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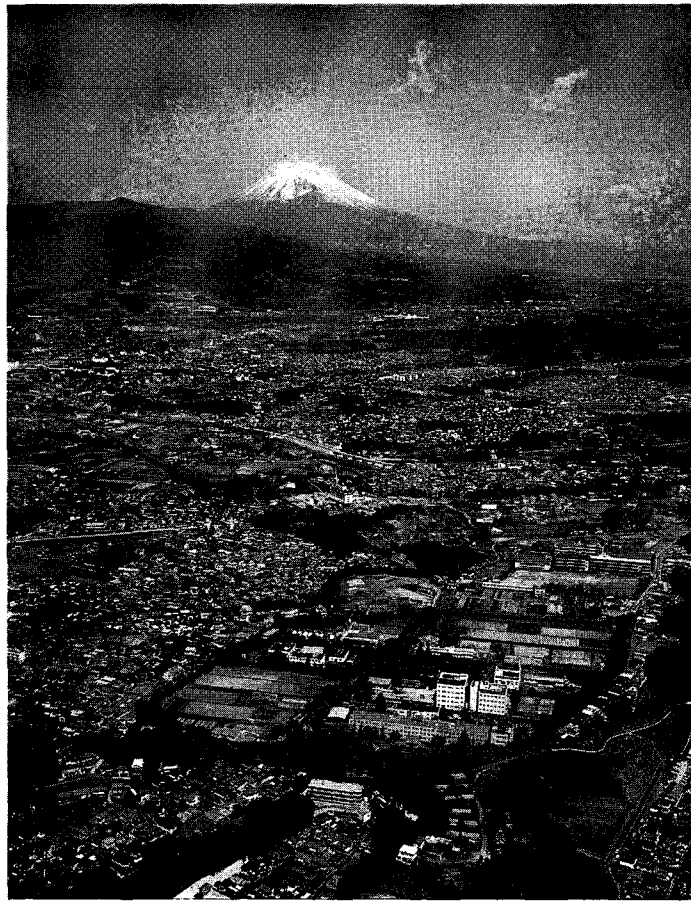

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GENERAL STATEMENT

Our institute was established 43 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 biology, 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. In addition, together with seven inter-university research institutes, we formed the Graduate University for Advanced Studies, in 1988. Our institute has admitted graduate students in the Department of Genetics of the Graduate School of Life Science. We have now about 30 such students and thus maintaining a steady flow of young scientists that is vital for our research activities.

Recent rapid progress in the field of genetics with newly developed approaches has greatly shifted the course of study in biology. Accordingly, researches in our institute are focused more on molecular studies on various aspects of genetics. This is not to ignore the importance of more traditional approaches on genetics of various organisms. Our institute is uniquely suitable to pursue cooperative works by scientists of various disciplines. Through interactions between these lines of researches, our institute will flourish.

We have been carrying out several research related services. We house the DNA Data Bank of Japan (DDBJ) that gather, annotate, store and distribute information on DNA sequences. As one of the three banks in the world, DDBJ will continue to serve the world-wide community of biological science and technology. Our institute also stores and distributes various organisms with genetically characterized traits. Among them services on mice, rice and *Escherichia coli* are particularly significant. These service activities will be advanced. However, as the responsible manager of these activities, I honestly feel pain in running service operations in the conditions of understaffing, underpaying and underfunding. Nation-wide recognition of 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 supports from governmental and private sources, I could lead the institute into a more successful future.

In the past year, Associate Prof. S. Hirose was promoted to the professor of the Laboratory of Phenogenetics and Dr. F. Tajima (Laboratory of Population Genetics) was promoted to the rank of associate professor. Associate Prof. T. K. Watanabe was transferred to Kyoto Institute of Technology, Associate Prof. N. Takahata to the Graduate University for Advanced Studies both as professor, Dr. H. Tezuka to Yamanashi University School of Medicine and Dr. H. Tachida to Kyushu University both as associate professor. Dr. T. Toyoda joined us in the Laboratory of Molecular Biology and Dr. M. Hatta in the Laboratory of Developmental Genetics. The DNA Research Center gained the following four as research members: Drs. H. Nagai, T. Ishihara, Y. Andachi and K. Ikeo.

It is a pleasure to note that Dr. M. Kimura, Emeritus Professor, was honored by the Darwin Medal by the British Royal Society for his work on the role of stochastic events in determining the rate of molecular evolution, and Dr. T. Shiroishi the Encouragement Award of the Genetics Society of Japan for his study on recombinational hotspots of the mouse major histocompatibility complex.

Junichi Tomizawa

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MORIWAKI, Kazuo, D. Sc.

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Adviser

MORIWAKI, Daigoro; Honorary member, National Institute of Genetics

PROJECTS OF RESEARCH FOR 1992

1. DEPARTMENT OF MOLECULAR GENETICS

Division of Molecular Genetics

Regulatory mechanisms of gene transcription in prokaryotes (ISHIHAMA, FUJITA and YAMAGISHI)

Molecular architecture of transcription apparatus from eukaryotes (ISHIHAMA and YAMAGISHI)

Molecular mechanisms of transcription and replication of animal and plant viruses (ISHIHAMA, TOYODA and NAKAMURA)

Division of Mutagenesis

Molecular and genetic studies of cell-cycle associated genes (SENO, YAMAO and KANEDA)

Genetic consequences of thymidylate stress in mammalian cells (SENO and YAMAO)

Radiation sensitivity in mammals (TEZUKA)

Division of Nucleic Acid Chemistry

Mechanism of mRNA capping (Mizumoto)

Transcription of the Sendai virus genome (Mizumoto)

Cell-cycle regulation by cyclin and CDC2 kinase (YASUDA)

2. DEPARTMENT OF CELL GENETICS

Division of Cytogenetics

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

* Genetic stock research center.

Recombinational hotspots in the mouse MHC (SHIROISHI and MORIWAKI)
Theoretical and experimental bases for karyotype evolution (IMAI)
Study of adrenal function in mouse development by transgene (GOTOH, 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)
Studies on bacterial plasmids and transposons (OHTSUBO)

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, TACHIDA and TAJIMA)
Theoretical studies on the evolution of multigene family (OHTA)
Theory of gene genealogy (TAJIMA)
Population genetical studies on quantitative characters (TACHIDA)
Statistics for DNA polymorphisms (TAJIMA)

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 (MATSUMOTO and IKEMURA)
Molecular evolutionary analysis of nucleotide sequence data (SAITOU)
Studies on the genetic affinity of human populations (SAITOU)
Studies on molecular evolution on viruses (MORIYAMA)
Studies on molecular evolution of Drosophila genes (MORIYAMA)

Division of Theoretical Genetics

Population immunogenetics (TAKAHATA)
Molecular anthropology (TAKAHATA)
Computer analysis of genetic information (YASUNAGA)

5. DEPARTMENT OF INTEGRATED GENETICS

Division of Human Genetics

Genetic and physical mapping of human genome (IMAMURA and NAKASHIMA)
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. RESEARCH FACILITIES

