI* digestion. The digested DNA are circularized by ligation, and are subsequently used as the template for inverse PCR with two primers #ah1 and #ah2, which carry base sequences found in the segment between the *Sau3AI* site and the end of Tn10 (see Fig. 1).

In a model experiment, two genetic markers *str^R* and *lacZ*, a selectable and a non-selectable mutation, respectively, were easily mapped to their known sites using the present method.

Molecular Mechanism in Determination of Timing of Cell Division in *Escherichia coli* K12

Akiko NISHIMURA

Cell division in normally growing cells strictly takes place 20 minutes after DNA replication is terminated. This periodic process is not disturbed under extensive growing conditions. Cells lacking DNA are found at a remarkably low frequency in cultures of wild type strains. This fact suggests that the cell must have mechanisms coordinating the timing of cell division to DNA replication. I isolated novel mutants, *cfcA, B, C, D, E, F*, which uncouple the DNA replication and cell division. The *cfc* mutants divide 1.3 to 1.5 times more frequently per round of DNA replication. The *cfcA1* mutation was

within the α -subunit region of glycine-tRNA synthetase. From the genetic and physiologic analysis of the *cfcAI* mutant, the *cfcA* gene was found to be specifically involved in the regulation of cell cycle by a pathway different from stringent response or SOS induction. The *cfcBI* mutation was mapped at 1 minute on the *E. coli* genetic map and was complemented by a plasmid carrying the *apaGH* region. The *apaH* gene encodes diadenosine tetraphosphate (AppppA) hydrolase and the *apaH*⁻ mutants show a ≥ 16 -fold increase in AppppA. It has been suggested that AppppA could 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 neither aminoacyl-tRNA synthetase mutants, inhibitory treatments of aminoacylation, nor structural mutants of tRNA are known to produce AppppA *in vivo*. The *cfcAI* mutation was complemented by an *apaH*⁺ plasmid. This suggests that the *cfcAI*, the mutant of glycine-tRNA synthetase, could synthesize AppppA *in vivo* and that the AppppA nucleotide is involved in determining the timing of cell division in a manner which couples cell division to DNA replication. I am analyzing other *cfc* mutants to search for the targets of AppppA.

Correlation between Cell Division and the Nature of the Cell Membrane

Kengo KANAMURA, Hideyuki TAKAGI and Akiko NISHIMURA

In cell division, two main characters participate in the dramatic reaction, one is a division machinery which divides the cell into two and the other is the cell membrane structure to be divided. For a systematic understanding of cell division, biochemical studies should focus not only on the former character but also the latter one. However, so far, no one has studied the problem of the correlation between cell division and the nature of the cell membrane. Recently, we isolated seven temperature sensitive cell division mutants (*fts*) of *E. coli* which might have defects in the correlation. The *fts* mutations were complemented by the 1.3 kb *HindIII*-*PvuII* DNA region at 27 min, including a previously reported *kdsA* gene which encodes 3-deoxy-D-mannoctulosonic acid (KDO) 8-phosphate synthetase (Woisetschlager, M., and Hogenauer, G. (1987) *Mol. Gen. Genet.* **207**, 369–373). KDO 8-phosphate is converted

to KDO by KDO 8-phosphatase and supplied as a part of the lipopolysaccharides (LPS). LPS is a major component of the outer membrane. Several researches have reported that LPS is essential for maintaining the barrier property of the outer membrane. It has also been suggested that a mutation in KDO 8-phosphate synthetase in *Salmonella typhimurium* causes a defect in the translocation of the lipid A precursor to the outer membrane and inhibition of the growth (Rick, P. D. and Osborn, M. J. (1977) *J. Biol. Chem.* **252**, 4895–4903). Accordingly, it is likely that these *fts* mutants of *E. coli* inhibit cell division by somewhat altering the membrane structure, causing instability in the membrane, or through deficient translocation of membrane proteins, at the non-permissive temperature. Because there have been no reports of any *E. coli* mutants in which a defect in membrane structure causes inhibition of cell division, new findings about cell division can be gained from this research. We are now analyzing the mutation points of the seven mutants to ensure that the *fts* mutations are actually within the *kdsA* gene.

III. MAMMALIAN GENETICS

Establishment of New Strains As a Tool For Analyzing Tumor Susceptibility Genes in Mice

Nobumoto MIYASHITA, Mika FUJII*, Akihiko MITA,
Toshihiko SHIROISHI and Kazuo MORIWAKI

To determine the locus in the *H-2* complex, which affects susceptibility to the development of pulmonary adenomas in mice, *H-2* congenic and recombinant strains were subjected to treatment with urethane. The differences between these strains in susceptibility are probably due to polymorphism in the two kinds of class II genes *Eβ* and *Eα*, which encode the heterodimeric glycoprotein expressed on the cell surface: the congenic strains with *Eβ^k* and *Eα^k* alleles show more sensitivity than strains with other alleles. Using the backcross method, we established a new *H-2* congenic strain, designated as A. B(4R). This new strain carries the *H-2^{h4}* haplotype with an A/Wy genetic background. The *H-2^{h4}* haplotype is a recombinant haplotype between *a* and *b*, and a combination of alleles *Eβ^k* and *Eα^b*. As the *Eα^b* gene has a deletion including all parts of the *Eα* promoter and the first exon, the E molecule cannot be expressed on the cell surface. To clarify whether the *Eβ* and *Eα* genes themselves are responsible for adenoma susceptibility or not, we will try to establish *Eα^k*-transgenic mice using the A.B(4R)/Ms strain as a donor.

The development of tumors is multigenic, complex trait. Strains derived from Asian wild mice have shown dominant, resistant alleles in their pulmonary adenoma susceptibility genes. To map these tumor suppression genes, we established a new series of recombinant congenic (RC) strains, named AXBG. These strains are made from the parental strains A/Wy (background) and BGR (wild-derived, donor). As we backcrossed only one generation, each RC strain contains 25% (average) of the alien genome brought onto the genetic background and a different subset of genes from the donor. These new tools will be useful in analyzing the multigenic quantitative polymorphisms which affect tumor development.

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Genetical Study on the Skeleton Mouse Mutant, *Tail short* (*Ts*)

Toshihiko SHIROISHI, Akihiko MITA, Sigeharu WAKANA
and Kazuo MORIWAKI

The mutation *Tail-short* (*Ts*) arose spontaneously in the BALB/c inbred strain in 1946 (Morgan, 1950). Mice heterozygous for *Ts* have shorter kinked tails and smaller bodies than normal mice. Other skeletal abnormalities of various kinds scattered over the whole body have been observed. It was reported that *Ts* homozygotes were prenatal lethal. Abnormalities in homozygous mice can be traced back to 31/2 days p.c. by several criteria including low cell number and absence of cavitation. All these indicate the indispensable function of the *Ts* gene not only in skeletal development but in the early development of mice. The *Ts* gene was mapped to the teromeric region of chromosome 11. In the past year, the gene for human campomelic dysplasia (*CMPDI*) which displays features similar to the *Ts* was mapped to the homologous syntenic region on human chromosome 17q24.3-q25.1 (Tommerup *et al.*, 1993). Morgan has already reported that mutant mice fell short of the Mendelian expectation by 30–40% in *Ts/+ × +/+* matings. This indicated that some fraction of mice heterozygous for *Ts* died at the time of birth, rendering genetic analysis difficult. We have started the fine mapping of *Ts*, toward positional cloning of this gene. In this study, we conducted backcross matings between the *Ts/+* strain and the MSM inbred strain, derived from Japanese wild mouse. The *Ts* heterozygotes from this cross were less defective and the litter-size was relatively large, compared to the cross between the *Ts/+* strain and C57BL/6 strain. Furthermore, penetrance of the *Ts* phenotype was almost complete in this cross. Thus far, one microsatellite marker, *D11Mit128* has been tightly linked to the *Ts* gene.

Genetic Analysis of a New Mouse Mutant *Rim4* Affecting Limb Pattern Formation

Hiroshi MASUYA, Tomoko SAGAI, Kazuo MORIWAKI
and Toshihiko SHIROISHI

The complex architecture of the vertebrate limb cartilage pattern probably develops under the control of positional signalling provided by the zone of polarity activity (ZPA) and the apical ectodermal ridge (AER). Signalling

from ZPA controls the development along the anteroposterior axis and the signalling from AER, along the proximodistal axis. The signaling may be interpreted by the *Hoxd* and *Hoxa* genes to certain positional values. *Rim4* (Recombination Induced Mutant 4) is a polydactyly mutant derived from an intra-MHC recombinant. Heterozygotes of *Rim4* show preaxial polydactyly and hyperphalangy of hindfeet. Homozygotes show preaxial polydactyly and hyperphalangy of forefeet and hindfeet, reduction of the tibia and a decrease in number of vertebrae. These phenotypes closely resemble the phenotypes of the another polydactyly mutant, *lx*. Many polydactylous mutants including *lx*, show hyperphalangy and tibial hemimelia. This indicates that the affected genes in these mutants function coordinately in a process of limb development (probably affecting production of certain positional values). We carried out linkage analyses of the *Rim4* crossing with DBA/2J and NZB. As a result, *Rim4* was mapped on chromosome 6 and was tightly linked to *D6Mit16*, *D6Mit17*, and *D6Mit94*. This position does not overlap with the loci of other polydactylous mutants. Several recombinants were observed between the *Hoxa* and *Rim4* loci. When C57BL/10J-*Rim4*/+ mice were crossed with other laboratory strains, the frequency of the *Rim4* phenotype varied according to the strain with which the *Rim4* was crossed. Thus, penetrance was incomplete in heterozygotes, ranging from 0% in the cross with the MSM strain to almost 100% in the cross with the NZB strain. The order of mouse strains with respect to penetrance was; NZB > B10 = CBA > DBA > C3H > BALB/c > MSM. The differences in penetrance indicates that the *Rim4* phenotype is controlled by allelic forms of the *Rim4* locus or other genes, which are not linked to *Rim4*.

Gene Mapping toward Positional Cloning of the *Rim3* Gene

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

Rim3 is a hairless mutant derived from an intra-MHC recombinant, showing hyperkeratinization in the cornea. This gene has been mapped to the *Cola-1* (collagen-1) locus on chromosome 11. In the present study, we extended the mapping of the *Rim3* gene, using a cross between the C57BL/10J-*Rim3* and MSM strains. As a result, the *Rim3* gene was tightly linked to a microsatellite marker *D11Mit14*. We also mapped the *Krt-1* (keratin type

I) locus to this position. We carried out PCR-screening of the YAC library provided by Research Genetics, USA, using *D11Mit14* primers, and identified a single positive clone. Now we are characterizing this clone.

**Germ-line Transmission of ES Cell Lines Established from
Mouse Strains Enhancing Meiotic Recombination
at the *LMP-2* Hotspot in the MHC**

Tomoko SAGAI, Norio NAKATSUJI, Toshihiko SHIROISHI
and Kazuo MORIWAKI

The *wm7* MHC haplotype derived from Japanese wild mouse enhances meiotic recombination specifically in female at the hotspot located to the 3'-end of the *LMP-2* gene. Molecular characterization of this hotspot and previously identified hotspots located in the *Eb* gene revealed that these two hotspots share a similar molecular organization, consisting of three molecular elements, consensus sequence of mouse middle repetitive MT-family, tetramer (TCTG) repeated sequence and LTR repeat of mouse retro virus.

In order to elucidate the relationship between the location of the recombinational breakpoints and these elements, we intended to carry out the gene targeting experiment in order to modify the structure of the hotspot. For this purpose we needed the ES cell lines derived from the mouse strain which enhances the meiotic recombination at the hotspot. Because pluripotent ES cell lines available now were produced mostly from 129 strain which is not capable of enhancing recombination at the hotspot, we isolated the new ES cell lines from B10.A(R209) strain, which exhibits high recombinational frequency at the hotspot. These ES cell lines were tested for the chimera formation and germ-line transmission through the microinjection into 8 cell stage embryos of ICR strain. As the result, we obtained several chimera mice with the various coat color contribution. Finally, three ES cell lines established from B10.A(R209) strain and one from (R209 × CBA)F1 were transmitted to the germ-line of the chimera mice. We are now preparing the targeting vector and are planning to use these ES cell lines for the analysis of the molecular mechanism of meiotic recombination at the *LMP-2* hotspot.

Genetical Analysis of rim2 Coat Color Mutant

Tomoko SAGAI, Shigeharu WAKANA, Kazuo MORIWAKI
and Toshihiko SHIROISHI

The rim2 is one of the RIMs (recombination induced mutants) which arose spontaneously in the B10.A(R201) strain. The rim2 is recessive coat color mutant and is characterized by the diluted pigmentation on the entire hair, the ears and tail. The progressive depigmentation of the pelage is frequently observed in the young individuals. The amount of pigment in their eyes at birth is smaller than the original B10.A(R201) strain, but the eye color becomes darker in adult animals and cannot be distinguished from the wild type.

Chromosomal mapping of this mutation was carried out using several micro-satellite markers. (MSM × rim2)F1 × rim2 back-cross mating resulted that the rim2 mutant gene was tightly linked with D13MIT108 marker located between D13MIT27 and 36. In this region, old coat color mutation, *pe* (pearl), was mapped. As the phenotype of the pearl mutant resembles that of rim2, it is possible that the rim2 is allelic to the *pe*.

Mutagenesis with Intra-MHC Recombinant Strains

Masayasu YOSHINO, Tomoko SAGAI, Kazuo MORIWAKI
and Toshihiko SHIROISHI

In our laboratory, hundreds of intra-MHC recombinants have been established from thousands of progeny of crosses between a wild-derived MHC congenic strain, B10.MOL-SGR, and standard inbred strains. We found that some of these intra-MHC recombinants have spontaneously yielded multiple visible mutations. The mutation rate of these intra-MHC recombinants seems to be much higher than the standard values, 3.8×10^{-6} for recessive and 3.4×10^{-7} for dominant mutations, which were estimated from laboratory mouse strains. In order to understand what mechanism causes the high rate of mutation in the intra-MHC recombinants, we have screened mutants at specific loci which can be analyzed at the molecular level. We used the PT/7af strain as a tester in genetic crosses with the intra-MHC recombinant, as it carries recessive mutations at seven specific (*a, b, c^{ch}, d, p, s, se*) loci. In practice, we irradiated the B10.BR.(R228) mouse strain, from which a dominant visible mutation of the hairless phenotype (*Rim3*) was obtained,

and mated it with testers of the PT strain. We have detected one mutant with the *short ear* (*se*) locus and one visible mutant with a phenotype of dominant white spot, in 976 offspring from 5 Gy-irradiated females of B10.BR.(R228). In addition to these experiments, one spontaneous visible mutation with a phenotype of dominant white spot was obtained among 480 offspring from non-treated B10.BR.(R228). We are planning to analyze the *se* mutant with DNA markers located near the *se* locus and to test whether or not the dominant spot mutants are affected at the *W* or *Silocus*.

Chromatin Structure of the Recombinational Hotspot in the Mouse MHC

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

It has been established that reciprocal recombination occurs at a high frequency in the mouse major histocompatibility complex (MHC) when some wild-derived mouse strains are crossed with laboratory strains. The breakpoints of these recombinations are clustered at specific sites termed hotspots. The *wm7* MHC haplotype derived from Japanese wild mouse enhances the recombination at the hotspot located in the 3'-end of the *Lmp2* gene between the *Pb* and *Ob* genes (*Lmp2* hotspot). The breakpoints of the recombinants turned out to be confined to a DNA fragment of 2 kb. The molecular mechanism that controls the site-specificity of the recombinational breakpoints has yet to be determined. One possibility is that hotspots have an open chromatin structure leading to enhanced accessibility of DNA to the recombination machinery. In yeast, there are reports that the chromatin structure contributes to the initiation of meiotic recombination. We attempted to examine this hypothesis focusing on the *Lmp2* hotspot. First, we fractionated testicular germ cells from several mouse strains through centrifugal elutriation after EDTA and trypsin treatment of testes. Using this method, we enriched pachytene cells in which meiotic recombination is thought to occur. In order to analyze the high-order structure of chromatin around the hotspot, we tried to analyze DNase I-hypersensitivity sites (DHSSs) in the chromosomal region surrounding the hotspot. As a result, clear DHSS was not detected in the 2 kb segment of the hotspot, but DHSSs were detected at a distance of 1.5 kb proximal to the hotspot. On the other

hand, we detected faint DHSSs in the hotspot located in the second intron of the *Eb* gene in the MHC as reported previously by another group. It appeared that the presence or absence of the DHSSs was not directly related to recombinational frequency in the crosses. In this study, the DHSSs were similarly present in all mouse strains examined. In addition testis cells, we analyzed the chromatin structure in somatic cells. We detected clear DHSSs in the *Lmp2* hotspot both in spleen and liver cells. Results showed that recombination at the hotspots during meiosis occurs independently on the chromatin structure. Thus, the molecular mechanism of meiotic recombination on hotspots in the mouse genome is different from that in the yeast genome.

Polymorphism of the *Lmp2* Gene Encoding a Subunit of the Mouse Proteasome

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

Exogenous antigenic proteins, such as viral antigens, are digested to small octomeric or nanomeric peptides before being presented in the context of the MHC class I molecules to the T-cell receptors (TCR). A multisubunit protein complex, proteasome, produces this protein degradation in an energy-dependent manner. In mouse, the poly A signal of the *Lmp2* gene which encodes a subunit of proteasome is mapped at a distance of only 2 kb from tetramer repeats identified in the recombinational hotspot. Recently, it has been shown that non-synonymous substitutions at the antigen recognition site of class I and class II genes in the MHC is more rapid than synonymous substitutions. It has been also reported that the *Lmp2* genes in laboratory mice are polymorphic at both amino acid and nucleotide levels. We are interested in whether the *Lmp2* gene evolved in the trans-species mode as the antigen recognition site of the class I and II genes. To clone the *Lmp2* genes in various species and subspecies of mice and determine these sequences, we extracted mRNA from mouse spleens and cloned the genes using the RT-PCR method. We are presently sequencing the clones of the genes.