Genetic Stock Research Center

Analysis of morphogenesis in postimplantation mouse embryos (NAKATSUJI and SHIRAYOSHI)
Manipulation of embryogenesis using mammalian embryonic cells (NAKATSUJI and SHIRAYOSHI)
Molecular analysis of cell differentiation in postimplantation mouse embryos (SHIRAYOSHI and NAKATSUJI)
Genetic mechanisms for regulating tumor development in the laboratory and wild mice (MIYASHITA and MORIWAKI)
Evolutionary genetics of *Drosophila* (WATANABE)
Molecular genetics of insect development (UEDA and HIROSE)
Molecular mechanism of cell division in *E. coli* (NISHIMURA)
Theoretical studies in molecular phylogeny (TATENO)

DNA Research Center

Interaction between proteins and nucleic acids (SHIMAMOTO and NAGAI)
Developmental and behavioral genetics of *C. elegans* (KATSURA and ISHIHARA)
Computer analysis of DNA sequences (GOJOBORI and UGAWA)
Construction of DNA sequence database (GOJOBORI, UGAWA, IKEO and TATENO)
Molecular evolution of DNA sequences (IKEO and GOJOBORI)
Molecular genetics of *Drosophila* development (HAYASHI and HIROSE)
Molecular biology of *C. elegans* development (KOHARA and ANDACHI)
Genome analysis of *C. elegans* (KOHARA)
Large-scale genetic information analysis project (KITAKAMI and YAMAZAKI)

Radioisotope Center

Radiation genetics of *Caenorhabditis elegans* (SADAIE)
Molecular mechanisms of sporulation in *Bacillus subtilis* (SADAIE)

Experimental Farm

Molecular genetics of morphological mutants in rice (NAKAMURA)
DNA fingerprinting of plant species (NAKAMURA)

RESEARCH ACTIVITIES IN 1992

I. MOLECULAR GENETICS

The Promoter Selectivity Control of *Escherichia coli* RNA Polymerase: Mapping of the Contact Sites on RNA Polymerase with Transcription Factors

Akira ISHIHAMA, Chao ZOU, Ashok KUMAR*, Nobuyuki FUJITA, Hitomi SAKURAI, Kozo MAKINO** and Richard S. Hayward*

The promoter selectivity control of RNA polymerase plays a key role in global regulation of gene transcription in prokaryotes. A number of accessory transcription factors have been identified in *Escherichia coli*, which interact with RNA polymerase and modulate its promoter selection pattern. One group factors form RNA polymerase complexes in the absence of DNA, while the other group factors bind to DNA and interect with RNA polymerase when both are aligned along the same DNA strand (Ishihama, A. (1988) *Trends Genet.* **4**, 482–486). Regardless of apparent difference in the order of molecular assembly, ternary complexes are formed between RNA polymerase, transcription factors and DNA signals (promoter and enhancer) prior to transcription initiation.

Since the finding of the presence of at least two contact sites on *E. coli* RNA polymerase for CRP (cAMP receptor protein) (Igarashi, A. and Ishihama, A. (1991) *Cell* **65**, 1015–1022; Igarashi *et al.* (1991) *Proc. Natl. Acad. Sci. USA* **88**, 8958–8962), our effort has been focussed towards the classification of various transcription factors on the basis of the contact site selection on RNA polymerase. In collaboration with a large number of laboratories, various transcription factors have been examined as to their abilities to activate mutant RNA polymerases contsisting of C-terminal truncated α subunits which lack the contact site I for class-I factors (review-

* On leave of absence from Institute of Cell and Molecular Biology, University of Edinburgh, Edinburgh, UK.

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Table 1. Classification of transcription factors

Factor	Contact site*	Publications
Ada	I	Sakumi, K. *et al.* (1993) *J. Bacteriol.* **175**, 1162–1167.
CRP	I/II	Igarashi, K. and Ishihama, A. (1991) *Cell* **65**, 1015–1022.
		Igarashi, K. *et al.* (1991) *Proc. Natl. Acad. Sci. USA* **88**, 8958–8962.
		Kolb, A. *et al.* (1993) *Nucleic Acids Res.* **21**, 319–326.
		Zou, C. *et al.* (1992) *Mol. Microbiol.* **6**, 2599–2605.
IHF	I	Giladi, H. *et al.* (1992) *J. Mol. Biol.* **227**, 985–990.
λcI/cII	II	Gussin, G. N. *et al.* (1992) *J. Bacteriol.* **174**, 5156–5160.
NtrC	II	Lee, H.-S. *et al.* (1993) *J. Bacteriol.* **175**, 1568–1572.
OmpR	I	Igarashi, K. *et al.* (1991) *Proc. Natl. Acad. Sci. USA* **88**, 8958–8962.
OxyR	I	Tao, K. *et al.* (1993) *Mol. Microbiol.* **7**, 859–864.
PhoB	II	Igarashi, K. *et al.* (1991) *Proc. Natl. Acad. Sci. USA* **88**, 8958–8962.

* Contact site I = α subunit; contact site II = σ subunit

ed in Ishihama, A. (1992) *Mol. Microbiol.* **6**, 3283–3288; Ishihama, A. (1993) *J. Bacteriol.* **175**, 2483–2489). Some of the results have been published as listed in Table 1.

Fine mapping of the contact site I region was carried out for CRP (Zou *et al.*, 1992) and OxyR (Tao, K., unpublished). Taken together with results from other laboratories, it turned clear that multiple contact sites exist within the contact site-I region in the C-terminal one-third of RNA polymerase α subunit, each interacting with a different set of transcription factors and each consisting of a structure formed by a short peptide of about 10 amino acid residues (reviewed in Ishihama, A. (1992) *Mol. Microbiol.* **6**, 3283–3288; Ishihama, A. (1993) *J. Bacteriol.* **175**, 2483–2489).

Recently two trascription factors, PhoB (in the case of *pstS* transcription) and CRP (in the case of *gal*P1 transcription), were found to make contact with σ^{70} subunit at upstream from the region 4 (the recognition domain of promoter −35) (Makino, K. *et al.* (1993) *Genes Devel.* **7**, 149–169; Ashok, K. *et al.* (1993) *J. Mol. Biol.*, in press). Analysis of C-terminal deletion of the σ^{70} subunit indicated that these two class-II transcription factors interact at different sites within this contact site-II region (Ashok, K. *et al.* (1993) *J. Mol. Biol.*, in press).

The Promoter Selectivity Control of *Escherichia coli* RNA Polymerase: Modulation of RNA Polymerase at Stationary Growth-Phase

Hitomi SAKURAI, Kan TANAKA*, Nobuyuki FIJITA and Akira ISHIHAMA

Under starved environmental conditions, some bacterial species such as *B. subtilis* maintain cell viability by forming dormant spores. Even in non-differentiating bacteria such as *E. coli*, however, genetic programs operate as to establish a metabolically less active and more stress-resistant state. Results of our previous studies led us to postulate that the modulations of both RNA polymerase and ribosomes play major roles in this switch of the genetic program (Ozaki, M. *et al.* (1991) *Mol. Gen. Genet.* **230**, 17–23; Ozaki, M. *et al.* (1992) *Nucleic Acids Res.* **20**, 257–261; Wada, A. *et al.* (1990) *Proc. Natl. Acad. Sci. USA* **87**, 2657–2661). The marked decrease in the net charge for the stationary-specific core enzymes itself indicated protein modification with moieties of highly negative charge. Preliminary experiments indicated the attachment of phosphate moieties in this modification. The chemical nature of this modification is being analyzed, by making a large amount of stationary-specific RNA polymerase at different phases of cell growth.