**Genetic Diversity and Geographic Distribution of Asiatic *Mus musculus*
Subspecies Based on the Polymorphism of Mitochondrial DNA
with Special Reference to the Origins of Japanese Mouse
'*M. m. molossinus*'**

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Wild mice of the species, *Mus musculus*, were collected from Asiatic countries and their mitochondria DNA(mtDNA) was analyzed by restriction enzymes. *M. m. musculus* and *M. m. castaneus* were commonly found in this area with clear geographical distribution patterns: *M. m. musculus* from Western China, Russia, Korea to Japan, and *M. m. castaneus* from Southeast Asia to Southern China. As reported previously, mice with *castaneus* mtDNA could also be found in northern Japan and newly in eastern Russia. However, it was difficult to find intrasubspecific RFLVs (Restriction Fragment Length Variations) in the *musculus* mice as well as in the *castaneus* mice. We thus determined the DNA sequences of the D-loop region to clarify the fine structure of both subspecies. At least one mouse was used for this analysis in each collection locality. The total number of mtDNA haplotypes sequenced so far is 106. As expected, intrasubspecific DNA variations (mainly base substitutions) were found in the region. Pair-wise comparison was performed using their DNA sequences, their genetic diversity values were calculated, and then phylogenetic trees were constructed based on the values. The cluster of *M. m. musculus* is clearly separated from that of *M. m. castaneus*. Furthermore, several distinct subclusters exist in the *musculus* cluster and the same is true in the *castaneus* cluster. New lines of evidence that we found in this year are that at least two components exist both in the Japanese *musculus* populations and in the *castaneus* ones. In the *musculus* population, one component is closely related to *musculus* mice collected in Korea and northern China. In the *castaneus* mice, one is related to mice trapped in Palau and the other with those of eastern Russia.

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Recombination in the Class III Region of the Mouse MHC

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and Toshihiko SHIROISHI¹

Sites of meiotic recombination in the class II region of the mouse MHC are not randomly distributed, but are clustered within short segments known as recombinational hotspots. The MHC consists of three linked gene clusters. The class I and II regions contain genes encoding highly polymorphic cell surface antigens which are involved in the presentation and recognition of foreign antigens during immune responses, whereas the class III region of the MHC contains miscellaneous genes, trapped in the middle of the MHC, that are not polymorphic and do not necessarily play a role in immune responses. The class III region contains genes encoding steroid 21-hydroxylase (*Cyp21*), members of the complement cascade (*C4*, *Slp*, *C2*, *Bf*), the heat shock proteins *Hsp70* and *Hsp68c*, and the tumor necrosis factor cytokines (*Tnfa*, *Tnfb*). At present, it is not known if recombinational hotspots are a general feature of the MHC or specific to the class II region. To answer this question, we characterized 79 *Ab:H2-D* recombinants using 11 DNA markers and mapped recombinational breakpoints. In addition, we determined the gene order in the MHC class III region. We used two DNA marker genes and one SSLP (simple sequence length polymorphism) marker which had not previously been mapped precisely. They are *Tnx*, the gene for an extracellular matrix protein, *tenascin X*, the *Notch*-related *Int3* gene, and a microsatellite marker, *D17Mit13*. The results gave the gene order of *Eb-61.1-Int3-Tnx-Cyp21/C4-Bf-Hsp68c-D17Mit13-Tnfa/Tnfb-D*. The crossover sites in 40 of the 79 recombinants were confined within the *Eb/Int3:Tnx/Cyp21* interval. The results demonstrated that unequal distribution of recombination is a general feature of the mouse MHC, suggesting the presence of a novel

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recombinational hotspot within the *Int3:Tnx* interval.

Linkage Analysis of the *js* (Jackson shaker) Locus on Mouse Chromosome 11

Sigeharu WAKANA¹, Hiromichi YONEKAWA², Hideki KANEDA²,
Mieko OKAMOTO², Toshihiko SHIROISHI³
and Kazuo MORIWAKI⁴

Jackson shaker (*js*) is a recessive mutant in the mouse, which exhibits circling, head-shaking and deafness. These abnormalities are caused by the disarray of stereocilia of the outer hair cells in inner ear. Thus, *js* would be a useful model for human congenital deafness. The *js* gene has been mapped on the distal part of mouse chromosome 11. To search for DNA markers closely linked to this locus, we employed a detailed linkage analysis using 300 segregants of an intersubspecific backcross between C57BL/6-*js/js* and *MSM*, an inbred strain established from Japanese wild mice *Mus musculus molossinus*. The analysis was performed by ordinary PCR and PCR-SSCP using many microsatellite markers mapped on the the distal end of the chromosome. We identified *D11MIT128* as being very closely linked to the *js* locus. We started screening the YAC clone with this microsatellite marker.

The Relationship between Recombinational Frequency and Sequence Homology at the Hotspot in the Mouse MHC

Masayasu YOSHINO, Tomoko SAGAI, Kazuo MORIWAKI
and Toshihiko SHIROISHI

The mouse major histocompatibility complex (MHC) is one of the most useful genetic regions for studies of the molecular mechanism of meiotic recombination in mammalian genomes. The class I and II genes of the MHC encode highly polymorphic cell-surface antigens, the genotypes of which can be readily determined serologically, facilitating the rapid identification of intra-MHC recombinants. Furthermore, the MHC region has been well characterized at the molecular level, so that we can map the recombinational

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breakpoints at the nucleotide level. Sites of meiotic recombination in the proximal region of the mouse MHC are not randomly distributed but are clustered within short segments known as recombinational hotspots. The *H2* congenic strain of B10.MOL-SGR whose MHC haplotype of *wm7* were derived from Japanese wild mouse, *Mus musculus molossinus*, enhances meiotic recombination at the *Lmp2* hotspot that are located in the *H2-K* to *Ab* interval. Heterozygotes of crosses between B10.MOL-SGR and standard *H2* congenic strains, B10, B10.A or B10.BR have yielded high frequencies of recombination (2.1%) in this region, and these frequencies are 100-fold higher than the frequencies estimated for standard crosses. Mating experiments between B10.MOL-SGR and B10.CAS4(R28) or B10.CAS3, which were derived from Thai wild mouse, *Mus musculus castaneus*, revealed that recombination occurred at the *Lmp2* hotspot at lower frequencies (0.3–0.6%) than in the crosses between B10.MOL-SGR and the standard B10 strain (2.5%). The results indicated that high recombinational frequencies in crosses with B10.MOL-SGR depend on the counterpart strain in the cross. In the present study, we focused on the question of what mechanism is responsible for differences in the rate of recombination among different crosses. For this purpose, three test crosses were made. First, B10.MOL-SGR was crossed with the 129 strain which has the same *b* haplotype as B10. Second, B10.MOL-SGR was crossed with another standard laboratory MHC-congenic strain of B10.D2. Third, B10.MOL-SGR was crossed with B10.M or B10.MOL-TEN1 whose MHCs are characteristic of *Mus musculus castaneus*. The rates of recombination in these crosses were compared with that in the cross of B10/B10.MOL-SGR. Summarizing our results, 129 enhanced recombination (1.8%) as well as B10 but the frequency in the cross of B10.D2/B10.MOL-SGR was lower (0.3%) than that in the cross of B10/B10.MOL-SGR. Moreover, neither B10.M nor B10.MOL-TEN 1 produced recombination in the *K-Ab* interval in crosses with B10.MOL-SGR. We sequenced the *Lmp2* hotspot of these strains and the sequence data indicated that B10 had the highest homology with B10.MOL-SGR and that B10.M and B10.MOL-TEN1 had the lowest. These results suggest that recombination frequency is parallel with the extent of sequence homology at the *Lmp2* hotspot.

M-phase Specific Phosphorylation of Mouse p84^{wee1} Kinase

Hideyo YASUDA

I have been working on the mechanism of regulation in mammalian cell cycle progression, especially on the mechanism of regulation through protein phosphorylation. Phosphorylation and dephosphorylation are important mechanisms for cell proliferation. In mammalian cells, cyclin dependent kinases (cdks) play key roles in cell cycle regulation, whose activation and inactivation are also regulated by phosphorylation and dephosphorylation (Norbury, C. and Nurse, P. (1992) *Annu. Rev. Biochem.* **61**, 441-470). CAK (cdk activating kinase) phosphorylates Thr161 or Thr160 of cdc2 or cdk2 kinase, resulting in activation of these kinases (Poon, R. Y. C., Yamashita, K., Adamczewski, J., Hunt, T., Shuttleworth, J. W. and Shuttleworth. (1993) *EMBO J.* **12**, 3321-3329.).

On the other hand, phosphorylations of mammalian cdc2 kinase on Thr14 and Tyr15 negatively regulate kinase activity (Norbury, C., Blow, J. and Nurse, P. (1991) *EMBO J.* **10**, 3321-3329.). Phosphorylation at Tyr15 of cdc2 kinase is catalyzed by wee 1 kinase, though the kinase responsible for Thr14 phosphorylation has not been identified so far (Honda, R., Ohba, Y. and Yasuda, H. (1992) *Biochem. Biophys. Res. Commun.* **186**, 1333-1338), (Parker, L. L. and Piwnicka-Worms, H. (1992) *Science* **257**, 1955-1957).

We have isolated a cDNA clone of a mouse wee 1 kinase which is about 1.5-fold larger than that of *S. pombe* wee 1. Its carboxyl terminal domain, which contains catalytic sites of the kinase, resembles the entire human wee 1 kinase. In a cell cycle, this mouse wee 1 kinase is phosphorylated at M phase and this phosphorylation occurs at the N-terminal domain of the kinase in M phase extract, not G1 phase extract. These results suggest that the N-terminal and C-terminal domains of the wee 1 kinase are of regulatory and catalytic, respectively, and that the human wee 1 kinase reported previously is not an entire molecule but only a catalytic domain.

IV. DEVELOPMENTAL AND SOMATIC CELL GENETICS

An Exhaustive Screening of Peptide Signal Molecules Affecting Gene Expression in Hydra

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Osamu KOIZUMI¹, Yojiro MUNEOKA², Toshio TAKAHASHI²,
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Past studies have provided evidence which suggests that hydra tissue contains morphogens which play major roles in regulating regeneration and other developmental processes in this organism. Attempts made to isolate these morphogens have not been very successful. Head-activator (see below), originally isolated as a factor stimulating head formation (Schaller and Bodenmueller, 1981), has a broad spectrum of activity, and its real *in vivo* function is uncertain. Adopting a new strategy, we started a new project to isolate peptide signal molecules in hydra.

Hydra was mass cultured to produce about 150 g of fresh tissue. Peptides were extracted from the tissue with 5% acetic acid, and separated into 15 crude fractions. One fraction was selected as representative, and individual peptides present in this fraction were isolated through multiple steps of ion-exchange and reverse-phase HPLC. A total of 61 peptides were obtained in quantities ranging from 1 to 10 nmoles, suggesting that more than 300 peptides will be present in the remaining 14 fractions. Amino acid sequences of all 61 peptides were determined, and chemical synthesis was carried out for 4 of them based on the structures determined. Synthetic and natural peptides were compared and judged to be identical by HPLC and MS analyses. One of the synthetic peptides, Hyd-23, has amino acid residues identical to Head-activator in 5 of the 10 C-terminal positions as shown:

Hyd-23: K WVQGKPTGEVKQIKF

Head-activator: <pEPPGGSKVILF

The synthetic peptides were examined for their capacity to function as signal molecules. Hydra were treated with the peptides ($10^{-7}M$), RNA was

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extracted from the treated and untreated control animals, and subjected to Random Primer PCR Differential Display analysis (Liang and Pardee, 1992). This procedure is very powerful in displaying small differences in gene expression (poly-A RNA synthesis) in different tissue samples. Using this assay, Hyd-23 was found to be active in altering gene expression in the treated polyps. This suggests that this peptide may serve as a signal molecule by binding to a specific receptor on cells and, controlling gene expression in the cells, via the signal transduction pathway. On the basis of these results, we intend to carry out a larger screening program as follows; (1) Peptides present in the remaining 14 fractions (> 300) will be purified by HPLC. (2) Half of the purified peptides will be used to treat hydra ($> 10^{-7} M$), and changes in gene expression produced by the treatment will be examined by RPPDD. (3) The remaining half will be used for structure determination of the peptides found to be active in RPPDD. (4) Chemical synthesis will be carried out for the peptides whose structures are determined. (5) Synthetic peptides will be used in biological studies to examine their effect on morphogenesis, cell proliferation, cell differentiation and other developmental and physiological processes in hydra.

Screening of Cell Adhesion Molecules in Hydra

Yasuyuki KISHIMOTO, Masayuki HATTA, Engelbert HOBMAYER*
and Tsutomu SUGIYAMA

A project has been started to isolate cell adhesion molecules involved in regeneration and other morphogenetic processes in hydra. Hydra body column tissue consists of the ectodermal and the endodermal epithelial cell layers. These 2 layers were separated from each other through Procaine treatment, and cut into small pieces. When two tissue pieces thus produced were brought into contact in 3 combinations (ectodermal-ectodermal, endodermal-endodermal and ectodermal-endodermal), firm adhesion was established within 1–2 hours in all cases. In the case of ectodermal-endodermal contact, an “epiboly”-like process occurred following the initial adhesion. The ectodermal epithelial cells gradually spread over the endodermal tissue, and eventually formed a continuous single ectodermal epithelial cell layer to cover the entire endodermal tissue mass.

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Cell adhesion molecules located on the cell membrane probably play a major role in these tissue interacting systems. Several approaches were taken to isolate these molecules. In one approach, a soluble extract was produced from a hydra membrane fraction through trypsin digestion, and its effect was examined. The tryptic extract (< 0.1 mg/ml) was found to specifically inhibit the epiboly-like process; it inhibited the spreading of the ectodermal cells over the endodermal tissue, without affecting the initial ectodermal-endodermal (or ectodermal-ectodermal or endodermal-endodermal) adhesion. The inhibition was reversible; The epiboly-like process, stopped midway by adding the extract, could be resumed by removing the extract through washing.

The effect of the tryptic extract can be explained as follows. The extract contains a tryptic fragment of an adhesion molecule. The original intact molecule is located on one partner of the ectodermal-endodermal interaction, and its binding to the ligand located on the other partner is a crucial step in the epiboly-like process. The fragment containing the adhesion domain of this molecule can bind to the ligand, thereby preventing the normal ectodermal-endodermal interaction in the epiboly-like process. Other possibilities can also be considered.

The active factor in the tryptic extract has an MW of about 3 KD. HPLC is being used currently to purify the factor. The purified factor, when obtained, will be used to isolate a cDNA clone for the original intact molecule. Specific antisera also will be produced. Using them, we intend to examine the expression and roles of the molecule in hydra development.

In another line of study, we are using PCR to isolate hydra homologues of known vertebrate cell adhesion molecules. We have obtained PCR fragments showing homology to vertebrate cadherin (protocadherin), integrin and β -catenin, respectively. When hybridized to hydra poly-A RNA in Northern analysis, all fragments produced clear signals at expected sizes. Attempts to isolate cDNA clones from hydra cDNA libraries have yielded 3 overlapping β -catenin clones (80% identity in amino acid sequences to human counterpart). cDNA clones of hydra cadherin and integrin are presently being searched for.

Underwinding of DNA on Binding of Yeast TBP to the TATA Element

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The TATA box-binding factor TBP is an essential component in the initiation of transcription by eukaryotic RNA polymerase II. We investigated the effect of DNA supercoiling on TBP: promoter interactions using recombinant yeast (ry) TBP. DNase I footprinting analysis showed that ryTBP has a higher affinity for the adenovirus major late promoter in the negatively supercoiled state than in the relaxed state. On the contrary, its affinity for the *Drosophila* hsp70 promoter is constant irrespective of DNA topology. Binding of ryTBP to these promoters induced underwinding of duplex DNA. The functional TATA box and active ryTBP are essential for the underwinding. The step was facilitated by negative supercoiling of DNA on the adenovirus major late promoter but not on the *Drosophila* hsp70 promoter. For details, see *Biochem. Biophys. Res. Commun.* **192**, 1432–1438, 1993

DNA Sequence Requirement of a TATA Element-Binding Protein from *Arabidopsis* for Transcription *in vitro*

Fujio MUKUMOTO**, Susumu HIROSE, Hidemasa IMASEKI**
and Ken-ichi YAMAZAKI**

We analyzed the DNA sequence requirements for the functioning of TATA elements by examining transcriptional activities associated with 24 promoters, including representatives of each of the 21 point mutations in the consensus sequence from plants, TATATATA, both in a HeLa *in vitro* system and in a chimeric *in vitro* system in which human TATA-binding protein (TBP) was replaced by purified TBP of *Arabidopsis*. Although the relative transcriptional activities varied among these promoters, both systems gave virtually identical results. Among the mutant TATA elements, those with the sequences TAGAGATA and GAGAGAGA did not show any detectable activity. The rest had activity that ranged from 7% to 130% of the activity associated with the consensus element. These results suggest the functional conservation of TBP between plants and animals. For details, see

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Plant Mol. Biol. **23**, 995–1003, 1993.

BmFTZ-F1 Activates *in vitro* Transcription of the *fushi tarazu* Gene by Binding to the FTZ-F1 Recognition Site

Feng-Qian LI, Hitoshi UEDA and Susumu HIROSE

To study the mechanism involved in transactivation by BmFTZ-F1, we used an *in vitro* transcription assay. Because the target genes of BmFTZ-F1 in the silkworm are unknown, we used the *Drosophila fushi tarazu (ftz)* gene as a model template. A single binding site for FTZ-F1 is present at around 280 bp upstream from the initiation site. We also prepared mutant templates carrying base substitutions within the FTZ-F1 recognition site. These mutations abolished the binding of FTZ-F1 and BmFTZ-F1 and markedly reduced the expression of the *ftz-lacZ* fusion gene in transformed *Drosophila* embryos.

When the wild-type template was incubated with the posterior silk gland extract containing BmFTZ-F1, the *ftz* gene was transcribed as efficiently as the fibroin gene added as an internal control. The mutant templates produced five fold fewer transcripts than did the wild-type construct. When we used posterior silk gland extract, from which BmFTZ-F1 had been depleted by treatment with latex beads coupled with the FTZ-F1 site DNA, transcription of the *ftz* gene was severely reduced while that of the fibroin gene was unaffected. Addition of purified BmFTZ-F1 to the depleted extract restored *ftz* gene transcription to the level before depletion. These results show that BmFTZ-F1 activates transcription of the *ftz* gene in the posterior silk gland extract by binding to the FTZ-F1 recognition site.

Molecular Cloning of a cDNA for BmFTZ-F1, a Silkworm Counterpart of *Drosophila* FTZ-F1

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. We isolated a cDNA encoding BmFTZ-F1. Amino acid sequences in the zinc finger DNA-binding region and the putative ligand-binding domain of BmFTZ-F1

showed a strong similarity to not only FTZ-F1 but also its mammalian homologues, LRH-1, ELP, and Ad4BP. 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.

Genetic Studies on the Duration of Pupal Lifespans in *Bombyx mori*

Akio MURAKAMI

Information on pupal lifespans in the domesticated silkworm, *B. mori* (L.) is vague: the average pupal lifespan is said to range from eleven to thirteen days, but some pure stocks and hybrid lines lifespans of more than 20 days. Also, certain decapitated pharate pupae in certain F₁ hybrid lines have lived 40 days (cf., Kobayashi, 1957). It is of interest that the C115 stock itself, which is one of parent stocks above the hybrid lines, also showed a long pupal life or "dauer (long-lived) pupa". Similar surgical extirpations of the brain in the many other lines did not produce the so-called the long-lived pupa.

In recent observations certain long-lived pure lines have been found among NIG preserved stocks, the J115 stock as well as the Ascoli stock and other with a lifespan of 18 days. Moreover, the average duration of pupal lifespan among temperate varieties is estimated to be 10–12 days and that of tropical varieties is 9 days. In any case, the duration of pupal lifespan in *B. mori* differs from stock to stock and is peculiar to each stock, showing that the biological phenomenon is a genetically based. The duration of pupal lifespan of F₁ hybrid lines obtained from a reciprocal cross between the standard life (or short) and long life stocks are much the same as the short-lived parent stock regardless of sex. This suggests that the short-lived trait for pupal lifespan is not dominant but recessive.

In short, there are two types of stocks of *B. mori*: one of them has a pupal lifespan ranging from nine to twelve days and the majority of stocks belong to this type, and the second type has a pupal lifespan of more than 18 days and it rather rare.

The current concept of the humoral pathway leading to adult emergence from pupal form in *B. mori* is that the prothoracic glands are stimulated by the neurosecretory system of the brain, PTTH (prothoracicotropic hormone) or brain hormone, and then secrete the moulting hormone or ecdysone which finally leads to pupal ecdysis to form the adult. PTTH is released into body

fluid via the corpora cardiaca. Ecdysone stimulates the tissue cells to initiate mitosis and the epidermal cells to begin elaboration of adult cuticular matters. If the ecdyson titer in the pupal body-fluid is low, ecdyson titres need to accumulate above the threshold dose, required for pupal periods or prolongation of lifespan. Surgical removal of the brain or cutting off the supply of PTTH in the pharate pupae results in prolongation of lifespan (or "dauer-pupa"). If this is the case, some stocks, which are relatively longer lived pupal lifespans than the standard stocks, can be regarded as having a low titer of PTTH and/or ecdyson, while the majority of standard stocks and lines, which showed a short duration of pupal lifespans, have a sufficient dose of both the neurosecretory substances.

It seems likely that the duration of some pupal lifespans *B. mori* is under genetic control of the secretory activities of the neurosecretory complex, PTTH and ecdyson of copora allata.

Ecogenetic studies on the Duration of Pupal Lifespans in Bombycidae

Akio MURAKAMI and Osamu NINAKI*

The majority of Lepidopteran insects including *Platysamia (Hyalophora) Secropia*, *Philosamia cynthia ricini*, *Attacus cynthia*, *Caligura japonica*, *Chilo supressalis*, etc. are of a pupal diapause type. However some species including *Bombyx mori* (L.) and its putative ancestor, *Bombyx mandarina* (M.) are of an egg diapause type and are said to enter the diapause state at a slightly later stage, when the embryo is dumbbell-shaped but as yet unsegmented. As stated in the preceding report, some long-lived (ca. 17–18 days at 25°C) pupal stocks of *B. mori*, J115, Ascoli, and others are preserved in our institute. Pupal durations extended ca. 60% over the average term for *B. mori*. No selective effects on pupal lifespans for almost 15 generations were observed, indicating that these biological phenomena might be stabilized or established and are under the control of genetic mechanisms. A similar observation was found in a pure stock, C115 (Kobayashi, 1959). It was also demonstrated that when the extirpation of the brain either at a later stage of the last instar larvae, or at an early stage of pupae in a certain F₁ hybrid line was done, pupal durations tended to be extended up to more than 40 days.

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The present findings and others suggest that the domesticated silkworm, *B. mori* has the potential to some extent to suspend pupal development or extend its lifespan.

In *B. mandarina*, the duration of pupal lifespan varies widely. One of the present authors, Ninaki and his collaborators (1982) showed that the lifespan of pupae, which have been cultured in insectaria for several generations collection from fields, should considerable variation among individuals, ranging from, 11 to 44 days regardless of rearing season. This indicates that the wild silkworm, *B. mandarina*, can shorten its pupal durations by means of selective pressures. It is of interest to note that the shortest pupal lifespan in *B. mandarina*, 11 days, is similar to that of *B. mori*. However, the pupal lifespan of *B. mandarina* newly collected in autumn attained almost four months, from the middle of September to the middle of January of the next year, indicating that these pupae might be passing through not only a metamorphic phase but also a hibernating phase. Some eggs laid by the mated female moths immediately resumed embryonic development without the egg-diapause form, but others entered the diapause form. Accordingly, it seems likely that the former type pupae, which showed a remarkably long pupal lifespan and whose female moths laid a high frequency of non-diapausing eggs, are a kind of pupal diapause type instead of an egg diapause type, while the latter, which also showed longer pupal lifespans but whose female moths laid diapausing eggs, are still both an egg and pupal diapause type. Thus, it can be said that in *B. mandarina* neither egg or pupal diapause form are clearly established, while in *B. mori* the egg diapause form is clearly established as the main surviving strategy against unfavorable environmental conditions throughout a long evolution and/or domestication process. Some *B. mori* stocks with a relatively long-lived pupal lifespan appear to be a remnant form of the pupal diapause form of *B. mandarina*. Diapause is usually confined to one stage in the lifecycle. However, both the obligatory egg and very weak facultative pupal diapause forms seem to exist in Bombycidae, *B. mori* (L.) and *B. mandarina* (M.) an exceptional case among lepidopteran species along, with the winter moth, *Operophtera brumata* [I. W. Kozhantshikov, *Ent. Oboz.* 31: 178 (1950)].

Embryonic Process of Holometabolous Insects

Kiyoshi MINATO

Among animals of classis insecta, there are a large number of species which are holometabolous or completely metamorphic compared to those that are hemimetabolous and ametabolous. One of the reasons is thought to be their larval forms relatively simple and different from adult ones, which enable their larvae to obtain various newly-found habitats, additionally without competition from adult.

The development of various holometabolous embryos was investigated through available literature and compared to that of hemimetabolous and ametabolous embryos. As a result, little evidence was found showing that the more simple larval forms of holometabolous insects was a result of the hatching of these embryos at relatively less developed stages or "neoteny", as suggested by Berlese (1913). It is more likely to be a result of the suppressed expression of some external morphology of the embryo such as the compound eye, the more developed legs, and the relatively complex form of the body, and also the increased expression of some internal morphology such as the abdomina motive-muscle system. These active regulation mechanisms of expression of the embryonic features in holometabolous insects and the genetic background are now under investigation.

Isolation of a cDNA Encoding the Largest Subunit of TFIIA Reveals Functions Important for Activated Transcription

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Kiyoshi OGURI, Xiaqing SUN*, Tadashi WADA, Takeshi IMAI,
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Transcription factor IIA has been shown to interact with the TATA-binding protein and to act early during preinitiation complex formation. The human factor is composed of three subunits (α , β , γ). A human cDNA clone encoding the largest subunit of TFIIA (α) was isolated. The recombinant α polypeptide, together with the β and γ subunits, was capable of reconstituting

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TFIIA activity. Studies using antibodies raised against recombinant α polypeptide demonstrate that FTIIA can be an integral component of the preinitiation complex. We demonstrated that TFIIA not only interacts with TBP but also can associate with the TFIID complex. Functional assays established that TFIIA has no apparent role in basal transcription but plays an important role in activation of transcription. Interestingly, amino acid sequence analyses of the β -subunit demonstrate these residues to be entirely contained within the carboxyl terminus of the cDNA clone encoding the α -subunit. For details see *Genes & Dev.* 7, 2246–2257, 1993.

cDNA Cloning of Transcription Factor E4TF1 Subunits with Ets and Notch Motifs

Hajime WATANABE, Jun-ichi SAWADA, Kei-ichi YANO*, Kazuo YAMAGUCHI*,
Masahide GOTO and Hiroshi HANDA

E4TF1 was originally identified as one of the transcription factors responsible for adenovirus E4 gene transcription. It is composed of two subunits, a DNA binding protein with a molecular mass of 60 kDa and a 53-kDa transcription-activating protein. Heterodimerization of these two subunits is essential for the protein to function as a transcription factor. In this study, we identified a new E4TF1 subunit, designated E4TF1-47, which has no DNA binding activity but can associate with E4TF1-60. We then cloned the cDNAs for each of the E4TF1 subunits. E4TF1 was purified, and the partial amino acid sequence of each subunit was determined. The predicted amino acid sequences of each cDNA clone revealed that E4TF1-60 had an ETS domain, which is a DNA binding domain common to ets-related transcription factors. E4TF1-53 had four tandemly repeated notch-ankyrin motifs. The putative cDNA of E4TF1-47 coded almost the same amino acid sequences as E4TF1-53. Three hundred and thirty-two amino acids of the N termini of E4TF1-47 and -53 were identical except for one amino acid insertion in E4TF1-53, and they differ from each other at the C terminus. These three recombinant cDNA clones were expressed in *Escherichia coli*, and the proteins behaved in the same manner as purified proteins in a gel retardation assay. Nucleotide and predicted amino acid sequences were highly homologous to GABP- α and - β , which is further supported by the

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observation that the GABP-specific antibody can recognize human E4TF1. For details see *Mol. Cell. Biol.* **13**, 1385–1391, 1993.

Genetics of Fluoride-resistance in the Nematode *Caenorhabditis elegans*

Minoru KAWAKAMI, Takeshi ISHIHARA and Isao KATSURA

Sodium fluoride (NaF) is toxic to almost all organisms. Biochemical reactions *in vitro* suggest that it may interfere with signal transduction systems, because it depletes Ca^{2+} , inhibits phosphatases, and activates G-proteins. We are trying to elucidate such a system through the isolation and characterization of *C. elegans* mutants resistant to NaF.

We isolated 13 recessive fluoride-resistant mutants that map in 5 new genes, *ftr-1 X*, *ftr-2 V*, *ftr-3 IV*, *ftr-4 X* and *ftr-5 V*. They are grouped into two categories, class 1 and class 2. Class 1 mutants (*ftr-1*, *ftr-3*, and *ftr-4*) are resistant to 10 mM NaF, but they grow twice as slowly as wild type worms and have a small brood size even in the absence of NaF. In contrast, class 2 mutants (*ftr-2* and *ftr-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 the strong fluoride-resistance of class 1 mutations. (Katsura, I. *et al.* (1994) *Genetics* **136**, 145–154).

To understand the biochemical reactions for fluoride-sensitivity we cloned *ftr-1* and *ftr-3* genes from transposon-insertion mutants, using the transposon Tc1 as the probe. We also cloned partial-length cDNAs of these genes from a *C. elegans* cDNA library using fragments of the genomic clones as the probes and determined their nucleotide sequences. The predicted amino acid sequence of the *ftr-1* protein showed a weak homology with that of some ion channels, but further evidence is required to draw a decisive conclusion. In contrast, the *ftr-3* protein has almost all the domains of protein kinases (Hanks, S. K. *et al.* (1988) *Science* **241**, 42–52). However, since it does not have a close relationship to any special family of protein kinases, we think it encodes a protein kinase belonging to a new family.

***Caenorhabditis elegans* Larval Lethal Mutations That Cause Gross Morphological Changes**

Ryuichi HISHIDA, Takeshi ISHIHARA and Isao KATSURA

We have been analyzing mutants that die as larvae with abnormal shapes, in order to study essential functions in late embryonic and post-embryonic development (Katsura, I. (1993) *Genetica* **88**, 137–146). Among those mutants, we concentrated our study on those in which the outer surface of the intestine is detached from the inner surface of the body wall. We call them *clr-1*-like mutants, since the phenotype resembles that of *clr-1* among known mutations. Ten such mutants have been 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 act also 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 and determined the nucleotide sequence, which encodes a novel protein. We plan to test whether the phenotypes of various signal-transduction mutants are suppressed by microinjection of the wild-type clone of *let(ut40, ut102)*. We will also check the time and location of gene expression using a *lacZ* fusion of *let(ut40, ut102)*.

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 moves anteriorly instead of posteriorly during larval development. Since we isolated a transposon-insertion mutant in *hch-1*, we cloned the gene by the transposon-tagging method. No significant homology to known genes has been found. We plan to clone the cDNA for further investigation.

Construction of *lacZ* Markers for Various Nerve Cells in the Head of *C. elegans*

Takeshi ISHIHARA and Isao KATSURA

To analyze the formation and function of the neural circuit in the head of *C. elegans*, we are preparing markers for various nerve cells using the “promoter trapping” method (Hope I. (1991) *Development* **113**, 399–408). We first made a library consisting of about 20,000 independent clones by ligating genomic DNA fragments of *C. elegans* to a *lacZ* fusion vector. Then, we divided it into about 200 pools, injected the DNA constructs of each pool into the ovary of *C. elegans*, and stained the transgenic worms with Xgal. About 20% of the transformants expressed *lacZ* in some nerve cells in the head. In these cases we isolated single positive clones from the pools and inserted them into a chromosome of *C. elegans*. Thus we obtained stable *C. elegans* strains in which almost all neurons or only one pair of head neurons, for example, express *lacZ*. To prepare markers for subsets of neurons, we also used promoters of genes that probably code for neuronal proteins as suggested by the *C. elegans* genome project. In many cases *lacZ* in the vector was replaced by the cDNA of the green fluorescent protein of the jellyfish *Aequorea victoria* (Chalfie, M. *et al.* (1994) *Science* **263**, 802–805). Such worms showed fluorescence in a defined set of neurons as live animals without any treatment.

Analysis of the Head Neural System of *C. elegans* as Assayed by the Formation of Dauer Larvae

Norio SUZUKI, Misako URASAKI, Takeshi ISHIHARA and Isao KATSURA

If larvae of *C. elegans* are in crowded conditions just after hatching and if only a small number of bacteria are present around them, they develop into enduring, non-feeding larvae called dauer larvae instead of third-stage larvae (L3). The decision to form dauer or L3 seems to be regulated by the head neural circuit with pheromone and the smell of bacteria as key inputs, which act mainly on a pair of sensory organs in the head called amphids. Since the assay of dauer formation is much less time-consuming than behavioral assays such as that of chemotaxis, we started functional analysis of the head neural circuit by checking dauer formation as the output.

It is known that some double mutants in movement or behavior (*unc-31; unc-3*, *unc-31;aex-3*, *unc-104;osm-1*, *unc-104;che-11* and *unc-104;che-13*), but not any of the single mutants, are constitutive in dauer formation. Namely they form dauer larvae even if they are not crowded and are fed well with bacteria. This phenomenon can be explained if we assume parallel pathways for a dauer-inhibiting signal in the head neural circuit, where each of the mutations blocks only part of the pathways. We then looked for other combinations of known mutations that have a synthetic dauer-constitutive phenotype. The following double mutants were found to have such a phenotype: *egl-4;unc-3*, *egl-32;unc-3* and double mutants between either *unc-31* or *unc-3* and one of the following, *che-3*, *che-11*, *daf-6*, *daf-10*, *osm-1*, *osm-5* or *osm-6*. Strangely enough, it is known that the latter mutations change the structure of amphids and have a dauer-defective phenotype by themselves. We discovered also that some other chemotaxis mutations that do not affect the amphid structure as well as most of the fluoride-resistant (*flr*) mutations (see above) have a dauer-constitutive phenotype, if they are combined with *unc-31* or *unc-3*. These results are especially interesting to us, because it shows that the *flr* mutations probably affect neural functions.

We also isolated more than 40 new mutations that have a dauer-constitutive phenotype in combination with an *unc-31* mutation but not by themselves. For this purpose we made a strain *unc-31 Ex[unc-31(+)]* by microinjection of a clone of the wild-type *unc-31* gene into an *unc-31* mutant. Since the injected clone is maintained as an extrachromosomal array, it is somewhat unstable, and the transformant worms produce both Unc^+ and *Unc-31* progeny. We mutagenized such worms with ethyl methanesulfonate and selected among the F2 progeny for those producing *Unc* dauers but not Unc^+ dauers. We are now mapping those mutants using a method based on polymorphic sequence-tagged sites (Williams, B. D. *et al.* (1992) *Genetics* 131, 609–624).