In addition to the core enzyme modification, replacement of the σ subunit has been proposed. In collaboration with Dr. K. Tanaka *et al.* (Univ. Tokyo), we succeded the detection *in vitro* of sigma activity for expressed and purified σ^{38} (or σ^{S}; the *rpoS*(*katF*) gene product) when bound to the core enzyme isolated from exponentially growing cells (Tanaka, K. *et al.* (1993) *Proc. Natl. Acad. Sci. USA*, **90**, 3511–3518). A systematic comparison of the promoter selectivity between Eσ^{70} and Eσ^{38} holoenzymes indicated that Eσ^{70} recognizes a set of promoters associated with highly expressed genes in exponentially growing *E. coli* while Eσ^{38} initiates transcription from promoters of stationary phase-specific genes. However, a group of promoters are recognized by both holoenzymes, indicating cross-talk between two sigma factors.

* Institute of Applied Microbiology, University of Tokyo, Tokyo, Japan.

Starvation-Coupled Modulation of *Escherichia coli* Ribosomes: Regulation of the *rmf* Gene Encoding the Ribosome Modulation Factor

Masahiro YAMAGISHI, Hiroshi MATSUSHIMA*, Akira WADA** and Akira ISHIHAMA

Ribosome modulation factor (RMF) is a protein specifically associated with 100S ribosome dimers which start to accumulate in *Escherichia coli* upon growth transition from exponential to stationary phase (Wada, A. *et al.* (1990) *Proc. Natl. Acad. Sci. USA* **87**, 2657–2661). The gene, *rmf*, encoding RMF was cloned from 21.8 min region of the *E. coli* genome, and sequence analysis indicated that *rmf* codes for a small basic protein of 55 amino acid residues (Yamagishi, M. *et al.* (1993) *EMBO J.* **12**, 625–630). By Northern blot analysis, it was found that *rmf* was silent in rapidly growing cells at exponential phase, but became highly expressed concomitantly with the growth transition to stationary phase.

Under slow growth conditions, however, *rmf* was expressed even at the exponential growth phase, and an inverse relationship was observed between the expression of *rmf* and the cell growth rate. Thus, the expression profile of *rmf* is contrary to that of the genes for components of translational appratus such as ribosomal proteins and ribosome-associated proteins. Disruption of *rmf* resulted in loss of ribosome dimers and reduction of cell viability during stationary phase.

Molecular Composition of Fission Yeast RNA Polymerase II: Analysis of the *S. pombe rpb2* Gene for RNA Polymerase Subunit 2

Makiko KAWAGISHI, Masahiro YAMAGISHI and Akira ISHIHAMA

RNA polymerase plays a central role in gene expression. Analysis of the structure and function of RNA polymerase is essential to understand the molecular mechanisms of transcriptional regulation. In eukaryotes, three classes of nuclear RNA polymerases, I, II and III have been identified, each responsible for transcription of large ribosomal RNA, mRNA, and small ribosomal RNA and tRNA, respectively. Each class RNA polymerase is

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composed of two large polypeptides and about 10 smaller subunits. However, the minimum essential subunits have not been identified for anyone of the three RNA polymerases. In order to extend our knowledge of the subunit composition of RNA polymerase II and the role of each subunit in transcription, we started biochemical and genetical studies of RNA polymerase II from the fission yeast, *Schizosaccharomyces pombe*. Last year, we cloned and sequenced the *rpb1* gene for the largest subunit (subunit 1) using the corresponding gene (*RPB1*) of the budding yeast, *Saccharomyces cerevisiae*, as the hybridization probe (Azuma, Y. *et al.* (1991) *Nucleic Acids Res.* **19**, 461–468).

This year we cloned the *S. pombe rpb2* gene by cross-hybridization with the *S. cerevisiae RPB2* gene. The *rpb2* gene encodes the subunit 2 (the second largest subunit) of RNA polymerase II with 1210 amino acid residues and a calculated molecular weight of 138 kDa (Kawagishi, M. *et al.* (1993) *Nucleic Acids Res.* **21**, 469–473). From the result of gene disruption experiment, we concluded that the *rpb2* gene is essential for cell viability.

Because of a local sequence similarity between a part of the subunit 2 of RNA polymerase II and bacterial RNase such as barnase, we tried to test whether this RNase-like region of the subunit 2 carries RNase activity or not. For this purpose, we expressed in *E. coli* a GST-fusion protein including this RNase-like region of the subunit 2. In a preliminary experiment, the partially purified GST-fused protein showed RNase activity *in vitro*, even though its activity was much weaker than the authentic barnase. As an attempt to confirm this observation, we introduced a mutation at an essential site for the catalytic activity of barnase. The GST-fusion protein containing this mutant *rpb2* segment showed weaker activity of RNA hydrolysis than the unmodified fusion protein. Further confirmatory experiments are being carried out using a highly purified preparation of the fusion proteins. The specificity of this subunit 2-associated RNase, and the biological role(s) of this RNase-like region all remain unsolved.

Molecular Composition of Fission Yeast RNA Polymerase II: Analysis of the *S. pombe rpb3* Gene for RNA Polymerase Subunit 3

Yoshinao AZUMA, Masahiro YAMAGISHI and Akira ISHIHAMA

In order to improve our understanding of the structure-function relationship of RNA polymerase II, we continued the cloning, sequencing and characterization of the genes encoding proteins identified in a highly purified RNA polymerase II fraction from the fission yeast *S. pombe*. Up to the present time, we have isolated the genes, *rpb1* and *rpb2*, coding for the largest (subunit 1) and the second-largest (subunit 2) subunits, respectively, of RNA polymerase II (Azuma, Y. *et al.* (1991) *Nucleic Acids Res.* **19**, 461–468; Kawagishi, M. *et al.* (1993) *Nucleic Acids Res.* **21**, 469–473). This year we cloned and sequenced the gene, *rpb3*, coding for the third-largest subunit (subunit 3).

Since the *RPB3* gene for the RNA polymerase II subunit 3 of the budding yeast *Saccharomyces cerevisiae* did not cross-hybridize with *S. pombe* DNA, we tried to clone the subunit 3 gene using synthetic probes designed based on the knowledge of amino acid sequence. For this purpose, we first purified RNA polymerase II from *S. pombe*. By SDS-gel electrophoresis, a highly purified RNA polymerase II fraction gave at least 11 bands. The sizes of two large subunits were estimated to be about 210 and 150 kDa, respectively, close to those calculated from the nucleotide sequences of the cloned *rpb1* and *rpb2* genes and similar to those of subunits 1 and 2 of *S. cerevisiae* RNA polymerase II. We isolated the third-largest subunit with approximate molecular weight of 40 kDa and subjected it to amino acid microsequencing. With use of three different oligonucleotide primers designed from the partial amino acid sequences, we cloned the gene encoding the putative subunit 3 polypeptide. Using the genomic clone as a probe, we also cloned cDNA for this polypeptide. Sequence determination indicated that this gene, interrupted by two introns, codes for a polypeptide of 307 amino acid residues with a calculated molecular weight of 34 kDa. This protein contains five conserved structural domains of the third largest subunits from other eukaryotic RNA polymerase II. Thus, we concluded that the gene we cloned is *S. pombe rpb3* encoding the subunit 3 of RNA polymerase II.