Systematic Analysis of *C. elegans* cDNA —Towards an Expression Map of the Genome—

Yuji KOHARA, Tomoko MOTOHASHI, Akiko NISHIGAKI,
Akiko SUGIMOTO and Hisako WATANABE

Aiming to ultimately understand the network of gene expression in devel-

opment of the worm, we are trying to construct an expression map of the 100 Mb genome through the identification and characterization of all of its cDNA species, whose number is estimated to be around 15,000.

cDNA were made from the mRNA of a mixed-stage population of the *him-8* strain and were size-fractionated before library construction. This time, a set of some 4,400 cDNA clones was selected from a library of >2 kb cDNA by picking up cDNA clones at random (using λ ZAPII as a vector) and then removing abundant cDNA clones (some 20 species which occupied ca. 20% of the library). cDNA inserts of individual clones were PCR-amplified using vector primers and then subjected to (1) tag-sequencing, (2) mapping onto the genome, and (3) the surveying of expression patterns.

(1) Tag sequencing: Single reads have been made from both 5'-ends (using vector primers) and 3'-ends (using anchored oligo-dT primers in order to minimize the effect of the long poly-A stretch) on ABI sequencers. Thus far, we have finished the tag-sequencing for almost all the clones. Out of them, 3,089 clones gave clean 3'-tag sequences, which were used to classify the clones into 1,478 species (808 standing-alone clones and 670 groups of 2–23 clones) by comparing the tags. This search also detected pairs of clones which appeared to be generated by alternative splicing. A database search showed that the set of 1,478 species hit 20% (36 genes) of the *C. elegans* gene entries in GenBank and 23% of the clones showed significant similarities with genes of other organisms. At least 49 cDNA hit the 2 Mb genomic region which has been sequenced by the genome sequencing consortium. Analysis of the 5'-tags are in progress.

(2) Mapping onto the genome: Most of the clones have been mapped using the ordered YAC filter. Recently a supplementary filter (Suppoly) was made available by Alan Coulson at the Sanger Center, U.K. Using these, about 700 cDNA species have been mapped onto the genome; the central regions of chromosomes were much denser for cDNA than other regions.

(3) Surveying expression patterns: Since *in situ* hybridization analysis has confirmed the validity of the membrane strips on which aliquots of the 3'-end region of all cDNA species, amplified from single embryos at various developmental stages, have been dot-blotted (see Tabara & Kohara, in this issue), we are using the membrane strips for the surveying. Thus far, 126 cDNA species mapped on chromosome 3 have been analyzed; briefly, 52 cDNA showed ubiquitous expression throughout embryogenesis, 19 appeared maternal since they gave signals only on the dot of the 0 hr embryo, 14 cDNA

appeared zygotic since they gave signals on the dots from later stage embryos but not from the 0 hr embryo, and the remainder gave no signals at all, probably due to expression after embryogenesis or a very low level of expression.

Part of these data are already in an integrated *C. elegans* genome database, ACEDB version 2 (constructed and managed by Richard Durbin at the Sanger Center, U.K.), and the remaining data will be submitted to the database as soon as possible. The tag-sequences were also submitted to DDBJ.

As a next step, we have picked up altogether 30,000 cDNA clones at random from 3 different cDNA libraries; the same library of >2 kb cDNA, a library of unfractionated cDNA, and an embryonic cDNA library. We are now making membrane blots of high-density grids of the clones, which will be used for probing with the already characterized cDNA to make a subset of the clones for unidentified cDNA species. We are going to continue the above analyses on the subset.

***In situ* Hybridization on *C. elegans* Embryos—Analyses of Candidate Clones for Differentially Expressed Genes—**

Hiroaki TABARA¹⁾ and Yuji KOHARA

Previously, we have developed a method to amplify the 3'-end region (0.5–1 kb) of all mRNA (cDNA) species from a single embryo or a single blastmere quantitatively. The amplified cDNA have been used for two types of differential screening which have given several candidate clones for differentially expressed genes; (1) The cDNA were used as probes in conventional screening of cDNA libraries, (2) aliquots of the amplified cDNA from various stages of single embryos were dot-blotted on strips of nylon membrane and then probed by individual cDNA clones. The validity of the amplification was confirmed by the fact that consistent hybridization signals were obtained in model experiments in which the strips of the dot-blot were probed with stage-specific gene probes; *glp-1* for very early stage, *unc-54* and *myo-2* for mid-late stage and *vit-2* for no expression in embryogenesis. However, the PCR amplification involved in the method could produce potential uncertainty with respect to quantitiveness, and information about

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the distribution of mRNA within an embryo is not available from these analyses. Thus, we have developed a method for in situ hybridization on embryos.

The *in situ* method is based on our protocol for separating blastmeres, in situ protocol for *Drosophila* embryos and that of G. Seydoux (Carnegie Institute, Baltimore). The main point is that the egg shell is removed by chitinase treatment and then the vitelline membrane is partially broken by shearing force before fixation, which takes a bit of time but allows us to regulate the extent of fixation and proteinase digestion closely, leading to high signal-to-noise ratio for early embryos. The results of a model experiment using the stage-specific gene probes (*unc-54*, *myo-2* and *glp-1*) verified the method. As to *glp-1*, both AB and P1 blastmeres were positive, which was consistent with observations by Evans and Kimble.

The results of in situ analysis on 4 cDNA clones were as follows;

(1) Clone 4-1: This clone was obtained through differential screening between the amplified cDNA from single embryos at 1.5 hr and 9.5 hr after the 1st cleavage. It showed positive signals the dots from the 9.5 hr embryo in the dot-blot analysis. In situ analysis showed that this gene gave signals on the embryos from the comma to the 2-fold stages, which exactly coincided with the period expected from the dot-blot analysis, and more precisely, the expression was limited in cells placed along the worm body.

(2) Clone 4-3: This was obtained from the same screening as with clone 4-1, but was specific for the 1.5 hr embryo. The results of in situ analysis were very interesting. No expression was observed in embryos earlier than the 4-cell stage or later than the end of gastrulation. The expression was first detectable at the 8-cell stage in all blastmeres except for P3, and culminated at the beginning of gastrulation except that one cell, probably P4, was still free from the expression.

(3) *ant-1*: This clone was obtained from differential screening using cDNA amplified from AB and P1 blastmeres. However, in situ analysis showed no clear localization of the mRNA at the 2-cell stage and showed rather ubiquitous expression during early embryogenesis. Since this gene has a similarity to the cell cycle related gene, CKS1 (*cdc28* kinase subunit), the differential screening might have detected the difference between the stages of cell cycle in the AB and P1 cells from which the cDNA were amplified. We are going to continue the differential screening.

(4) *skn-1*: Although the *skn-1* gene product was reported to be localized

in P1 and therefore a good example of a maternal gene which showed localized expression in early embryos, *skn-1* probes hybridized equally to dot-blot of the cDNA amplified from AB and P1 blastmeres, suggesting there is no localization of the mRNA. In situ analysis also showed the existence of the mRNA in both AB and P1 cells. These data suggest that the expression of the *skn-1* gene is regulated post-transcriptionally.

Gene Disruption in *C. elegans*— Application of Transposon Insertion Mutant Banks

Yoshiki ANDACHI and Yuji KOHARA

An efficient method for targeted gene disruption is essential to understanding gene function. A so-called gene targeting method is not available in *C. elegans* due to the lack of appropriate selection markers, but mutator strains of high-hopper transposon Tc1 have been used to obtain insertion mutants for many genes. Once a Tc1 insertion mutant is obtained, deletion mutants can be generated from it, taking advantage of illegitimate excision of Tc1, and, it was reported recently that gene replacement was also possible. Building on these strategies, we developed an efficient strategy for isolating Tc1 insertion mutants in a desired gene from a bank of frozen mutant worms.

The main point of our method is a pooling strategy. Pools of worms of a mutator strain, each of which contains about 100 worms, are cultured to the F1 generation. Part of the F1 worms of each pool is stored frozen to construct a mutant bank. Genomic DNA are extracted from the remaining part of the F1 worms and subjected to PCR assay using primers derived from the target gene and Tc1. When a positive pool is found, frozen worms from the pool are cultured individually and a portion of the progeny are subjected to the same PCR analysis. In this fashion, a strain with the Tc1 insertion can be isolated.

We have constructed mutant banks of two mutator strains; 768 pools (about 80,000 independent worms) of RW7097 (*mut-6*) and 192 pools (about 20,000 independent worms) of MT3126 (*mut-2*). To test the quality of the banks, insertion mutants were screened for several genes. Screening of the RW7097 bank for 13 genes revealed 9 mutant strains for 7 genes, and screening of the MT3126 bank for 6 revealed gave 5 mutant strains for 4 genes. The MT3126 bank appears more efficient than the RW7097 bank since

the number of pools in the MT3126 bank is 1/4 of that of the RW7097 bank. These figures provide an estimation for one strain having a Tc1 insertion in a given 4 kb genomic region obtained from the MT3126 bank.

Seven strains out of the 14 insertion strains turned out to have the Tc1 insertions in exons. One strain with the insertion in an exon of the *unc-22* gene showed a typical twicher phenotype. However, the others didn't show any abnormality in viability, morphology and mobility even when they were homozygous with respect to the Tc1 insertion. Recently it was reported that a Tc1 sequence which was inserted in an exon of a gene was spliced out to generate wild-type mRNA. Therefore, we tried to isolate deletion mutants from a Tc1 insertion mutant taking advantage of the fact that excision of a Tc1 frequently accompanies deletion of the flanking region. Two worms of a Tc1 insertion homozygous strain were cultured in each of 50 plates to starvation. DNA was extracted from a part of the progeny worms of each plate, and subjected to PCR analysis using primers flanking the Tc1 insertion site with a distance of about 3 kb. A pool containing deletion mutants gives a PCR fragment shorter than the wild-type fragment (about 3 kb). A strain of the deletion mutation can be isolated by repeating sub-division of the worms of the positive pool and performing PCR analysis.

We have applied the procedure to the *kin-16* gene which is one of the protein kinase homologues, in collaboration with W. Morgan (The College of Wooster, USA), and have isolated 3 deletion mutant strains. One of them turned out to have a deletion of the entire coding region of the gene, and the homozygous strain did not show any abnormality in viability, morphology and mobility. This suggests that the *kin-16* gene is a non-essential gene or that there are functionally exchangeable genes. Isolation of deletion mutants for other genes is in progress.

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 and primordial germ cells (PGCs). PGCs migrate to fetal gonads, become the gonial cells, and take different courses of differentiation in male

and female embryos. We are studying 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 proliferation of PGCs at early migration stages when added to the culture medium (Kawase, E. *et al.* (1994) *Devel. Biol.* **161**, 91–95). We also found that a medium conditioned with buffalo rat liver (BRL) cells has a similar effect on PGCs, but it is effective not only on the younger PGCs but also on those at later stages, thus indicating the presence of different factor(s).

We are currently studying differentiation of PGCs into gonial cells after they have settled in gonads. After developing culture conditions to allow survival and differentiation of PGCs at these stages, we are now analyzing molecular and cellular changes of germ cells at this stage. In addition to the end of proliferation and cell shape changes, we have found differences in response to growth factors and cell surface antigens at these critical stages. Our aim is to develop experimental methods to study developmental processes including meiosis and gamete formation in culture, and to enable manipulation of germ cells, such as gene transfection, at various developmental stages to facilitate 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 a molecular aspect. One area of experimentation involves identifying important genes in the determination and differentiation of the central nervous system during the postimplantation period. We have constructed a cDNA library from 7.5–8.5 days old mouse embryos, in which neural plate and neural tube formation is initiated, and screened for important genes relating to CNS development.

We are currently characterizing one such gene to find out its possible function in embryogenesis. For such a purpose, we are going to analyze the expression pattern using in situ hybridization method. Further, we will study

their roles in development either by ectopically expressing the gene in transgenic mice or chimaeras with transfected embryonic stem (ES) cells, 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.

Analysis of Migration Patterns of Neurons in Morphogenesis of the Mouse Central Nervous System

Norio NAKATSUJI

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. Such a perpendicular pattern occurred even on an artificially fabricated microstructure on the quartz surface (Nagata, I. *et al.* (1993) *Development* **117**, 401–408). We also 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 roles of perpendicular contact guidance in the actual morphogenesis of brain cortices, together with the high resolution optical analysis of growth cone motility to find out the molecular mechanisms of perpendicularity.

V. POPULATION GENETICS

Pattern of Nucleotide Substitutions in Growth Hormone-Prolactin Gene Family: A Paradigm for Evolution by Gene Duplication

Tomoko OHTA

The growth hormone-prolactin gene family in mammals is an interesting example of evolution by gene duplication. Divergence among members of duplicated gene families and among species was examined by using reported gene sequences of growth hormone, prolactin and their receptors. Sequence divergence among species was found to show a general tendency in which a generation-time effect is pronounced for synonymous substitutions but not so for nonsynonymous substitutions. Divergence among duplicated genes is characterized by the relatively high rate of nonsynonymous substitutions, *i.e.*, the rate is close to that of synonymous ones. In view of the stage- and tissue-specific expression of duplicated genes, some of the amino acid substitutions among duplicated genes is likely to be caused by positive Darwinian selection. For details, see *Genetics* **134**, 1271–1276.

Amino Acid Substitution at the *Adh* Locus of *Drosophila* is Facilitated by Small Population Size

Tomoko OHTA

The number of amino acid replacement substitutions and that of synonymous substitutions are examined by using DNA sequences of the *Adh* locus of *Drosophila*. The ratio of replacement to synonymous substitutions is higher in sequence comparisons between species than in polymorphisms within species. The ratio for the between-species comparisons is highest in the Hawaiian group and lowest in the obscura group. These observations suggest that amino acid substitutions are facilitated by small population size. The result is in accord with the nearly neutral theory of molecular evolution. For details, see *Pro. Natl. Acad. Sci., U.S.A* **90**, 4548–4551.

An Examination of the Generation-time Effect on Molecular Evolution

Tomoko OHTA

By using DNA sequences of 17 mammalian genes, the generation-time effect is estimated separately for synonymous substitutions and non-synonymous 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, by using the methods of (i) Nei and Gojobori, (ii) Li, and (iii) Ina. The proportion of accepted amino acid substitutions in evolution is estimated to be almost twice as large in the primate lineage as in the rodent lineage. This result is in accord with the nearly neutral theory of molecular evolution. For details, see *Proc. Natl. Acad. Sci. U.S.A.* **90**, 10676–10680.

Unbiased Estimation of Evolutionary Distance between Nucleotide Sequences

Fumio TAJIMA

The number of nucleotide substitutions between nucleotide sequences is one of the fundamental quantities for the study of molecular evolution. The proportion of different nucleotide pairs between two nucleotide sequences can be converted into the number of nucleotide substitutions per nucleotide site (i.e., evolutionary distance) if an appropriate method is used. Although there are many methods for such transformation, these methods have some problems. First, these methods give an overestimate when the length of nucleotide sequence is short. Second, they cannot be applied when the argument of a logarithm of the estimation formula becomes negative; such inapplicable cases occur quite frequently when two distantly related nucleotide sequences are compared. That is, when x is a random variable, the expectation of $-\log_e(1-x)$ is larger than $-\log_e\{1-E(x)\}$ and x can be larger than unity even if $E(x)$ is smaller than unity, where $E(x)$ is the expectation of x .

I have obtained a new algorithm without logarithm for estimating the evolutionary distance between two nucleotide sequences. This algorithm can be applied to many estimation methods, such as Jukes and Cantor's method, Kimura's transition/transversion method, and Tajima and Nei's method. Unlike ordinary methods, this algorithm is always applicable. Numerical

computations and computer simulations indicate that this algorithm gives an almost unbiased estimate of the evolutionary distance unless the evolutionary distance is very large. This algorithm should be useful especially when we analyze short nucleotide sequences. It can also be applied to amino acid sequences for estimating the number of amino acid substitutions. For details, see *Mol. Biol. Evol.* **10**, 677–688.

Simple Methods for Testing the Molecular Evolutionary Clock Hypothesis

Fumio TAJIMA

Whether the molecular evolutionary clock hypothesis holds or not is one of the most important issues in molecular evolution. This hypothesis may not hold if natural selection is operating, or if mutation rate is not constant per year.

Although there are several methods for testing this hypothesis, all methods have some problems: (i) Absolute rate of molecular evolution cannot be estimated unless we know the divergence time. (ii) We must know the pattern of substitution rates to estimate the number of nucleotide (or amino acid) substitutions per site. (iii) We must also know the variation of substitution rates among different sites to estimate the number of substitutions per site. (iv) The phylogenetic relationship among nucleotide (or amino acid) sequences must be known before the test is performed.

I have developed new statistical methods for testing the molecular evolutionary clock hypothesis, which overcome above problems. These methods are based on the chi-square test and are applicable even when the pattern of substitution rates is unknown and/or the substitution rate varies among different sites. Furthermore, some of the methods can be applied even when the outgroup is unknown. Using computer simulations, these methods were compared with the likelihood ratio test and the relative rate test. The results indicate that the powers of the present methods are similar to those of the likelihood ratio test and the relative rate test, in spite of the fact that the latter two tests assume that the pattern of substitution rates follows a certain model and that the substitution rate is the same among different sites, while such assumptions are not necessary to apply the present methods. The present methods are so simple that they can be easily applied to both nucleotide and

amino acid sequences. For details, see *Genetics* **135**, 599–607.

Statistical Analysis of DNA Polymorphism

Fumio TAJIMA

A large amount of genetic variation can be maintained in natural populations. In order to understand the mechanism maintaining genetic variation, we must first estimate the amount of genetic variation. There are two measures for estimating the amount of DNA polymorphism, i.e., the average number of pairwise nucleotide differences and the number of segregating sites among a sample of DNA sequences. Using these two measures, we can test the neutral mutation-random drift hypothesis (the neutral theory). The expectation of the amount of DNA polymorphism has been studied under several models, including population subdivision, change in population size, and natural selection. When a population is subdivided, a large amount of DNA polymorphism can be maintained in the population if the migration rates among subpopulations are small. In this case the amount of DNA polymorphism in the subpopulation with lower migration rate is expected to be smaller than that of higher migration rate. When the population size changes, the number of segregating sites changes more rapidly than does the average number of nucleotide differences. When purifying selection is operating, the number of segregating sites is more strongly affected by the existence of deleterious mutants than is the average number of nucleotide differences. On the other hand, when balancing selection is operating, the effect of the selection on the average number of nucleotide differences is larger than that on the number of segregating sites. A mutant under natural selection affects the amount of DNA polymorphism at linked sites (hitchhiking effect). DNA sequences are not random sequences and there may be conservative and variable regions in them. For details, see pp. 37–59 in *Mechanisms of Molecular Evolution* (N. Takahata and A. G. Clark, eds, Japan Sci. Soc. Press, Tokyo/Sinauer Associates, Sunderland, MA.) and *Jpn. J. Genet.* **68**, 567–595.

The Molecular Descent of the Major Histocompatibility Complex

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

In the last few years, more than 500 primate major histocompatibility complex (*Mhc*) genes have been sequenced. This extraordinary sequence information was used to draw conclusions about the manner of *Mhc* evolution. *Mhc* genes were found to evolve at a relatively slow rate with the regularity of a clock. It took from 1 to 6 million years for a new mutation to be incorporated into an *Mhc* allele, and the mutation rate is comparable to that of most other primate genes. The nonsynonymous sites coding for the peptide-binding region (PBR) are under relatively weak positive selection pressure (selection coefficient of a few percent only); the nonsynonymous non-PBR sites are under moderate negative selection pressure. The positive pressure is probably provided by parasites and is responsible for the trans-species persistence of allelic lineages at functional *Mhc* loci for more than 40 million years. (For details, see *Annu. Rev. Immunol* **11**, 269–295, 1993.)

Allelic Genealogy and Human Evolution

Naoyuki TAKAHATA

Genetic variation at most loci examined in human populations indicates that the (effective) population size has been $\sim 10^4$ for the past 1 million years (Myr) and that individuals have been genetically united rather tightly. Also suggested is that the population size has never dropped to just a few individuals, even in a single generation. These impose important requirements for a hypotheses on the origin of modern humans: a relatively large population size and frequent migration if populations were geographically subdivided. Any hypothesis that assumes a small number of founding individuals throughout the late Pleistocene can be rejected. Extraordinary polymorphism at some loci of the major histocompatibility complex (*Mhc*) rules out the past action of severe bottlenecks, or the so-called founder principle, which invokes only a small number of founding individuals when a new species emerges. This conclusion may be extended to the 35-Myr-old history of the human lineage, because some polymorphism at *Mhc* loci seems to have lasted that long. Furthermore, although the population structure prior to the late Pleistocene is less clear, owing to the insensitivity of *Mhc*

alleles, even at low levels of migration, the nature of *Mhc* polymorphism suggests that the effective size of populations leading to humans was as large as 10^5 . Hence, the effective population size of humans might have become somewhat smaller in most of the late Pleistocene. The reduction could be due either to the then adverse environment in the Old World and/or to the increased migration rate. It can also be argued that population explosion fostered by the agriculture revolution had significant effects on incorporating new alleles into human populations. (For details, see *Mol. Biol. Evol.* **10**, 2–22, 1993.)

Effective Population Size, Genetic Diversity, and Coalescence Time in Subdivided Populations

Masatoshi NEI and Naoyuki TAKAHATA

A formula for the effective population size for the finite island model of subdivided populations was derived. The formula indicates that the effective size can be substantially greater than the actual number of individuals in the entire population when the migration rate among subpopulations is small. It was shown that the mean nucleotide diversity, coalescence time, and heterozygosity for genes sampled from the entire population can be predicted fairly well from the theory for randomly mating populations if the effective population size for the finite island model is used. (For details, see *J. Mol. Evol.* **37**, 240–244, 1993.)

The Synonymous Substitution Rate of the Major Histocompatibility Complex Loci in Primates

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

Because the divergence of many allelic lineages at the major histocompatibility complex (*MHC*) loci predates species divergence, standard methods of calculating synonymous substitution rates are not applicable to this system. We used three alternative methods of rate estimation: one based on the minimum number of substitutions (D_m), another on the nucleotide difference (D_{xy}), and the third on the net nucleotide difference (D_n). We applied these methods to the protein-encoding sequences of primate *MHC* class I (*A*, *B*, and *C*) and class II (*DRB1*) genes. To determine the reliability of the

different estimates, we carried out computer simulations. The distribution of the estimates based on D_{xy} or D_n , is generally much broader than that based on D_m . More importantly, the D_m -based method nearly always has the highest probability of recovering true rates, provided that D_m is not smaller than 5. Because of its desirable statistical properties, we used the D_m -based method to estimate the rate of synonymous substitutions. The rate is 1.37 ± 0.61 for *A*, 1.84 ± 0.40 for *B*, 3.87 ± 1.05 for *C*, and 1.18 ± 0.36 for *DRBI* loci, always per site per 10^9 years. Hence despite the extraordinary polymorphism, the mutation rate at the primate *MHC* loci is no higher than that of other loci. (For details, see *Proc. Natl. Acad. Sci. USA* **90**, 7480–7484, 1993.)

***MHC* Polymorphism and Human Evolution**

Jan KLEIN, Naoyuki TAKAHATA and Francisco J. AYALA

The diversity of the human major histocompatibility complex (*Mhc* or *MHC*), referred to as the human leukocyte antigens (*HLA*), was generated long before *Homo sapiens* emerged. The diversity that now shields humans from parasites is a heritage that has been handed down through innumerable generations over 65 million years. *HLA* polymorphism helps to solve various problems concerning the origin of humans. In this article, we focused on one convincing message of *HLA* polymorphism about the founding population of *Homo sapiens*. (For details, see *Scientific American* **269**, 46–51, 1993.)

Trans-specific *Mhc* Polymorphism and the Origin of Species in Primates

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

The major histocompatibility complex (*Mhc*) is a cluster of loci controlling the specific immune response in vertebrates. *Mhc* alleles often differ by a large number of nucleotide substitutions, some of which began to accumulate before the emergence of extant species. We applied the theory of allelic genealogy to the primate *Mhc* genes with the aim of estimating the size of the founding populations. The calculations indicated that the long-term effective population size of the studied species was between 10^4 and 10^5 individuals and that it most likely never dropped below 10^3 individuals. (For details, see *J. Med. Prematol* **22**, 57–64, 1993.)

**SPECIAL FEATURE On the Occasion of the 25th Anniversary
of the Neutral Theory (I) Introductory Comments
on Major Papers by Professor Motoo Kimura**

Naoyuki TAKAHATA

Professor Motoo Kimura is best known for his neutral, random drift theory of molecular evolution which was proposed 25 years ago. This theory has revolutionized the way we think about molecular evolution. Yet, population geneticists are more impressed by the power, originality, and ingenuity of his research in theoretical population genetics. The topics that he has treated during the past 40 years are diverse. Except for a few areas such as those in evolutionary stable strategies and applied quantitative genetics, he has indeed covered most of the problems that have been raised in modern evolutionary biology. Because of this diversity, however, it may not be easy to fully appreciate his papers, particularly for students and young researchers. Therefore it is my hope to establish the background for his papers and point out their relationship to other works and to subsequent developments.

Here, 57 papers are grouped into 18 subjects. This grouping obviously does not follow the chronological order and is neither completely unambiguous nor mutually exclusive. Nevertheless, I have adopted this compromise to save space (because the same topic sometimes appears repeatedly in several papers, with a different focus), but more importantly, to emphasize the prospective value of Professor Kimura's work. (For details, see *Jpn. J. Genet.* **68**, 353–394, 1993.)

**SPECIAL FEATURE On the Occasion of the 25th Anniversary
of the Neutral Theory (II) Relaxed Natural Selection
in Human Populations During the Pleistocene**

Naoyuki TAKAHATA

Available genetic data reveals that the human population is more variable than the chimpanzee population at the protein level, whereas the opposite is the case at the DNA level. The lower level of silent polymorphism in the human population suggests that its long-term breeding size is smaller than the chimpanzee's. The neutral theory suggests that natural selection has been

relaxed in the human population under an improved environment. The possibility that the relaxation began with the emergence of *Homo sapiens* was examined, because it is known that *H. habilis* underwent for the first time, dramatic changes in brain size, way of life, and culture, and that the childhood of *H. erectus* was already twice as long as that of chimpanzee. The relaxation hypothesis predicts that, relative to chimpanzee, some 20% of deleterious mutations became harmless under the changed environment throughout the Pleistocene. More extensive study of genetic variation in non-human primates is necessary not only to confirm the hypothesis, but also to better understand the human genome itself. (For details, see *Jpn. J. Genet.* **68**, 539–547, 1993.)

Decay of Linkage Disequilibrium in a Finite Island Model

Hidenori TACHIDA

Time-dependent behavior of linkage disequilibrium when there was initial linkage disequilibrium was studied in a finite island model assuming neutrality. Explicit expressions for linkage disequilibrium parameters were obtained. From these expressions, the initial and the ultimate decay rates of linkage disequilibrium parameters were found to increase and decrease, respectively, due to finiteness of the population when recombination rate, migration rate and inverse of subpopulation size were of comparable order. Also, differentiation of the gametic parameter of linkage disequilibrium among subpopulations was found to diminish quickly. Implications of these results for the interpretations of linkage disequilibria in natural populations were discussed.

Genetic Diversity in Partially Selfing Populations with the Stepping Stone Structure

Hidenori TACHIDA and Hiroshi YOSHIMARU*

Plant species are sometimes partially self-fertilizing. Thus, it is necessary to incorporate self-fertilization in the modeling of plant genetic diversity. Here, we developed a method to derive equilibrium identity coefficients of two genes in partially selfing populations using those in haploid populations. Since explicit expressions are available for the identity coefficients of two

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genes in haploid stepping stone models, we can obtain those in the partially selfing population with the same geographical structure as that of the haploid population using this method. If the mutation rate, population size and migration rate are small, we showed that the identity coefficients for a selfing population can be approximately obtained by putting $N(1-s/2)$ in place of N in the identity coefficients of the corresponding haploid population where N and s are subpopulation size and selfing rate, respectively. Applicability of this approximation and its extension are also discussed.

VI. EVOLUTIONARY GENETICS

**Three Human MHC Class III Genes Near the Junction with
the Class II Locus: Gene for the Receptor of Advanced
Glycosylation End Products, PBX2 Homeobox Gene
and a Notch-homolog, Human Counterpart of
Mouse Mammary Tumor Gene *int-3***

Kimihiko SUGAYA*, Tatsuo FUKAGAWA*, Ken-ichi MATSUMOTO,
Kazuei MITA**, Ei-ichi TAKAHASHI**, Asako ANDO***,
Hidetoshi INOKO*** and Toshimichi IKEMURA

Cosmid walking of about 250 kb from the MHC class III gene CYP21 to class II was conducted. The gene for the receptor of advanced glycosylation end products of proteins (RAGE, a member of immunoglobulin superfamily molecules), PBX2 homeobox gene designated HOX12 and human counterpart of mouse mammary tumor gene *int-3* were found. The contiguous RAGE and HOX12 genes were completely sequenced, and the human *int-3* counterpart was partially sequenced and assigned to a Notch homolog. This human Notch homolog, designated NOTCH3, showed both the intracellular portion present in the mouse *int-3* sequence and the extracellular portion absent in the *int-3*. It thus corresponds to the intact form of a Notch-type transmembrane protein. About 20 kb of dense *Alu* clustering was found just centromeric to the NOTCH3. For details, see *Genomics*, **23**, 408–419 (1994).

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**Pseudoautosomal Boundary (PAB1X and PAB1Y)-like Sequence
Existing Near Boundary of Long-range G+C% Mosaic
Domains in the Human MHC Locus**

Tatsuo FUKAGAWA¹⁾, Kimihiko SUGAYA¹⁾, Ken-ichi MATSUMOTO,
Katsuzumi OKUMURA²⁾, Asako ANDO³⁾, Hidetoshi INOKO³⁾
and Toshimichi IKEMURA

The human genome is composed of long-range G+C% mosaic structures related to chromosomal bands. We found the human MHC locus to be an example of megabase-level G+C% mosaic structures. Chromosome walking of the 450 kb region bridging MHC classes II and III and base-compositional analysis could precisely locate the boundary of the mosaic domains, disclosing a sharp G+C% transition. Near the transition point there was a sequence highly homologous with the pseudoautosomal boundary of short arms of human sex chromosomes (PAB1X and PAB1Y) which is the interface between sex-specific and pseudoautosomal regions. Many PAB1 XY-like sequences (PABLs) were detected by hybridization against genomic DNA, and the new sequences defined the complete form of PABLs of about 650 nt. Homologous sequences in the bovine genome were detected by hybridization, suggesting their evolutionarily stable maintenance and biological significance. For details, see *Genomics* 1995, in press.

**The Distribution of Tenascin-X is Distinct and often Reciprocal
to that of Tenascin-C**

Ken-ichi MATSUMOTO, Yumiko SAGA³⁾, Toshimichi IKEMURA,
Teruyo SAKAKURA⁵⁾ and Ruth CHIQUET-EHRISMANN⁴⁾

We have isolated a cDNA encoding mouse tenascin-X (TN-X), a new member of the family of tenascin genes. The TN-X gene lies in the major histocompatibility complex (MHC) class III region, as it is the case for its human counterpart. On Northern blots we detected a TN-X mRNA of

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approximately 13 kb in most tissues analyzed, whereas in various mouse cell lines mRNAs of 11 and 13 kb were detected, suggesting the possibility of alternative splicing of TN-X transcripts. We raised antibodies against mouse TN-X fragments expressed in bacteria and used these antibodies to identify the TN-X protein in heart cell extracts and in the conditioned medium of a renal carcinoma cell lines. The subunit molecular size of TN-X is approximately 500 kD, suggesting that the protein may contain up to 40 fibronectin type III repeats, making it the largest tenascin family member known yet. TN-X in conditioned medium, as well as the purified protein bind to heparin, but no binding to tenascin-C (TN-C), fibronectin, laminin or collagens could be detected. Thus the heparin-binding activity may be a common feature of the tenascins. The TN-X mRNA as well as the protein are predominantly expressed in heart and skeletal muscle, but the mRNA is found in most tissues at a low level. Immunostaining showed the protein to be associated with the extracellular matrix of muscle tissues and with blood vessels in all of the tissues analyzed. Although the TN-X gene lies in the MHC class III locus, it is not expressed in the lymphoid organs analyzed, except for the staining around blood vessels. In skin and tissues of the digestive tract often a reciprocal distribution of TN-X and TN-C was observed. For details, see *J. Cell Biol.* **125**, 483–493, 1994.

Interfamilial and Intrafamilial Genomic Diversity of Human T Lymphotropic Virus Type I Strains from Papua New Guinea and the Solomon Islands

V. R. NERURKAR*, N. SAITOU and R. YANAGIHARA*

To determine the interstrain genomic diversity and molecular phylogeny of the recently identified variants of human T-cell lymphotropic virus type I (HTLV-I) in Melanesia, we enzymatically amplified, then directly sequenced representative regions of the gag, pol, and env genes of HTLV-I strains from 10 members of four families, including one family from Papua New Guinea and three families from the Solomon Islands. When aligned and compared to a Japanese strain of HTLV-I (ATK), the Melanesian HTLV-I strains differed by 7.6 to 8.7% in the gag, 7.1 to 9.3% in the pol, and 7.3 to 8.2% in the env gene regions. Based on 931 nucleotides, the overall sequence divergence of

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the 10 Melanesian HTLV-I strains from HTLV-I ATK was 7.3 to 8.1% (68 to 75 base substitutions). The intrafamilial genetic heterogeneity among these virus strains was nil to 0.2%, while the interfamilial sequence variation between HTLV-I strains from the Solomon Islands and those from Papua New Guinea was 3.4 to 4.2%, and the genetic heterogeneity among virus strains from the three Solomon Islands families was 0.2 to 0.9%. Using the maximum parsimony and neighbor-joining methods, phylogenetic analysis indicated that the HTLV-I strains from Papua New Guinea and the Solomon Islands formed a monophyletic group and that the Melanesian and cosmopolitan strains of HTLV-I have evolved along two major geographically dependent lineages. For details, see *Virology* **196**, 506–513, 1993.

Population Genetic Study in Hainan Island, China. I. Distribution of Blood Genetic Markers

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

Results obtained in a joint Japanese-Chinese field study in Hainan Island are presented. Three national minority groups, Li, Miao and Hui, together with Han as control, were studied. A total of 23 blood genetic markers comprising 9 blood groups, 7 red cell enzymes and 7 serum proteins, and also alpha and beta thalassemias were examined, and phenotype and allele frequencies were tabulated. The distributions of allele frequencies differed markedly among the four groups, although falling in general within that of southern Chinese. Two sample groups of Li, i.e., Qi-Li and Benti-Li, are more similar to each other than the two sample groups of Miao, probably reflecting different backgrounds in migratory history of Li and Miao. Judging from the high frequencies of both alpha and beta thalassemias and G-6PD deficiency in Li and Miao, it was considered that malaria played a major role in determining these characteristics. For details, see *Anthropological Science* **101**, 1–24, 1993.

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Genetic Variability of Major Histocompatibility Complex in Human Populations

Takashi GOJOBORI and Tadashi IMANISHI

Human major histocompatibility complex (MHC) molecules are called human leukocyte antigens (HLA). A variety of knowledge about HLA structures, functions, and genes has been obtained particularly at the molecular level. The HLA gene complex in human is 4 Mbp long and is located on chromosome 6. HLA genes are classified essentially into two groups, class I and class II, depending on their structure and function. Genes in each class exist as a multigene family in the MHC gene region. It is well known that several HLA genes exhibit an extremely high degree of polymorphisms. In particular, HLA-DR and HLA-DQ genes in class II are highly polymorphic in various ethnic groups. Thus, these genes are very useful for tracing an evolutionary history of human populations. In this study, we described the outline of our phylogenetic analysis of 80 ethnic groups by use of HLA typing data that were collected in the 11th International MHC Workshop at Yokohama.