Molecular Anatomy of Influenza Virus RNA Polymerase: *in vitro* Reconstitution Studies

Makoto KOBAYASHI, Yukiyasu ASANO, Kiyohisa MIZUMOTO* and Akira ISHIHAMA

Influenza virus RNA polymerase catalyzes multiple step reactions in transcription and replication of the genome RNA. The core enzyme is composed of each one of the three P proteins, PB1, PB2 and PA (Honda, A. *et al.* (1990) *J. Biochem.* **107**, 624–628). Subunit PB1 is involved in RNA polymerization, while PB2 is involved in capped RNA recognition. The function of PA protein is totally unknown. For detailed analysis of the role of each P protein and functional domains on each P polypeptide, we expressed individual P proteins in insect cells infected with recombinant baculoviruses carrying cDNA for each P protein (Kobayashi, M. *et. al.* (1992) *Virus Res.* **22**, 235–245). RNA polymerase reconstituted by mixing three purified P proteins was able to transcribe short model RNA templates containing the conserved terminal sequences of influenza virus RNA segments. The mixture of any two of the three P proteins did not exhibit transcription activity, confirming that the RNA polymerase is composed of the three P proteins. We started to make a set of mutant RNA polymerases containing mutant P proteins. For this purpose, we established expression systems of each P protein in *Escherichia coli* using T7 RNA polymerase/promoter system. In parallel, we started to identify functional sites on each P protein subunit by chemical cross-linking with analogues of substrates, primers, templates and products. As expected, a GTP analogue was cross-linked to the PB1 protein.

In addition to the three P proteins, NP protein is required for efficient elongation of RNA chains (Honda *et al.* (1988) *J. Biochem.* **104**, 1021–1026). In order to construct *in vitro* transcription system of long RNA templates, the NP protein was prepared from *E. coli* expressing cDNA for the NP gene. A set of deletion mutants of the NP protein was made in order to identify the functional domain required for RNA elongation.

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Molecular Anatomy of Influenza Virus RNA Polymerase: *in vitro* Replication Studies

Tetsuya TOYODA, Susumu NAKADA* and Akira ISHIHAMA

RNA-dependent RNA polymerase associated with influenza virions is composed of each one of the three P proteins (PB1, PB2 and PA) (Honda, A. *et al.* (1990) *J. Bichem.* **107**, 624–628; Kobayashi *et al.* (1992) *Virus Res.* **22**, 235–245). In influenza virus-infected cells, the RNA polymerase is invloved in both transciption and replication of eight genomic RNAs (vRNAs). In order to reveal the difference in the molecular architecture between the two RNA polymerase complexes, *i.e.*, transcriptase and replicase, we prepared nuclear extracts from influenza virus A/PR8-infected MDCK and HeLa cells. Both extracts catalyzed the synthesis of not only mRNA but also cRNA (the first step reaction of replication). We also detected these two activities for cell extracts of the clone 76 cell line, which expresses influenza virus three P and NP proteins (Nakamura *et al.* (1991) *J. Biochem.* **110**, 395–401). Theses observations indicates that three P proteins and NP are enough for transcription and replication.

We reconstituted an *in vitro* system of primer-independent RNA synthesis using high salt-treated RNP from virus-infected cells and nuclear extracts of uninfected cells. Identification of the host factor(s) required for replication of the influenza virus genome is in progress.

Regulatory Role of the Matrix Protein on Virus Growth

Jiro YASUDA and Akira ISHIHAMA

The genome of influenza A virus is composed of eight RNA segments of negative polarity. RNA segment 7 encodes two molecular species of the M protein. The M1 protein forms matrix (or membrane) of virions, while M2 forms an ion channel in virus-infected cell membranes. M1 is an amphiphilic protein, interacting with both RNP cores at its hydrophilic inside surface and viral envelope at the hydrophobic outside surface. The activity of RNA polymerase associated with RNP cores is repressed by the M1 protein (Hankins, R. W. *et al.* (1989) *Virus Genes* **3**, 111–126; Hankins, R. W. *et al.* (1990) *Res. Virol.* **141**, 305–314).

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Influenza virus A/WSN/33 forms large plaques (>3mm diameter) on MDCK cells whereas A/Aichi/2/68 forms only small plaques (<1mm diameter). Fast growing reassortants (AWM), isolated by mixed infection of MDCK cells with these two virus strains in the presence of anti-WSN antibodies, all carried the M gene from WSN (Yasuda, J. *et al.*, (1993) *Arch. Virol.*, in press). Upon sinlge-round infection, these reassortants showed rapid appearance and high yield of progeny viruses. The fast-growing reassortants overcame the growth inhibitory effect of lignins. Pulse-labeling experiments at various times after virus infection showed that the reassortant AWM started to synthesize viral proteins earlier than Aichi. Taken together, we conclude that upon infecting MDCK cells, the reassortant viruses advance rapidly into the growth cycle, thereby leading to elevated levels of viral gene expression and progeny viruses in the early period of infection.

Molecular Assembly of Influenza Virus: Association of the NS2 Protein with Virion Matrix

Jiro YASUDA, Susumu NAKADA*, Atsushi KATO**,
Tetsuya TOYODA and Akira ISHIHAMA

The genome of influenza A virus is composed of eight RNA segments of negative polarity, which altogether encode ten different viral proteins. RNA segment 8 (vRNA 8) codes for two non-structural proteins, NS1 and NS2. NS1 is translated from a colinear transcript (mRNA 8) of vRNA 8, while NS2 is a translation product of an RNA generated from mRNA 8 after splicing. NS1 seems to be invloved in control of this splicing. Nothing is known on the function of NS2.

The NS2 protein was found to exist in virus particles (Yasuda, J. *et al.*, (1993) *J. Virol.* **196**, 249–255). By immunochemical method, the average number of NS2 in a virus particle was estimated to be 130–200 molecules. After solubilization of the viral envelope, NS2 was still associated with ribonucleoprotein (RNP) cores, but was later dissociated upon removal of the M1 protein.

A filter binding assay *in vitro* indicated direct protein-protein contact between M1 and NS2. Following chemical cleavage of the M1 protein, NS2

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bound only a C-terminal fragment of M1. By immunoprecipitation, NS2-M1 complexes were also detected in virus-infected cell lysates. These observations altogether indicate specific molecular association between M1 and NS2. Possible involvement of NS2 in the control of M1 functions is being tested.

Structure and Function of Mouse Mx1 Protein: Inhibition Mechanism of Influenza Virus Growth

Yukiyasu ASANO*, Tetsuya TOYODA, Satoshi SATO** and Akira ISHIHAMA

The mouse Mx1 protein, a host factor invloved in resistance against influenza virus infection is an interferon-inducible nuclear protein. The Mx1 protein does not affect virus adsorpotion, penetration, uncoating, and transport of RNP complexes into nuclei, but appears to block primary transcription of the parental influenza viral genome. Previously, we found that the Mx 1 protein was 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). By electron microscopic observation, Mx1 formed self-assembled polymers consisting of more than 30 monomers (Nakayama, M. *et al.* (1993) *J. Biol. Chem.*, **268**, 15033–15038). Deletion analysis revealed that the main motif for self-assembly was mapped between amino acid residues 51–99 of the Mx1 protein.