For more details, see *Human Population Genetics: A Centennial Tribute to J. B. S. Haldane*, Plenum Publishing Co., pp 67-74.

Cloning of Human, Mouse and Fission Yeast Recombination Genes Homologous to RAD51 and recA

Akira SHIOBARA*, Hideaki OGAWA*, Yoichi MATSUDA**,
Noriko USHIO*, Kazuho IKEO and Tomoko OGAWA*

Rad51 of *Saccharomyces cerevisiae* is a homologue of recA of *Escherichia coli* and plays crucial roles in both mitotic recombination and in repair of double strand breaks of DNA. We have cloned genes from human, mouse and fission yeast that are homologous to rad51. The 339 amino acid proteins predicted for the two mammalian genes are almost identical and are highly homologous with the yeast proteins. The mouse gene is transcribed at a high level in thymus, spleen, testis and ovary and at a lower level in brain and other tissues. The rad51 homologues fail to complement the DNA repair

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defect of rad51 mutants of *S. cerevisiae*. The mouse gene is located in the F1 region of chromosome 2 and the human gene maps to chromosome 15.

For details, see *Nature Genetics* **4**, 239–243.

An Evolutionary Role of Kringle Structures

Kazuho IKEO, Kei TAKAHASHI* and Takashi GOJOBORI

Serine proteases involved in the system of blood coagulation and fibrinolysis consist of “kringle” and protease domains. A kringle domain represents a conspicuous secondary structure of about 80 amino acids with three sets of intrachain disulfide bonds. Recently, a new member of kringle family was found in the cell surface protein (ROR) which is a kind of tyrosine kinases. To understand the evolutionary origin and the evolutionary process of the kringle family in the ROR gene, we constructed the phylogenetic trees by use of the nucleotide sequences of kringle structures and tyrosine kinase domains. Our molecular evolutionary analysis shows that the kringle structure sequence of the ROR gene diverged from its ancestral gene about 400 million years ago. Because the ancestral gene of ROR appeared about 1,500 million years ago, the kringle structure was considered to have been inserted into the ROR gene after the appearance of the ancestral gene of ROR-type tyrosine kinase. Our study also shows that the kringle structures play an important role as one of evolutionary and functional units.

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

Yumi YAMAGUCHI and Takashi GOJOBORI

The third variable (V3) region in the envelope protein of HIV is known to be one of major epitopes. It has been reported that some amino acid changes in the V3 region affected the binding ability of antibodies to a viral particle. To elucidate the evolutionary mechanisms of the V3 region within a single host, we conducted a phylogenetic analysis of HIV clones isolated from single hosts. In this analysis, we used two sets of the nucleotide sequence data for the V3 regions of HIV that were reported to have been isolated from single hosts at several time points after infection. In particular, we estimated the

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rates of synonymous and nonsynonymous substitutions during each time interval between different time points. The results obtained showed that the rates of both synonymous and nonsynonymous substitutions vary with time. In particular, we found that there was a time interval when the rate of nonsynonymous substitution became much higher than that of synonymous substitution. It appears that this time interval may be related to the time of onset of AIDS.

Search for Candidate Genes on Which Positive Selection Is Operating

Toshinori ENDO, Kazuho IKEO and Takashi GOJOBORI

It has been known that for most genes examined so far, the number of synonymous substitutions is much higher than that of nonsynonymous substitutions. It is due to less functional constraints against synonymous substitutions because these substitutions, by definition, do not affect the structure and function of a protein. If there is a gene in which the number of nonsynonymous substitutions is significantly larger than that of synonymous substitutions, we can reasonably assume that positive selection may be operating on the gene. Because only the MHC gene can be considered as a candidate gene of positive selection by the studies conducted so far, we performed the extensive search for such candidate genes in the DNA data base. By translating the coding regions of the genes in each entry of the DNA data base into the amino acid sequences, we made the amino acid sequence data base. We then conducted homology search by taking each entry as a query sequence to collect all homologous gene groups. The gene groups obtained were multiply aligned with each other. Finally, the so-called cDNA multiple alignment data base was constructed by replacing the amino acid sequences by the cDNA sequences. Using the cDNA multiple alignment data base, we estimated the numbers of synonymous and nonsynonymous substitutions for all possible pairs of cDNA sequences in all available gene groups. Although we have not obtained the final results yet, the preliminary results suggest that there are several gene groups in which the number of synonymous substitutions is much higher than that of nonsynonymous substitutions.

Relative Efficiencies of Various Methods for Estimating Molecular Phylogenetic Trees Such as the Maximum Likelihood, Maximum Parsimony, Neighbor-joining Methods, when Rate of Nucleotide Substitution Varies with Nucleotide Site

Yoshio TATENO, Naoko TAKEZAKI* and Masatoshi NEI*

The relative efficiencies of the maximum-likelihood (ML), neighbor-joining (NJ), and maximum-parsimony (MP) methods in obtaining the correct topology and in estimating the branch lengths for the case of four DNA sequences were studied by computer simulation, under the assumption either that there is variation in substitution rate among different nucleotide sites or that there is no variation. For the NJ method, several different distance measures (Jukes-Cantor, Kimura two-parameter, and gamma distances) were used, whereas for the ML method three different transition/transversion ratios (R) were used. For the MP method, both the standard unweighted parsimony and the dynamically weighted parsimony is more efficient than unweighted parsimony in obtaining the correct topology. (2) However, both weighted and unweighted parsimony methods are generally less efficient than the NJ and ML methods even in the case where the MP method gives a consistent tree. (3) When all the assumptions of the ML method are satisfied, this method is slightly more efficient than the NJ method. However, when the assumptions are not satisfied, the NJ method with gamma distances is slightly better in obtaining the correct topology than is the ML method. In general, the two methods show more or less the same performance. The NJ method may give a correct topology even when the distance measures used are not unbiased estimators of nucleotide substitutions. (4) Branch length estimates of a tree with the correct topology are affected more easily than topology by violation of the assumptions of the mathematical model used, for both the ML and the NJ methods. Under certain conditions, branch lengths are seriously overestimated or underestimated. The MP method often gives serious underestimates for certain branches. (5) Distance measures that generate the correct topology, with high probability, do not necessarily give good estimates of branch lengths. (6) The likelihood-ratio test and the confidence-limit test, in Felsenstein's DNAML, for examining the statistical significance of branch length estimates are quite sensitive to violation of the assumptions and are generally too

liberal to be used for actual data. Rzhetsky and Nei's branch length test is less sensitive to violation of the assumptions than is Felsenstein's test. (7) When the extent of sequence divergence is $< 5\%$ and when $> 1,000$ nucleotides are used, all three methods show essentially the same efficiency in obtaining the correct topology and in estimating branch lengths. Clearly, the simplest method, i.e., the NJ method, is preferable in this case.

XII. HUMAN GENETICS

Isolation and Fluorescence *in situ* Hybridization Mapping of 60 Cosmid Clones on Human Chromosome 18

Hitoshi NAKASHIMA, Masako SAKAI, Rie INABA and Takashi IMAMURA

We have mapped 60 new cosmids on the short and long arms of chromosome 18 either by R or by DAPI banding and simultaneous fluorescence *in situ* hybridization. These markers were isolated from a hybrid MS126-21, made from a human-rodent hybrid cell line that retained human chromosome 18. Twenty-two of the cosmid probes map on the short arm, and 31 probes cluster in the distal half of the long arm between bands 18q21.1 and 18q23 while 7 other probes are mapped more proximal to the centromere around bands 18q11.1-q12.3. The technique of fluorescence *in situ* hybridization has proven to be a very efficient methodology for gene mapping. These 60 probes will be useful in the elucidation of genetic alterations associated with diseases such as tetrasomy 18p syndrome, 18q- syndromes, and colorectal cancer.

Chromosome 18 is involved in tetrasomy 18p and trisomy 18. The latter is the second most common chromosomal abnormality after Down syndrome. To identify genes involved in these disorders, construction of high-resolution physical map of this chromosome is a rational strategy. A number of small DNA markers and genes have been physically mapped to chromosome 18 by the use of human-rodent somatic hybrids or human chromosome deletion panel. Using Cot-1 or total human DNA as competitor, the hybridization signals from genomic repetitive DNA sequences can be appropriately suppressed so that large DNA segments cloned in artificial yeast chromosomes (YAC) or cosmids can now be precisely localized to the corresponding regions of the chromosomes by fluorescence *in situ* hybridization (FISH). For characterization of disease-related genes, localization of many such large DNA fragments on chromosomes will be very useful. The resources of human 18 chromosome YAC libraries have recently available for mapping, but the localization of a large number of cosmids to this chromosome has not been reported.

Cosmid DNAs were labeled by nick translation with biotin-11-dUTP, hybridized to chromosome DNA as described, under conditions that suppress

signals from repetitive sequences. Hybridized probes were detected by means of fluorescein isothiocyanate (FITC) conjugated to avidin and biotinylated anti-avidin antibody. Signals on metaphase were viewed using a Zeiss Axiophot epifluorescence microscope equipped with a Cooled Charge Coupled Device (CCD) camera. A total of 60 cosmids have been mapped to metaphase chromosome 18. Twenty-two of the cosmid clones were mapped to the short arm, one mapped to the centromere, and 37 were mapped to the long arm of chromosome 18. None of the clones thus far analysed was fortuitously mapped to other chromosomes, suggesting that the library used contains DNA fragments derived only from human chromosome 18 as expected. However, possibility for those cosmids to contain some human-rodent chimeric DNA insert could not be excluded.

Our initial study indicated that cosmid probes derived from the Giemsa dark bands were difficult to hybridize to the R-banded chromosome spreads. However, they might well be localized on the DAPI-banded chromosomes. Since the thymidine synchronization to induce R-banding efficiently blocked the cells at the moment when the R-bands have finished their replication and before the G-bands have began their synthesis, the Giemsa dark bands, being late replicating, were BrdU substituted. Thus, the chromatin structure in G-bands might have undergone overly extensive damage by UV-exposure process resulting in loss of DNA. We thought that the loss of DNA from chromatin might have reduced the efficiency of *in situ* hybridization of the probe relative to G-bands. Since BrdU substituted chromosomal DNAs became more sensitive to UV-irradiation procedure, particular attention was given to the analysis of these probes by omitting UV and reducing denaturation time to the minimum for DAPI multiple banding as suggested. Probes that appeared locating at the junctional region between the G- and R-bands were tentatively mapped on the G-band when detected only by the DAPI banding and simultaneous FISH method, but not by the R-banding of the chromosomes. In any event, probes derived from R-bands were to be mapped clearly on either R- or DAPI-banded chromosomes.

**Characterization of the Supernumerary der(18) Chromosomes by
Fluorescence *in situ* Hybridization with
Single-copy DNA Probes**

Hitoshi NAKASHIMA, Masako SAKAI, Rie INABA, Tomoko HASEGAWA,
Yasuhiro NAKAMURA and Takashi IMAMURA

Many cases of extra marker chromosomes have been reported, including familial and 'de novo' events. In the inherited cases the extra chromosome may not appear to be causally related to the phenotype of the probands. The de novo cases present a variety of clinical pictures depending on the different origins of the markers. Constitutional supernumerary marker chromosomes cannot be easily identified using standard cytogenetic banding techniques because they lack a distinct banding pattern. Marker chromosomes may be associated with a wide range of phenotypes, making it important to identify the chromosomal origin of the markers so that correlations with a clinical phenotype can be evaluated. To develop a method that can differentiate between the i(18p) and other markers including the 18q- chromosome, cosmid probes were cloned from both arms of chromosome 18 using a somatic cell hybrids, which were then mapped to chromosome 18 by fluorescence *in situ* hybridization (FISH). The probe was labeled with biotin and used to identify rearrangements involving the chromosome 18. This approach was used to analyze two patients with the phenotype of tetrasomy (18p) or trisomy 18.

A set of chromosome 18-specific DNA probes were obtained from a cosmid DNA library derived from the cell line MS126, a somatic cell hybrid containing a normal chromosome 18 as its only human component (Nakashima *et al.* (1993) *Genomics* **19**, 577-580). Cosmid SCW0204F was hybridized specifically to the short arm at 18p11.2, SCW0207A hybridized to the pericentric region of chromosome 18, and SCW0205D hybridized to the long arm at 18q11.2. FISH was performed according to the methods described elsewhere. Cosmid DNAs were labeled by nick translation with biotin-11-dUTP and were hybridized to chromosome DNA under conditions that suppress signals from repetitive sequences. Hybridized probes were detected by means of fluorescein isothiocyanate (FITC) conjugated to avidin. Signals on both metaphase and interphase nuclei were viewed using a Zeiss Axiophot epifluorescence microscope equipped with a cooled charge coupled device (CCD) camera.

A 15-year-old boy was observed to have microcephaly, epicanthal folds, hypotelorism, hypotonia, a single simian crease, and mildly delayed development. Chromosome analyses revealed a karyotype, 47, XY, +mar, for all cells examined. The marker chromosome was metacentric and potentially an *i*(18p). A probe previously localized on the short arm of chromosome 18 was selected for FISH to determine the origin of the marker chromosome. Fluorescent signal of the probe SCW0204F DNA was present on the short arm at 18p11.2 of the normal copies of chromosome 18. Furthermore, the probe was hybridized to both arms of the metacentric marker chromosome. The results clearly indicate that the marker chromosome is the *i*(18p).

The other patient had phenotypic features characteristic of trisomy 18 at her birth. Cytogenetic analysis showed a karyotype, 47, XX, +18, t(2;18)(q11;q11), inv(9)(p11q13). The probe SCW0207A specific for the pericentric region of chromosome 18 hybridized to one of the rearranged chromosome t(2p18q), but not to the other t(18p2q) chromosome. It is noteworthy that the triplicated hybridization signals for the pericentric region of chromosome 18 could be obtained also from the interphase nuclei (data not shown). The probe SCW0205D hybridizing to the long arm at 18q11.2 of the normal copies of chromosome 18 situated on the long arm of rearranged t(2p18q) chromosome. Therefore, the break point on the chromosome 18 must be localized between the SCW0207A locus at pericentric region and the SCW0205D locus at 18q11.2.

One type of marker chromosome that has been reported is the isochromosome 18p. The *i*(18p) chromosome is difficult to distinguish from chromosome with a large deletion of 18q. Furthermore, translocation products of 18 with another chromosome may appear cytogenetically indistinguishable from the *i*(18p). In cases associated with multiple congenital anomaly, clinical features are variable and standard banding techniques allow only a likely diagnosis. Thus, the identification of *i*(18p) chromosome should use an 18p probe with attention paid to the distribution of signal on both arms of the marker chromosome as well as on normal copies of chromosome 18. While chromosome paints may identify the origin of markers or detect translocations, band-specific single-copy sequence probes will permit detailed analysis of chromosomal rearrangements when more precise information may be needed. To our knowledge this is about the first occasion that chromosome 18 trisomy has been reported in association with a reciprocal translocation between chromosome 2 and 18. Although it is important to detect the origin

of imbalance of chromosome 18, it remains to be clear whether this case is a de novo rearrangement or a malsegregant of a previously existing balanced karyotype in a parent.

A Dinucleotide Microsatellite Polymorphism on the Chromosome 18q22 Locus

Hitoshi NAKASHIMA, Masako SAKAI, Rie INABA
and Takashi IMAMURA

Cosmid SCW0205G containing the (CA)_n repeat was isolated from the chromosome 18-specific cosmid library that was made from a human-rodent hybrid cell line MS125-21 (Nakashima *et al.* (1993) *Genomics*, **19**, 577-580) with chromosome 18 as the only human chromosome materials. Colonies were gridded on nylon membranes and were screened with radio-labeled (CA)₂₀ oligonucleotide probe. The insert DNA was digested with Sau3AI and subcloned into pUC18 plasmid. CAW0205G, a 0.7 kb Sau3AI fragment was sequenced using primers designed for the sequence flanking the cloning site. The dinucleotide repeat polymorphism was typed using the polymerase chain reaction with one of the primer fluoro-labeled. Primer sequences were TAT GTA AAA ACT GAA AGA ATC CAC (CA strand) and TTT TAT TCT TTT TGA TGT TAT TGT (GT strand). The reaction products were analysed on a 6% denaturing acrylamide sequencing gel by the use of an automated laser fluorescent DNA sequencer detection system (Genesis 2000 DNA Analysis System; DuPont). Allele sizes were determined by comparison to M13mp18 sequencing peaks. Fourteen different allele-frequencies ranged from 0.02 to 0.23, which were calculated from the genotype of 50 unrelated Japanese. The heterozygosity was 0.87. Codominant segregation was observed in 4 informative two- to three-generation-families.

Peopling of the Americas, Founded by Four Major Lineages of Mitochondrial DNA

Satoshi HORAI, Rumi KONDO, Yuko NAKAGAWA-HATTORI, Seiji HAYASHI,
Shunro SONODA and Kazuo TAJIMA

Mongoloid descendants are now distributed over a wide area of the Pacific-Rim region, having adapted to a variety of environments. One of the

major issues for research into the prehistoric dispersal of Asiatic Mongoloid peoples is the question of the first Americans, the "Peopling of the New World". No authority doubts that the ancestral native Americans came from Northeast Asia across the Bering land bridge, and then dispersed and settled in various parts of the Americas, and finally reached the southernmost part of South America. However, when they came and with what genetic backgrounds and cultures, is not yet fully resolved.

Nucleotide sequence analysis of the major noncoding region of human mitochondrial DNA from various races was extended with 72 Native Americans from 16 different local populations (nine populations from Chile, four from Colombia, and one each from Brazil and from Maya and Apache Indians). The sequences were determined directly from the polymerase chain reaction products. On the basis of a comparison of the 482-bp sequences in the 72 Native Americans, 43 different types of mitochondrial DNA sequences were observed. The nucleotide diversity within the Native Americans was estimated to be 1.29% which is slightly less than the value of 1.44% from the total human population including Africans, Europeans, and Asians. Phylogenetic analysis revealed that most Native American lineages are classified into four major distinct clusters. Individuals belonging to each cluster share at least two specific polymorphic sites that are nearly absent in other human populations, indicating a unique phylogenetic position of Native Americans. A phylogenetic tree of 193 individuals including Africans, Europeans, Asians, and Native Americans indicated that the four Native American clusters are distinct and dispersed in the tree. These clusters almost exclusively consist of Native Americans with only a few Asians, if any. We postulate that four ancestral populations gave rise to different waves of migration to the New World. From the estimated coalescence time of the Asian and Native American lineages. We infer that the first migration across the Bering land bridge took place $\sim 14,000$ – $21,000$ years ago. Furthermore, sequence differences in all pairwise comparisons of Native Americans showed a bimodal distribution that is significantly different from Poisson. These results suggest that the ancestral Native American population underwent neither a severe bottleneck nor rapid expansion in population size, during the migration of people into the Americas. For details, see *Mol. Biol. Evol.* **10**, 23–47 (1993).

Evolution of Hominoid Mitochondrial DNA with Special Reference to the Silent Substitution Rate over the Genome

Rumi KONDO, Satoshi HORAI, Yoko SATTA
and Naoyuki TAKAHATA

Focusing on the synonymous substitution rate, we carried out detailed sequence analyses of hominoid mitochondrial(mt) DNAs of ca. 5-kb length. Owing to the outnumbered transitions and strong biases in the base compositions, synonymous substitutions in mtDNA reach rapidly a rather low saturation level. The extent of the compositional biases differs from gene to gene. Such changes in base compositions, even if small, can bring about considerable variation in observed synonymous differences and may result in the region dependent estimate of the synonymous substitution rate. We demonstrate that such a region dependency is due to a failure to take proper account of heterogeneous compositional biases from gene to gene but that the actual synonymous substitution rate is rather uniform. The synonymous substitution rate thus estimated is $2.37 \pm 0.11 \times 10^{-8}$ per site per year and comparable to the overall rate for the noncoding region. On the other hand, the rate of nonsynonymous substitutions differs considerably from gene to gene, as expected under the neutral theory of molecular evolution. The lowest rate is 0.8×10^{-9} per site per year for COI and the highest rate is 4.5×10^{-9} for ATPase 8, the degree of functional constraints (measured by ratio of the nonsynonymous to the synonymous substitution rate) being 0.03 and 0.19, respectively. Transfer RNA (tRNA) genes also show variability in the base contents and thus in the nucleotide differences. The average rate for 11 tRNAs contained in the 5-kb region is 3.9×10^{-9} per site per year. The nucleotide substitutions in the genome suggest that the transition rate is about 17 times faster than the transversion rate. For details, see *J. Mol. Evol.* **36**(6), 217-231 (1993).

Seroepidemiological Survey of Human T-lymphotropic Retrovirus Among Indigenous Plations in Taiwan

Takafumi ISHIDA, I-Hung PAN, Satoshi HORAI, Naruya SAITOU
and Chie-Shan SUN

Human T-lymphotropic retrovirus (HTLV) has been considered as the causative agent of adult T-cell leukaemia (ATL) and HTLV-I associated

myelopathy (HAM/TSP). HTLV showing endemic and non-endemic patterns of infection among human populations is not a common virus in man. Taiwan is situated between HTLV-endemic Ryukyu (Southwestern part of Japan) and non-endemic mainland China. Recent seroepidemiological studies showed several seropositive cases of HTLV infection in the Taiwanese population. In addition patients with ATL and HAM/TSP have been identified. The Taiwanese population consists of Han Chinese and indigenous populations. The Han Chinese migrated from Fujian and Canton in mainland China several hundred years ago and are to be found throughout the island. The indigenous Taiwanese who live in the mountainous areas, on the southeastern coastal region, and on an island between Taiwan and the Philippines, have been classified into nine ethnoculturally distinguishable populations. Ethnological data suggest that there is a close relationship between the indigenous Taiwanese and southeast Asian native groups. Data from two nationwide seroepidemiological studies of HTLV are available and both report positive cases in the Han Chinese with prevalence rates ranging from 0% to 1%. However, there is controversy over the presence of virus carriers in the indigenous populations. In one study seropositivity was reported in the north whereas in another no seropositive cases were found among eight indigenous population groups.

Because previous surveys did not cover all indigenous population groups, a serological survey of HTLV infection among all nine indigenous populations of Taiwan (Ami, Atayal, Bunun, Paiwan, Puyuma, Rukai, Saisiat, Tsuo, and Yami) was carried out. In all, 797 healthy subjects in Taiwan including Han Chinese and nine indigenous populations were examined for the presence of antibodies to HTLV by particle agglutination, indirect immunofluorescence, and Western blot test. Two seropositive cases were found in this screening. One Saisiat male and a Han Chinese female were seropositive for HTLV. The Western blot profile indicated the virus was type-1 HTLV. For details, see *Int. J. Epidemiol.* 22, 927-930 (1993).

Mitochondrial DNA Mutations at Nucleotide Positions 3243 and 3271 in Mitochondrial Myopathy, Encephalomyopathy, Lactic Acidosis, and Stroke-Like Episodes: a Comparative Study

Ryoichi SAKUTA, Yu-ichi GOTO, Satoshi HORAI and Ikuya NONAKA

An A-to-G transition mutation at nucleotide position (nt) 3243 in the mitochondrial (mt)-tRNA^{Leu(UUR)} gene (3243 mutation) is commonly found in patients with MELAS (mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes). In our previous study, 32 of 40 patients (80%) with MELAS had this mutation, the remaining 8 patients did not. We subsequently found a T-to-C transition in the same mt-tRNA gene but at a different position, at nt 3271 (3271 mutation), in three of the 8 remaining patients. In this communication, we report the analysis of 10 other patients with MELAS, which allowed us to estimate the incidence of the new 3271 mutation and to clarify the phenotypic differences between patients with the 3243 and 3271 mutations in a total of 50 patients.

Of 50 patients with the clinical characteristics of MELAS, 38 had a point mutation at nucleotide position (nt) 3243 in the tRNA^{Leu(UUR)} region in mitochondrial DNA and 6 at nt 3271 in the same tRNA^{Leu(UUR)} gene. Except for the later onset of the disease in the patients with the 3271 mutation, there were no clinical, biochemical and pathological differences between the two groups. Since the nt 3271 region is not located in the binding site for mitochondrial transcription termination (mTERM) factor, which has been proposed to be defective in the 3243 mutation, a functional defect in tRNA itself might be responsible for the enzyme defects in MELAS patients; however the mechanism by which the defective tRNA^{Leu(UUR)} induces the stroke-like episodes remains to be clarified. For details, see *J. Neurol. Sci.* **115**, 158-160 (1993).

An A-to-G Transition at Nucleotide Pair 11084 in the ND4 Gene May Be an mtDNA Polymorphism

Ryoichi SAKUTA, Yu-ichi GOTO, Ikuya NONAKA and Satoshi HORAI

Lertrit *et al.* (*Am. J. Hum. Genet.* **51**, 457-468, 1992) reported a new disease-related mutation of mtDNA in a patient with mitochondrial myopathy, encephalopathy, lactic acidosis, and strokelike episodes (MELAS).

They reported two Caucasian patients with the classical features of MELAS and found different mtDNA mutations in each patient: in one, an A-to-G transition at nucleotide pair (nt) 3243 in the tRNA^{Leu(UUR)} gene (3243 mutation), commonly seen in MELAS patients, and in the other, an A-to-G transition at nt 11084 in the ND4 gene (11084 mutation), a mutation which Lertrit *et al.* claimed is a novel disease-related mutation. In 50 MELAS patients studied in our laboratory, we found the 3243 mutation in 34 patients, a T-to-C transition at nt 3271 in the tRNA^{Leu(UUR)} gene (3271 mutation) in 4 patients, and the 11084 mutation in 3 patients. To determine whether the 11084 mutation was a disease-related mutation, we further analyzed 90 patients with a variety of other mitochondrial myopathies, as well as 105 normal Japanese individuals. The 11084 mutation was found in 9 patients (10%) with mitochondrial myopathies, including MELAS, chronic progressive external ophthalmoplegia (CPEO), and other miscellaneous mitochondrial disorders, and in 15 (14%) normal individuals. However, none of the 109 normal and diseased Caucasians, American blacks, or several patients with mitochondrial diseases, including 7 patients with Leber hereditary optic neuropathy, had the 11084 mutation. On the other hand, 1 of 10 Japanese patients with mitochondrial respiratory disorder showed the 11084 mutation. The high incidence of the A-to-G substitution at nt 11084 in mtDNA in the Japanese population suggests that this substitution may be a polymorphism prevalent in the Japanese. Although nt 11084 is an evolutionarily conserved site, and although the amino acid residues are highly invariant in various species, our findings suggest that an A-to-G substitution at nt 11084 may be a polymorphism and may not be responsible for inducing the clinical phenotype of MELAS. For details, see *Am. J. Hum. Genet.* 53, 964-965 (1993).

A T-to-G Mutation at Nucleotide pair 8993 in Mitochondrial DNA in a Patient with Leigh's Syndrome

Harumi YOSHINAGA, Tatsuya OGINO, Shunsuke OHTAHARA, Ryoichi SAKUTA,
Ikuya NONAKA and Satoshi HORAI

We studied a patient with Leigh's syndrome using neurophysiologic, radiologic, enzymatic, biochemical, and molecular analysis. Her clinical course had started with acute encephalopathic symptoms at 7 months of age. With repeated remission and exacerbation, she developed hypotonia and

symptoms of brainstem dysfunction, such as irregular respiration and swallowing difficulty. These symptoms were followed by epileptic seizures, including simple partial seizures and tonic spasms. Both serum lactate and serum pyruvate levels were elevated, and deficient activity was detected in cytochrome c oxidase in her quadriceps femoris muscle. From the early stages, we noted an abnormality in the auditory brainstem response and visual evoked potential, and an abnormal symmetrical low-density area in the basal ganglia on the computed tomographic scan. We found a mitochondrial DNA point mutation at 8993 in blood samples from both the patient and her mother using a simple polymerase chain reaction method. The ratio of wild and mutant mitochondrial DNA calculated densitometrically on polymerase chain reaction products was 56.6% in the patient's blood cells and 8.4% in her mother's. This patient's disorder was thought to be maternally inherited Leigh's syndrome. Her brother had died of the identical clinical features at 1 year 9 months of age. For details, see *J. Child. Neurol.* **8**, 129–133 (1993).

**Identification of HS1 Protein as a Major Substrate of
Protein-tyrosine Kinases Upon B-cell Antigen
Receptor-mediated Signaling**

Yuji YAMASHITA, Masato OKADA, Kumao TOYOSHIMA,
Daisuke KITAMURA and Takeshi WATANABE

Crosslinking of membrane-bound immunoglobulins, which are B-cell antigen receptors, causes proliferation and differentiation of B cells or inhibition of their growth. The receptor-mediated signaling involves tyrosine phosphorylation of cellular proteins and rapid activation of Src-like kinases. The amino acid sequences of five proteolytic peptides of p75, a major substrate of protein-tyrosine kinases in the signaling, showed that p75 is the human HS1 gene product. The HS1 gene is expressed specifically in hemopoietic cells and encodes p75^{HS1}, which carries both helix-turn-helix and Src homology 3 motifs. p75^{HS1} showed rapid tyrosine phosphorylation and association with a Src-like kinase, Lyn, after crosslinking of membrane-bound IgM. Thus, p75^{HS1} may be an important substrate of Lyn and possibly other protein-tyrosine kinases upon B-cell antigen receptor-mediated signaling. (*Proc. Natl. Acad. Sci., USA*, **9**, 3631–3635, 1993)

Regulation of the Expression of Human C ϵ Germline Transcript

Toshihiro ICHIKI, Wataru TAKAHASHI and Takeshi WATANABE

Transcriptional regulation of Ig H chain germ-line transcripts induced by cytokines, is a topic of recent interest in the understanding of the mechanism for class recombination. Among human B cell lines examined, we found that a human IgM-producing B cell line, DND39 (EBV negative), expressed germ-line transcripts of ϵ constant gene (C ϵ) when stimulated with IL-4. In our study, the regulatory element responsible for the expression of the IL-4 induced human C ϵ germ line transcript was determined using DND39 cells. To identify the IL-4 responsive promoter/enhancer element, deletion analysis of the upstream region of the germ-line exon (I ϵ) of the C ϵ -germ-line transcript located 5' to the switch region, was performed using a luciferase gene as a reporter. Deletion analysis showed that a DNA fragment which lies between -215 and -154 bp upstream from the most 3' transcriptional initiation site of the human I ϵ gene is fully responsible for the induction of germ-line transcripts by IL-4. From a mutations analysis, the DNA fragment between -163 and -152 bp, was identified as a novel IL-4 responsive element in a human C ϵ gene. Electrophoretic gel mobility shift assay showed the presence of an IL-4 induced nuclear factor that specifically bound to this IL-4 responsive element. This novel IL-4 responsive element and an IL-4 induced DNA binding protein may play an important role in the induction of the C ϵ germline transcript as well as class switching to Ig E (*J. Immunol.*, **150**, 5408-5415, 1993).

VIII. PLANT GENETICS

Bacterial Blight Resistance in Wild Rice Populations

Hiroko MORISHIMA and Toshie MIYABAYASHI

Population biology studies on bacterial blight resistance in wild and cultivated rice were undertaken to gain understanding of the evolutionary dynamics of a plant-pathogen system. We demonstrated that natural populations of wild rice as well as those of the rice landrace are polymorphic in resistance to bacterial blight disease (BB), and that wild rice is more resistant than cultivars, and the perennial ecotype of wild rice is more resistant than the annual ecotype. The following are experimental results recently obtained.

(1) Gene analysis of resistance in wild rice.

About 20 BB resistance genes have so far been identified through genetic research in rice. To investigate the genetic basis of resistance in wild rice (*Oryza rufipogon*), three strains showing different reaction patterns to four pathogen races (*Xanthomonas oryzae* pv. *oryzae*) were chosen from a polymorphic population of annual-type wild rice collected in Thailand and then crossed with each other. The segregation pattern of BB resistance observed in F₂ populations suggested that at least two dominant genes are responsible for resistance in the strains used. Most probably many more resistance genes are preserved in this population. Analysis of F₂'s derived from the crosses with tester lines indicated that the wild strains used, carry resistance genes different from either of the known genes (*xa-5* and *Xa-21*) which show similar reaction patterns.

(2) Mechanism of resistance polymorphism within populations.

We have found a high degree of BB resistance polymorphism within wild rice populations, especially in the annual type of wild rice. In general, resistance polymorphism can be explained by either of the following two models. 1) If resistant plants are less fit than susceptible ones in disease-free conditions ("resistance cost"), then a polymorphism can be maintained. 2) If pathogens are genetically heterogeneous with each specialized in different host genotypes, then a polymorphism can be maintained through frequency-dependent selection. We examined the relationship between degree of BB resistance and seed productivity efficiency in the absence of pathogens using

individuals derived from a few natural populations as well as F_2 populations derived from resistant and susceptible plants crosses. In experimental populations derived from natural populations of wild rice, the relationships between resistance and fitness did not show any consistency, being either positively correlated, negatively correlated or uncorrelated. In F_2 's, no correlation was found in any population. Thus, we failed to detect a trend to resistance cost in this experiment. We are now planning to examine genetic heterogeneity within a pathogen population which might be another possible mechanism of resistance polymorphism.

Life-History of Wild Rice Species Distributed in the Basin of the Amazon River

Hiroko MORISHIMA, Yoshiya SHIMAMOTO*, Masashi OHARA*,
Paulo S. MARTINS**, Akihiko ANDO** and
Giancarlo C.X. OLIVEIRA**

Under a Japan-Brazil cooperative project on Amazonian plant germplasm, we made study-trips to the Rio Negro in 1992 and to the Rio Solimões in 1993, focusing on the genus *Oryza*. Two wild rice species, *O. glumaepatula* and *O. grandiglumis* were found. A total of 103 sites inhabited by wild rice were studied, and seed samples were collected from 68 populations of *O. glumaepatula* and from 61 populations of *O. grandiglumis*. The former is a diploid species carrying the same genome as cultivated rice (AA), and the latter is an allotetraploid species carrying a different genome (CCDD).

1. *O. glumaepatula* was found in the Rio Solimões (water pH is nearly neutral) as well as in the Rio Negro (pH 4.5–5.5). *O. grandiglumis* was frequently distributed in the Rio Solimões, but rare in the Rio Negro. Interestingly, this species was found in a tributary of the Rio Negro (Rio Branco) which belongs to a high pH water system. This suggests that *O. glumaepatula* can be grown in water with a wide range of pH, but *O. grandiglumis* can not survive in low pH water.

2. Although during the present trip we could observe only the plant life in the aquatic phase, circumstantial evidence indicated that these wild rice species initiate their yearly growth in the terrestrial phase (dry season) from

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seedlings in *O. glumaepatula*, and from ratoons (asexual propagation) in *O. grandiglumis*.

3. When we traveled during the later stage of the aquatic phase, many populations of *O. glumaepatula* were forming free-floating vegetation, continuing to develop reproductive shoots, while plants of *O. grandiglumis* were standing erect, rooted on the river bottom. These two species seemed to have different strategies for coping with the drastic rise of water level; the former species through free-floating, and the latter through a high ability for internode elongation. *O. glumaepatula* proved to have a unique characteristic of brittleness of the stem which helped them to become free-floating plants.