To address questions concerning these biochemical activities and anti-viral function of the Mx1 protein, we constructed mutand Mx1 proteins carrying mutations in the GTPase and self-assembly domains and compared their biochemical activities and anti-viral function. In parrallel, we attemped to construct *in vitro* inhibition system of viral transcription by the Mx1 protein. For this pourpose, we prepared a 0.42 M NaCl extract of nuclei containing the Mx1 protein from interferon-treated A2G mouse embryonic cells. Western blot analysis using polyclonal antibodies against the Mx1 protein indicated that 20–30% of the Mx1 protein was extracted. Preliminary results indicate that this nuclear extract containing the Mx1 protein had an inhibitory activity of viral transcription *in vitro*.

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Ambisense Coding Strategy of Rice Stripe Virus

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Shigemitsu TORIYAMA* and Akira ISHIHAMA

Rice Stripe Virus (RSV), the prototype member of the tenuiviruses, has four-RNA segments of single-stranded RNA as the genome. Sequence analyses of the two small RNA segments revealed that both carry large open reading frames at 5′-proximal region of both genome-sense and anti-genome-sense RNAs. This year the sequence of RNA segment 3 was determined, which again suggested the ambisense coding strategy (Takahashi, M. *et al.* (1993) *J. Gen. Virol.* **74**, 769–773). To examine this ambisense nature of the geome, we synthesized *in vitro* RNAs of both porality by transcribing the cDNA clones for three RNA segments (RNA 2, 3 and 4) using T7 RNA polymerase and translated *in vitro* each RNA using two translation systems, reticulocyte lysate and wheat germ extract. Translation products were analyzed by SDS-gel electrophoresis and autoradiography. We detected all the proteins expected from the open reading frames on RNAs of both polarities (Hamamatsu, C. *et al.* (1993) *J. Gen. Virol.* **74**, 1125–1131).

By immunoprecipitation experiments, the product of anti-sense RNA of segment 3 was identified as the coat protein of RSV, while that of the genome sense RNA of segment 4 was the major non-structural protein (NS4). We also translated *in vitro* total virus RNA and detected the proteins generated from the open reading frames located in the 5′regions of vRNAs. The overall results are consistent with the prediction that RSV RNAs, at least up to the segment 2, are ambisense in coding strategy.

Regulatory Elements Located at the 5′ end of the First Intron of the Human Thymidylate Synthase Gene

Sumiko KANEDA and Takeshi SENO

Thymidylate synthase(TS) plays an essential role in regulating the balanced supply of the four DNA precursors for normal DNA replication. We isolated λ phage clones containing the functionally active human TS gene, determining the complete nucleotide sequence and the major transcriptional start sites. The biologically active unit spans about 16 kilo bases and is

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composed of seven exons and six introns. There is no typical promoter sequence. A human TS minigene containing 5′- and 3′-flanking sequences, all the exons and only intron 1 showed a normal frequency of stable transformation when transfected into TS-negative mutant cells, whereas minigenes in which intron 1 was replaced by intron 2 or deleted in the above construct, showed only less percent of the above frequency. Introduction of intron 1 into the above intronless or intron 2-minigene restored the transforming activities regardless of its position and orientation. Deletion analysis revealed two positive and one negative regulatory sequences in the 5′-end of intron 1, each of which seemed to bind specific proteins as shown by gel shift analysis. Intron 1 also stimulated expression of a TS promoter-CAT gene construct but not that of an SV40 promoter-CAT gene construct. These results clearly indicate that the intron 1 regulatory sequence cluster is a promoter specific enhancer which is basically different from known transcriptional enhancers. Intron 1 was also shown to be a major determinant of the cell cycle-dependent fluctuation of TS mRNA at the posttranscriptional step. Therefore, we postulated that there is a link between transcription and the processing of the precursor mRNA in the expression of human TS minigenes.

Cell Cycle Control by Ubiquitin System in Mammalian Cells; Is the Ubiquitin-activating Enzyme, E1, a Substrate of 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 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 temperature. Recently, other types of E1 mutants from the same cell line were also reported; some are arrested predominantly in the 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 the target molecules is associated with various functions of the cell cycle progression, presumably by way of 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/p34^{cdc2}. 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 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 were found at least five possible target sites of phosphorylation by the cdc2 kinase. The E1 enzyme from mouse cells was proved to be phosphorylated *in vivo*. It was also phosphorylated at least *in vitro* by the immunopurified cdc2 kinase. These results strongly suggest that the E1 enzyme is a substrate of cdc2 kinase in the cell.

Cell Cycle Control by Ubiquitin System in Mammalian Cells; Ubiquitin Conjugating Enzymes Specific for DNA Synthesis

Sumiko KANEDA, Yukiko NAGAI, Takeshi SENO and Fumiaki YAMAO

Ubiquitin 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 conjugation of ubiquitin to specific target proteins. There is increasing evidence that each E2 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 conferring the progression of each phase of the cell cycle, we examined *in vitro* ubiquitin transfer from E1 enzyme to E2 conjugating

enzymes in the extract of the various ts E1 mutants of the mouse cell line, FM 3A. Ubiquitin transfer to an E2 protein of approximately 35 kDa was found to be reduced specifically in lysates of mutant cell lines which were predominantly blocked in the S phase at restrictive temperature. 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 still could not at 39°C. The arresting points during the mitotic cell cycle of these revertants at the restrictive temperature were G1 and G2/M phases, and specifically suppressed the defect in S-phase progression of the parental mutant cell lines. In the extract of the partial revertants of the E1 mutants, the reduced ubiquitin transfer to the particular E2 was restored. Thus, E2 (35 kDa) appears to be involved specifically in DNA synthesis.

Pulverization of Chromosomal NORs in the Mouse Cell Mutant of Temperature-sensitive Ubiquitin-activating Enzyme with the Arrest Point at the G2 Phase of the Cell-cycle

Thangirala SUDHA, Sumiko KANEDA, Yukiko NAGAI, Fumiaki YAMAO and Takeshi SENO

The temperature-sensitive mutation of the ubiquitin-activating enzyme E1 in mammalian cells has given rise to unique phenotypes in which some mutants are arrested at the S phase of the cell-cycle but some others are arrested at the G2 phase. We found that mutant ts85 with the arrest point at the G2 phase shows a peculiar chromosomal aberration which is not observed in mutant tsFS20 which has the arrest point at the S phase. When ts85 cells were cultured at nonpermissive temperature for the indicated time, followed by culturing at permissive temperature in the presence of colcemid, we observed an interesting metaphase configuration, in which specific chromosomes arrayed radially together with pulverization shown only near the centromeric region of the chromosomes. This confuguration was observed at a frequency of 15–20%. We speculated that the configuration could be the result of interference in the dissociation of the interphase nucleolus. In fact, fluorescent signals of in situ hybridization using a segment of mouse rDNA gene as probe as well as silver staining specific for the nucleolar organizer region(NOR) coincided with the pulverized region. In addition, in situ hybridization using a mouse centromeric satellite DNA as probe demonstra-

ted that the chromosomes arrayed with centromeres towards the center. To identify the role of a particular ubiquitin pathway involved in the dissociation process of the nucleolus further analysis of the nucleolus in the arrested cells is essential. We collaborated with Dr. Hideo Tsuji and Dr. Yoichi Matsuda (the National Institute of Radiological Sciences, Chiba) in the latter part of this work. T. Sudha is a visiting scientist and on leave of absence from the Down's Research Society (India).

Mechanism of mRNA Capping Reaction

Kiyohisa MIZUMOTO

Most of the cellular as well as viral mRNAs in eukaryotes contain a 5′-terminal cap structure, m^7GpppN. The cap structure is required for efficient initiation of translation and for pre-mRNA splicing. It has also been suggested that the synthesis of the methylated cap structure may play an important role in transcription itself. The cap structure is synthesized at the initial stage of mRNA synthesis and conserved at the 5′-termini of RNAs while they are processed in the nucleus and transported to the cytoplasm. Elucidation of the mechamism of the cap structure is thus important for understanding the molecular mechamism of eukaryotic gene expression. We have been studying mRNA capping mechanism with purified capping enzymes from various eukaryotic cells (*Prog. Nucl. Acid Res. Mol. Biol.* **34**, 1, 1987).

The highly purified yeast mRNA capping enzyme is composed of two separate chains of 52 (α) and 80 kDa (β), responsible for the activities of mRNA guanylyltransferase (ppN-RNA + pppG → GpppN-RNA + PPi) and RNA 5′-triphosphatase (pppN-RNA → ppN-RNA + Pi), respectively (*J. Biol. Chem.* **262**, 1989, 1987). The gene encoding the α subunit, *CEG1*, has been isolated by immunological screening of a yeast genomic expression library with anti-capping enzyme antibody (*J. Biol. Chem.* **267**, 9521, 1992). The identity of *CEG1* was comfirmed by expressing the gene in *E. coli* to give a catalytically active guanylyltransferase. Gene disruption experiment indicated that the *CEG1* gene is essential for the growth of yeast. The α subunit protein synthesized in *E. coli* catalyzed the overall capping reaction with ppN-RNA as well as the formation of enzyme-GMP (E-pG) covalent reaction intermediate in the absence of the β subunit. The GMP was found

to be linked to the ε-amino group of a lysine residue of the enzyme. Location of the active site for E-pG formation was sudied by isolating a ^{32}P-guanylylated peptide after trypsin digestion of E-[^{32}P]pG.

Transcription of Sendai Virus (HVJ) Genome

Kiyohisa MIZUMOTO

Sendai virus (HVJ), a member of the paramyxovirus group, contains a single nonsegmented negative strand RNA genome of approximately 15 kb-long, encoding at least seven proteins, NP, P, C, M, F, HN and L. The genetic information of this negative strand RNA is expressed through monocistronic mRNAs that are transcribed by a virion-associated RNA-dependent RNA polymerase composed of P and L proteins.

We have developed a faithful *in vitro* transcription system with purified Sendai virus particles, in which RNA synthesis is almost entirely dependent on host cell proteins (host factors). The host factor activity was partially purified from bovine brain, and was shown to be separated into at least two complementary fractions. One of them could be replaced by highly purified tubulin.

Sendai virus particles were incubated with bovine brain extract in the absence of 4NTPs, and the viral nucleocapsid (RNP) was isolated by centrifugation through a glycerol cushion. The RNP complex thus obtained catalyzed the synthesis of viral mRNAs upon the addition of 4NTPs without any supply of host proteins, indicating that an active transcription initiation complex is formed. On the other hand, RNP isolated from Sendai virus not incubated with cell extract was completely inert.

When the active RNP was subjected to SDS-polyacrylamide gel electrophoresis, a single extra protein band, which was similar in size to tubulin, was observed, in addition to the protein components of viral nucleocapsid. The identity of this band as tubulin was confirmed by Western blot analysis. Highly purified tubulin from bovine brain also supported initiation complex formation. These rusults suggest that tubulin molecules are integrated into the initiation complex and may function in transcription initiation and/or elongation processes.

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 template as good as free DNA in transcription by *E. coli*, bacteriophage T7, T3, and SP 6 RNA polymerases. This technique enabled us to separate transcripts retained in the transcription complexes from those released by 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 a bacteriophage transcription system, we found a new kind of transcription complexes which intervenes the two complexes and abortive release, and named it the moribund complex. The moribund complex is formed from the active complex an obligatory intermediate in the abortive pathway, and elongate their RNA 100–1,000 times as slow as the active complexes, and is slowly converted into dead end complexes.

In the *E. coli* transcription system, we found the abortive release is greatly activated by the presence of polymerase in excess of the promoter. When translocation of a polymerase on DNA is inhibited by another preceding molecule stuck at 32nt from a promoter, a misincorporation at 5nt was found in addition to a further enhancement of the abortive release. The misincorporated transcripts never elongated into long RNA even when the stacked molecule was removed. Thus misincorporation was checked in the early steps in elongation.

Single-Molecule Dynamics of Transcription Using Immobilized Operons: Sliding of RNA Polymerase on DNA

Hiroyuki KABATA and Nobuo SHIMAMOTO

The first step of transcription is the search for and binding of RNA polymerase to a promoter, and this promoter search is the major determinant in selective expression of genes. The enzyme could directly bind to a promoter on a long DNA, or alternatively, first bind to DNA in a nonspecific

manner and then form the promoter complex by one-dimensional diffusion along the DNA chain. Unfortunately, kinetic evidence for the two conflicting models has been reported and further study should be based on more confident evidence. The immobilized operon provides an excellent method for determining which case is true. Linear DNA immobilized on a slide glass was prepared. Fluorescently labeled RNA polymerase was added to the linearly fixed DNA, and the movement of a single molecule of RNA polymerase was followed through microscopy and recorded on a video-tape. One-dimensional movement was detected and such movements were inhibited when the labeled polymerase was preincubated with heparin or free DNA fragment bearing a strong promoter.

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

Katsuhiko KAMADA, 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 called 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. To substantiate this hypothesis, we investigated the expression of the *ssb* gene under various conditions. The *ssb-lacZ* operon and protein fusions were employed for determination of the expression of the *ssb* gene *in vivo*. The results showed that neither overproduction of SSB protein nor addition of SOS-inducing agents (e.g. Mitomycin C) affect the expression of the ssb-lacZ fusion genes. The construction and examination of strains which deplete functional SSB protein is now in progress.

Structure analysis of native SSB protein is in the finishing stages. Crystals of the protein were reproducibly formed by a salting out with ammonium sulfate and a larger crystal suitable for X-ray crystallography was obtained. A diffraction pattern of 3–3.5 angstrom is now being analyzed. Isomorphous replacement with uranium was successful and the location of the metal was determined. We are continuing the effort to isolate ssDNA-protein co-

crystals or soak ssDNA into native crystals.

The Tissue Specific Expression of the *wx*$^+$ Gene in Transgenic Rice and Petunia

Hiro-Yuki HIRANO, Takeshi MATSUMURA*, Noriko TABAYASHI*, Masatoshi TANIDA*, Yoshibumi KOMEDA** and Yoshio SANO

The *wx* locus of rice controls amylose synthesis in the endosperm. We have reported that the *wx*$^+$ gene expressed in a tissue-specific manner, namely, it is expressed only in the endosperm and pollen (Hirano and Sano (1991) *Plant Cell Physiol.* **32**, 989–997).