4. It is not known how the flowering time of wild rice distributed in the Amazon is regulated where daylength as well as temperature are almost constant throughout the year. According to information obtained from local people, wild rice plants begin to flower during the period of rising river water. Our preliminary experiments showed that both species are photoperiod sensitive, inducing panicles in response to short day-length. In this experiment, *O. glumaepatula* showed a second peak of flowering under long day-length (*O. grandiglumis* did not show a second peak).

5. A preliminary study of isozyme variation indicated that *O. glumaepatula* showed little genetic differentiation among populations. This may be explained by the free-floating habit of this species. In contrast, *O. grandiglumis* which tends to persist at the same site seemed to develop a certain degree of population differentiation.

6. Seeds of both species were found to lack strong dormancy which is a general characteristic of wild rice. Further, they seemed to be able to survive in water longer than the seeds of related *Oryza* species distributed in other continents.

The present trip provided us with important information and germplasm collections of *Oryza* species in the Amazon Basin. They will surely contribute to our future ecological-genetic study of American wild rice. Further, the unique characteristics found in these wild rices are expected to be utilized in rice breeding work.

Genetic Erosion of Rice in the Tropics

Hiroko MORISHIMA, Yo-Ichiro SATO, Yoshiya SHIMAMOTO*,
Hiroshi YAMAGISHI** and Tadashi SATO***

Loss of genetic resources is called *genetic erosion*. Rapid progress of genetic erosion occurring in crop species and their wild relatives has been recognized as a serious problem in the field of agronomy. To estimate the rate of decline of the Asian common wild rice (*Oryza rufipogon*), biomass (estimated as the total area of population size \times rice plant cover in percent) of wild rice plants in 21 populations (nine annual and twelve perennial types) in northern and northeastern Thailand in 1983 and 1991 (or 1992) were compared. The biomass in the annual populations was reduced to 13 percent of the original value on the average during the last decade, suggesting that the annual type of wild rice will disappear by the end of this century. The biomass in the perennial populations was reduced to 73 percent. In perennial populations, the weedy type, which is most probably a derivative of natural hybridization between wild rice and the neighboring cultivars is increasing. Thus, pure perennial wild rice is now being replaced by the weedy type.

Genetic erosion occurs in cultivated rice also. The number of native varieties grown in northeastern Thailand has been reduced approximately to 50 percent during the last decade. Genetic diversity within a field has also been reduced along with the introduction of high-yielding varieties.

To prevent the progress of genetic erosion, gene banks at international and national levels were established for preservation of genetic resources for major cultivated plant species, such as rice, wheat, corn, barley, beans. In the case of rice, a gene bank was built at the International Rice Research Institute (IRRI). National sites were also built in various countries. Such conservation programmes have played important roles in maintaining genetic resources. The laboratory conservaton of seed stocks, however, is always subject to unconscious selection through man's manipulation and consequently potentially useful genes preserved in natural populations are endangered by various factors. The significance of *in situ* conservation (conservation of natural populations in their original habitats) of genetic resources, is now recognized. From the viewpoint of agronomy, low input sustainable

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agriculture (LISA) should be encouraged to maintain desirable agroecosystems.

Gamete Eliminator Adjacent to the *wx* Locus Detected in an Indica-Japonica Hybrid of *Oryza sativa*

Yoshio SANO, Mitsugu EIGUCHI and Hiro-Yuki HIRANO

A previous study of rice revealed that Wx^a (non-waxy endosperm) could not be transferred from PTB 10 (Indica type of *Oryza sativa*) to T65wx (Japonica type of *O. sativa*). To clarify this phenomenon, the F_1 hybrid was backcrossed with T65wx. Two semi-sterile BC_3F_1 plants were heterozygous for *wx*, but produced only waxy seeds on selfing. Pollen analysis revealed that non-waxy pollen grains were present at a frequency of 14.8×10^{-4} , indicating that most Wx^a pollen grains were eliminated during gametogenesis. The results showed that PTB 10 and T65wx carried $SIO^a(t)$ and $SIO(t)$, respectively, and that $SIO(t)$ induced abortion of gametes that carried the opposite allele, $SIO^a(t)$, which was linked to Wx^a . The frequency of recombination between *wx* and $SIO(t)$ suggests that the gamete eliminator detected is very close to the *wx* locus. Molecular analysis of the eliminator may thus be facilitated (J. Heredity 85: 310–312).

Procedures for Rejuvenating Plant Genetic Resource Accessions with Minimum Loss of Allelic Diversity

Katsuei YONEZAWA and Hiroko MORISHIMA

Allelic diversity in plant genetic resource accessions is reduced through cycles of rejuvenation. Strategies for minimizing the loss of allelic diversity were discussed based on effective population size and the probability that the initial allelic diversity within accessions is maintained after 10 and 50 cycles of rejuvenation. Population size (the number of plants grown for one cycle of rejuvenation) is the most manageable and important factor in determining the adequacy of maintenance. Population size for each accession, however, cannot be large in most maintenance programmes since many accessions must be regenerated within a limited field area. Maintaining more accessions with a smaller population size is more efficient than maintaining fewer with a larger population size. Optimum population size therefore is not uniquely

specified, but depends on the total number of accessions to be maintained and field capacity. When the population size is determined, a means for reducing variance in progeny number per plant is useful. The SSD (single seed descent) method is the most effective and practical means. Harvesting seeds from not all but only some sampled plants is not advantageous even if an equal number of seeds is taken from each of the sampled plants.

The effectiveness of the SSD method, however, is not sufficiently large in predominantly outcrossing accessions. In outcrossing accessions, controlled pollination such as selfing and sib-mating may be used combined with the SSD method if the accessions are self-compatible and sufficiently tolerant to inbreeding depression. Selfing by bagging would be the most handy and effective method. In species where controlled selfing is not practical, partitioned maintenance, in which each accession is maintained in a number of separate sub-groups, may be applied, though it is not highly rewarding. The biparental mating system (Gale and Lawrence 1984), which is highly effective in outcrossing accessions but resource-consuming, may be applied to accessions of special importance.

IX. DATABASE

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. We developed 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.

DNA Database Management System

Hajime KITAKAMI, Tadasu SHINI, Kazuho IKEO, Yoshihiro UGAWA,
Naruya SAITOU, Takashi GOJOBORI and Yoshio TATENO

A flat-file system is inadequate for building, integrating and searching a large-scale database in which the number of entries is increasing in an explosive manner. The DNA database of DDBJ (DNA Data Bank of Japan) has been built in AWB developed by LANL on behalf of a flat-file system since autumn 1991. AWB is a tool for building the DNA database with the so-called GenBank-schema based on a relational database management

system, SYBASE. Recently, we newly developed a *hierarchical relational schema* for effectively building, integrating, and searching the DNA database on the relational database management system, SYBASE, at DDBJ. The schema is named the “*DDBJ-schema*”. The schema allowed 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 with the *DDBJ-schema* has an interface, *YAMATO*, implemented in X-window and it can be used on UNIX based workstations over computer networks. The second type has an interface, *ASUKA*, implemented in the DB-Library of SYBASE and it can be used on character terminals over telephone networks. Both also have a window interface similar to the flat file format. Finally, we proposed a *structured SQL-programming method* to be used to implement the DNA database management system. The method is developed through the SQL expression and the control flow language (CFL) of SYBASE, and allows us to implement a restructuring tool and a tree search tool. The restructuring tool is needed to convert data with the GenBank-schema into those with the DDBJ-schema when we move AWB to the new system. The tree search tool is needed for repairing and searching the taxonomy database at DDBJ. For more details, please see “Molecular Bioinformatics,” IEEE Press, London pp. 61–69.

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ABSTRACTS OF DIARY FOR 1993

Biological Symposium

- 381st–Jan. 6 Molecular basis for the functional specificities of homeotic proteins (Katuo Tokunaga)
- 382nd–Jan. 11
1. Structure-function studies of RNA polymerase-sigma subunits (Ashok Kumar)
 2. How to tightly control transcription of a gene in *E. coli* and in mammalian cells (Hermann Bujard)
 3. New components of the yeast RNA polymerase II transcription initiation complexes (Richard A. Young)
- 383rd–Jan. 28 Insights into the peopling of Melanesia and Australasia from sequence and phylogenetic analysis of HTLV-I virus genes (Richard Yanagihara)
- 384th–Mar. 1 canceled
- 385th–Mar. 8 The evolution of mammalian mitochondrial DNA (Svante Pääbo)
- 386th–Mar. 8 Population dynamics of DNA fingerprinting patterns within and between populations (Ranajit Chakraborty)
- 387th–Mar. 10 Databases of EMBL and tools for finding protein functions (Rainer Fuchs)
- 388th–Mar. 18 Optimal growth in nature: The influence of bad times (G. C. Kurland)
- 389th–Mar. 31 A molecular genetic approach to protein folding: An intramolecular chaperone (Masayori Inouye)
- 390th–Mar 30 Molecular biology of rice tungro virus: Approaches to control by non-conventional protection (Roger Hull)
- 391st–May 13 Genetic and immunological dissection of RNA polymerase (Robert E. Glass)
- 392nd–June 16 TyrR mediated regulation of the tyrP gene (Blair Lawley)
- 393rd–July 6 Rules underlying DNA recognition by α -helices of transcription factors (Masashi Suzuki)
- 394th–July 21 Population genetics issues in the forensic uses of DNA

- (Bruce S. Weir)
- 395th–Aug. 4 The response of streptomyces coelicolor upon oxidative treatment (Jung-Hye Roe)
- 396th–July 22 Molecular analysis of head formation in hydra (Thomas Bosch)
- 397th–Sep. 3 Immortal genes: Several examples of interesting atavistic mutations (Susumu Ohno)
- 398th–Sep. 6 Streptomyces-curators of obsolete metabolic pathways? (Charles Thompson)
- 399th–Sep. 8 Estimation of origin times and substitution rates in multigene families (Marcy K. Uyenoyama)
- 400th–Oct. 4 The TCR β chain: CDR without HVR? and constancy of its thymocyte repertoire in man (Tommaso Meo)
- 401st–Oct. 7 canceled
- 402nd–Nov. 1 Mx-proteins: Interferon-induced GTPases with antiviral activity (Peter Staeheli)
- 403rd–Nov. 9 Loss of a yeast telomere: arrest, recovery, and chromosome loss (V. A. Zakian)
- 404th–Nov. 18 Sry and the molecular genetics of mammalian sex determination (Robin Lovell-Badge)
- 405th–Nov. 18 Paranemic states of DNA and their role in DNA unwinding (G. Yagil)
- 406th–Dec. 15 IGD-genome information management system (Otto Ritter)
- 407th–Dec. 20
1. Role of cell signalling and homeodomain proteins in the life cycle of the smut fungus *Ustilago maydis* (Flora Banuett)
 2. Control of the cell cycle and cell polarity in budding yeast (Ira Herskowitz)

Mishima Geneticists' Club

- 404th–Jan. 27 Functional analysis of primer protein in the protein primed DNA replication of Bacillus phages M2 and ϕ 29 (Tsutomu Kishi)
- 405th–Feb. 17 The necessity and the present situation of X-ray crystallography of proteins (Yasuo Shirakihara)
- 406th–Feb. 4 Examination of pattern formation mechanisms in *Dro-*

- sophila* imaginal disc by enhancer trap method (Satoshi Gotoh)
- 407th-Apr. 21 Zn-finger proteins expressed in the mouse germ line cells (Toshiaki Nose)
- 408th-Apr. 20 Molecular mechanisms of osmotic responses in *E. coli* and Cyanobacteria (Kengo Kanamaru)
- 409th-June 4 A functional analysis of Wnt-3A gene by gene targeting (Shinji Takada)
- 410th-July 8 Molecular mechanisms of the neural network formation in *Drosophila* RP neurons (Akira Chiba)
- 411th-Sep. 27 Color expression in Japanese morning glory and transposon (Shigeru Iida)
- 412th-Nov. 19 Molecular genetics of the embryonic tissue interaction in the spinal column formation (Kenji Imai)
- 413th-Nov. 25 Regulatory role of TFIIE on transcription initiation by eukaryotic RNA polymerase II (Yoshiaki Okuma)
- 414th-Dec. 13 Folk-head gene family and molecular mechanisms of development of the mouse body axis and central nervous system (Hiroshi Sasaki)

FOREIGN VISITORS IN 1993

Dec. 1, 1991– Jan. 6, 1993	Thangirala Sudha, Down's Research Society, India Katsuo Tokunaga, Zoological Institute, University of Basel, Switzerland
Jan. 11	Ashok Kumar, University of Edinburgh, U.K.
Jan. 11	Sankar Adhya, National Cancer Institute, NIH, U.S.A.
Jan. 11	Hermann Bujard, University of Heidelberg, Germany
Jan. 11	Richard A. Young, Whitehead Institute, Nine Cam- bridge Center, U.S.A.
Jan. 28	Richard Yanagihara, National Institute of Neurological Disorders and Stroke, NIH, U.S.A.
Mar. 8	Svante Pääbo, University of Munich and Zoological Museum in Munich, Germany
Mar. 8	Ranjit Chakraborty, University of Texas, U.S.A.
Mar. 10	Rainer Fuchs, European Molecular Biology Laborato- ry, Germany
Mar. 18	G. C. Kurland, Uppsala University, Sweden
Mar. 31	Masayori Inouye, University of Medicine and Dentistry of New Jersey, U.S.A.
May 8–19	Robert E. Glass, University of Nottingham, U.K.
May 20–June 19	Blair Lawley, University of Melbourne, Australia
May 26	Lin Dhung-Mao, National Chiayi Institute of Agricul- ture, Taiwan
May 26	Li Cheng-Chang, National Chung-Hsing University, Taiwan
May 26	Hu Mao-Lin, National Chiayi Institute of Agriculture, Taiwan
May 26	Liu Ching-Yung, National Taiwan University, Taiwan
June 10–11	Ju-Gyeng Kang, National Seoul University, Korea
July 6	Masashi Suzuki, MRC Laboratory of Molecular Biolo- gy, U.K.
July 20–22	Bruce S. Weir, North Carolina State University, U.S.A.
July 21–28	Charles N. David, University of Munich, Germany
July 21–28	Thomas Bosch, University of Munich, Germany

- July 25–30 Tang Ling-Hua, Institute of Food Crops, Jiangsu Academy of Agricultural Sciences, China
- July 26–30 Wang Xiang Kun, Beijing Agricultural University, China
- July 30–Aug. 1 Robert A. Steen, Cornell University, U.S.A.
- Aug. 2–5 Jung-Hye Roe, Seoul National University, Korea
- Aug. 6–7 Joe Inselburg, Dartmouth Medical School, U.S.A.
- Aug. 15–Nov. 14 G. Subramanya, Mysore University, India
- Aug. 17–Sept. 9 Chitrakon Songkran, Pathumthani Rice Research Center, Thailand
- Aug. 25–27 Deb Overath, University of Georgia, U.S.A.
- Aug. 27– Ashok Kumar, University of Edinburgh, U.K.
- Sept. 3 Susumu Ohno, Beckman Research Institute of the City of Hope, U.S.A.
- Sept. 3–8 Mercy K. Uyenoyama, Duke University, U.S.A.
- Sept. 6 Charles Thompson, University of Basel, Switzerland
- Sept. 6–8 Masatoshi Nei, Pennsylvania State University, U.S.A.
- Sept. 7–8 M. A. Zargar, Banaras Hindu University, India
- Sept. 7–8 T. K. Kundu, Indian Inst. Sci., India
- Sept. 7–8 P. K. Burma, Indian Inst. Sci., India
- Sept. 7–8 K. P. Gopinathan, Indian Inst. Sci., India
- Sept. 7–8 Xue Wu, Shanghai Inst. Biochem., China
- Sept. 7–8 Shin-Feng Tsai, Natl. Yang-Hing Med. College
- Sept. 7–11 D. C. Sy, University of Santo Tomas, Philippines
- Sept. 10–11 T. K. Kundu, Indian Inst. Sci., India
- Sept. 11–14 Kevin K. Murakami, University of Vermont, U.S.A.
- Sept. 27–Oct. 25 Supan Fucharoen, Khon Kaen University, Thailand
- Oct. 4 Tommaso Meo, Institut Pasteur, France
- Oct. 11–17 Wang Fengshan, Tianjin Institute of Industrial Hygiene and Occupational Diseases, China
- Oct. 5– Dzhanybek M. Adyshev, Institute of Biochemistry and Physiology, Kyrgys Academy of Sciences, Kyrgyz
- Oct. 29–Nov. 1 Ian Tessrey, University of Freiburg, Germany
- Oct. 29–Nov. 1 Martin Schwemmle, University of Freiburg, Germany
- Oct. 31–Nov. 3 Peter Staeheli, University of Freiburg, Germany
- Nov. 9 V. A. Zakian, Fred Hutchinson Cancer Research Institute, U.S.A.

- Nov. 16–19 Robin Lovell-Badge, National Institute for Medical Research, U.K.
- Nov. 18 G. Yagil, The Weizmann Institute of Science, Israel
- Nov. 22–26 Boriboon Somrith, Department of Agriculture, Thailand
- Nov. 25–26 Yoshiaki Ohkuma, The Rockefeller University, U.S.A.
- Dec. 1– Ritta H. Mikkola, Uppsala University, Sweden
- Dec. 1– Stefanie A. Margaron, University of Nottingham Medical School, U.K.
- Dec. 3– Dipankar Chatterji, Centre for Cellular and Molecular Biology, India
- Dec. 15 Otto Ritter, German Cancer Research Institute, Germany
- Dec. 19–21 Mark Stoneking, University of Pennsylvania, U.S.A.
- Dec. 20–21 Ira Herskowitz, University of California, U.S.A.
- Dec. 20–21 Flora Banuett, University of California, U.S.A.

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