In order to examine the promoter function for tissue specificity, we introduced the chimeric DNA consisting of the 5′ upstream region of the rice *wx*$^+$ gene and bacterial β-glucronidase (GUS) gene as a reporter into cells of rice and petunia and regenerated whole plants from them. The functions of the 5′ upstream region were monitored by measuring the enzymatic activities and/or histochemical analysis of the GUS gene in various tissues (organs) of transgenic plants. In the seed of transgenic rice, GUS activity was located in the endosperm and no activity was detected in the embryo. GUS activity was also detected in the pollen but was not in the leaves. These results indicated that the 5′ upstream region introduced was sufficient for tissue specific expression in the endosperm and pollen in transgenic rice. On the other hand, in the transgenic plants of petunia, the same chimeric gene was expressed in the pollen but was not properly expressed in the endosperm. These results suggest that the *cis*-acting elements that drive the gene in the pollen are common to both monocotyledonous and dicotyledonous plants but those for endosperm specific expression in rice, did not function in dicotyledonous plants.

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II. MICROBIAL GENETICS

Recognition of DNA Replication Origin of Bacteriophage f1 by RNA Polymerase

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Synthesis of the minus strand DNA of bacteriophage f1 is initiated by an RNA primer, which is synthesized by the host RNA polymerase. The minus strand origin contains two hairpin structures called [B] and [C] (Gray *et al.* (1978) *Proc. Natl. Acad. Sci. USA.* **75**, 50–53). The mechanism by which the primer RNA is synthesized at a specific site is not well understood. It has been generally assumed that primer RNA synthesis is initiated by some mechanism which is entirely different from the initiation of general transcription.

Previously, we constructed a number of deletion and insertion mutants in the hairpins [B] and [C], and analyzed them for the origin function (Higashitani *et al.* (1990) *annual rep.* **41**, 31–32). Furthermore, we have determined the start site of the primer RNA synthesis by sequencing the primer using chain-terminating ribonucleoside triphosphate analogues (3′-deoxyNTP's) (Higashitani *et al.* (1993) *J. viol.* **67**, 2175–2181). The results indicated that the size of the primer RNA was 20 nucleotides and that the sequence was complementary to the f1 DNA sequence of nucleotides 5736 to 5717. This meant that the primer synthesis started at a site 20 bases downstream from the site previously reported by Geider *et al.* ((1978) *Proc. Natl. Acad. Sci.* USA. **75**, 645–649). Since the newly determined start site was located within the hairpin [C], a possibility was raised that the primer synthesis is initiated by a mechanism similar to that for general transcription.

To examine the detailed mechanisms of recognition by RNA polymerase, we constructed a number of base substitution mutants in the hairpin [B]. These mutants were examined for the activity of the origin. The results indicated that a specific sequence which is required for the origin function exists within the stem of the hairpin [B]. In most of the other regions of the stem, double-strandedness, but not specific sequence, was required for the origin function.

Results of footprinting experiments of *E. coli* RNA polymerase on the

minus strand origin indicated that the bottom half of the stem in hairpin [B] and the bottom half of the stem in hairpin [C] which contains the initiation site of the primer RNA were protected by RNA polymerase from nuclease digestion. The binding of RNA polymerase was dependent on the presence of sigma (σ) factor.

These results suggest structural and functional similarity between the minus strand origin and transcriptional promoter. The recognition mechanism of RNA polymerase at the minus strand origin may be related to the mechanisms of transcriptional initiation. Indeed, the nucleotide sequence of the protected region showed weakly transcriptional promoter activity *in vivo*. Although we don't find a very prominent promoter consensus sequence in the hairpins, further mutational analyses may shed light on the relationship between the priming reaction and general transcription.

Requirement of the Correct Phasing within the Replication Enhancer of Phage f1

Satoshi ASANO, Atushi HIGASHITANI and Kensuke HORIUCHI

The plus-strand replication of filamentous coliphage is initiated by gp2, which is a phage-encoded initiator protein. gp2 binds to the plus-strand origin and introduces a specific nick in the plus-strand. The 3′-hydroxyl end of the nick serves as the primer for plus-strand rolling-circle-type replication.

The replication origin consists of the core region and the enhancer region. While the former is absolutely required for initiation and termination, the latter, located immediately downstream of the core, enhances initiation of replication. The enhancer region contains three binding sites (1, 2′ and 2″) for integration host factor (IHF). IHF binds strongly to site 1, but only weakly to sites 2′ and 2″. In vivo studies have revealed that either deletion of the enhancer region or lack of IHF reduces initiation of replication to one-hundredth of its normal level.

In this study, we investigated the mechanism of action of the enhancer region. The following results were obtained: 1) correct phasing between the core and the enhancer regions is important for replication; 2) correct phasing between IHF binding site 1 and site 2′, 2″ is important for replication; 3) when IHF site 1 was replaced by a sequence that IHF could not bind to, initiation efficiency was reduced; 4) when IHF site 2′, or both sites 2′ and 2″,

was replaced by a sequence that IHF could not bind to, initiation efficiency was reduced; 5) when IHF site 2″ was replaced by a sequence that IHF could not bind to, initiation efficiency was only weakly influenced. Our current working hypothesis is as follows: IHF binds to site 1 and introduces DNA bending in such a way that gp2 bound to the core region interacts with a yet unidentified factor that is bound to a distal portion of the enhancer region that includes sites 2′ and 2″.

Conformational Changes Induced in a Rolling Circle Replication Origin by Intiator Protein

Atsushi HIGASHITANI, Hideo HIROKAWA* and Kensuke HORIUCHI

Gene II protein (gpII) of the filamentous phage f1 is a multifunctional protein that participates in DNA replication. It introduces a single-strand break at a specific site on the plus strand of the negatively supercoiled replicative form (RF-I). The 3′ hydroxyl end of the nick serves as the primer for initiation of plus strand rolling circle replication. GpII binds to the origin and forms two complexes, I and II, that are separable by polyacrylamide gel electrophoresis (Greenstein & Horiuchi (1987) *J. Mol. Biol.* **197**, 157–174). The complex I results from interaction of two gpII molecules with the minimal binding sequence containing the inverted repeat, β and γ. In the second binding step, two additional gpII molecules bind to form complex II which is the functional complex for the nicking of replication initiation (Greenstein & Horiuchi (1990) *J. Mol. Biol.* **211**, 91–101). Bent DNA structures in these complexes were inferred from the gel mobility of a series of circularly permuted DNA fragments that differ in the placement of the gpII binding site (Greenstein & Horiuchi, unpublished result).

In this study, we characterized the bent structures of the complexes through electron microscopy. A 838 base pair *Hpa*I/*Hae*II restriction fragment containing the complete f1 plus strand origin was used. In the absence of gpII, the DNA fragment appeared extended. When gpII was mixed with the DNA in a molar ratio of 1 : 1, 34% of the DNA molecules formed bent structures, with a bending angle of about 90°. The bent occurred near the nicking site. Gel retardation assay indicated that approximately 30% of the DNA molecules formed complex I and 2% formed complex II under the

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conditions used. When gpII was mixed with the DNA in a molar ratio of 2:1, approximately 38% of the DNA formed complex I and 8% formed complex II. Under electron microscope, 43% of the DNA molecules were bent by approximately 90° and 12% were bent by approximately 150°. When the molar ratio of gpII to DNA was increased to 4:1, 45% of the DNA formed complex I and 22% formed complex II, and 41% of the DNA molecules were bent by 90° and 19% were bent by 150°. These results suggest that binding of gpII induces bending of the origin DNA and that the bending angles in complex I and complex II are approximately 90° and 150°, respectively.

We further investigated whether melting of the duplex structure is induced in the origin region by the binding of gpII. The potassium permanganate ($KMnO_4$) assay (Vos *et al.* (1991) *Cell* **65**, 105–113) was used to detect unpaired regions of DNA. Negatively supercoiled and linear DNA, both carrying the f1 origin, were incubated with gpII at 37°C in the presence or absence of Mg^{2+}. In the presence of Mg^{2+}, a half of the population of supercoiled DNA molecules was converted to the nicked form (RF-II), and the other half was converted to the relaxed form (RF-IV). The samples were subsequently treated with $KMnO_4$ to modify unpaired thymidine residues, and were cleaved with piperidine. The sites cleaved were determined by primer extension.

The results obtained indicated that, in supercoiled DNA, binding of gpII in the absence of Mg^{2+} made certain thymidine residues within the origin hyperreactive to $KMnO_4$. On the plus strand, the residues at positions -2, -1, and $+3$ in reference to the nicking site were made hyperreactive and the one at -3 became weakly reactive. On the minus strand, the thymidines at positions $+1$ and $+2$ became hyperreactive. In the reaction with the linear DNA, no signals of DNA melting by gpII were observed. These results indicate that a short region of duplex DNA around the nicking site becomes single-stranded upon binding of gpII and that this melting occurs only in supercoiled DNA.

To study which sequence element(s) are required for the DNA melting by gpII, we used deletion mutants $\Delta 76$ and $\Delta 83$. $\Delta 76$ lacks the a repeat sequence in the plus strand origin, which is essential for termination but not for initiation of DNA replication. $\Delta 83$ lacks the a sequence and additional 7 bases which include the gpII nicking site (Dotto *et al.* (1982) *J. Mol. Biol.* **162**, 335–343). Both the mutant origins can form complexes I and II. $\Delta 76$

origin is nicked specifically by gpII, but $\Delta 83$ is not. Our results showed that gpII binding caused DNA melting in the $\Delta 76$ origin in the same way as in the wild-type origin. This indicates that the DNA melting by gpII does not require formation of a hypothetical cruciform between the inverted repeats α and β. No melting was observed in the $\Delta 83$ origin.

In summary, we showed that DNA melting around the nicking site is induced by gpII binding on negatively supercoiled DNA, and that the sequence around the nicking site, but not the α repeat sequence, is required for the melting to occur.

Stabilization of Replication Initiator Protein DnaA of *Escherichia coli* by DnaK Chaperone

Seiichi YASUDA, Atsushi HIGASHITANI, Kensuke HORIUCHI and Yoshimasa SAKAKIBARA*

Dnak protein is a major heat shock protein of *Escherichia coli* and the homologue of eukaryotic hsp70. DnaK protein interacts with various proteins and activates them. *In vivo* studies of a *dnaK* mutant have shown that DnaK protein is involved in initiation of chromosome replication. *In vitro* studies using oriC plasmid replication systems have suggested that DnaK protein modulates the activity of DnaA protein which is essential for initiation of DNA replication. In this study we showed that DnaK protein stabilizes the heat-labile wild type DnaA protein so that it is fully active even after heating at 70°C for 10 min. DnaK protein cannot reactivate heat-inactivated DnaA protein in the presence or absence of ATP. DnaK protein can form a stable complex with DnaA protein; the complex consists of one or two molecules of each protein. The complex formation as well as the DnaK-dependent stabilization of DnaA protein is inhibited by ATP without its hydrolysis. These findings suggest that the function of DnaK protein in initiation of DNA replication is to stabilize DnaA protein by forming a complex with it.

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A 1.9-kb Upstream Sequence Is Required for Expression of the *ftsI* Gene Coding for Penicillin-Binding Protein 3 of *Escherichia coli*

Hiroshi HARA, Kensuke HORIUCHI and James T. PARK*

The *ftsI* gene of *Escherichia coli* codes for penicillin-binding protein 3 (PBP 3), a cytoplasmic membrane protein that functions as an enzyme for the formation of a septum of the murein sacculus and is the principal lethal target of β-lactam antibiotics. This gene is located at 2 min on the *E. coli* genetic map, a little distal to *leu*, and is among a large cluster of murein biosynthetic genes (*mur* genes and *mraY* and *ddl*) and cell division genes (*fts* genes and *envA*).

In an attempt to generate and characterize various *ftsI* mutant alleles on a plasmid, we realized that it would be useful to have a null allele of this gene on the chromosome. We replaced the 1.5-kb *Mlu*I-*Nar*I fragment within the cloned *ftsI* gene (nucleotides 124-1628 out of its 1764-nucleotide open reading frame [ORF]) with a chloramphenicol resistance (*cat*) gene in the same orientation as the *ftsI* gene, and exchanged it with a chromosomal wild-type allele in a *polA*(Ts) strain. This null allele (*ΔftsI*::*cat*) could be cotransduced with *leu* by P1 phage only if a recipient strain carried additional copies of wild-type *ftsI* on a plasmid. When the additional *ftsI* was under control of the *lac* operator-promoter, the cells grew normally in the presence of IPTG, but failed to divide and formed long filaments when incubated without the inducer. These results provided final proof that *ftsI* is an essential cell division gene.

It was unexpectedly found that a 2.6-kb *Pvu*II fragment containing *ftsI* and 0.5 kb of its upstream region could not complement *ΔftsI*::*cat* when cloned on a single-copy mini-F vector, although the fragment contains at least two promoter-like sequences and was shown to complement an *ftsI*(Ts) mutation when cloned on a multicopy vector. A 7.3-kb *Hin*dIII-*Eco*RI fragment containing *ftsI* and 1.9 kb of its upstream cloned on a mini-F vector could not complement the null allele either, and the addition of a further upstream 1.8-kb *Hin*dIII fragment was necessary for complementation by a single copy *ftsI*. (Complementation tests of *ftsI*-carrying mini-F plasmids using an *ftsI* (Ts) mutant did not give clear results because of thermosensitive mainte-

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nance of the F replicon.) Disruption of the *Hin*dIII site 1.9-kb upstream of *ftsI*, by the filling reaction of T4 DNA polymerase, resulted in loss of the complementation ability. Since this site was intact on the chromosome in the *ΔftsI*::*cat* strain, there seemed to be a sequence required in *cis*. A sequence with homology to the consensus promoter sequence recognized by RNA polymerase containing σ^{70} was found overlapping the *Hin*dIII site. This sequence was fused to a promoterless *lacZ* gene on a mini-F plasmid and shown to function as a promoter.

This promoter sequence lies near the 3′ end of a 0.6-kb region which contains no ORF of significant length and is followed by murein biosynthetic and cell division genes. These genes are tightly clustered in the same orientation without a recognizable transcription terminator. It was reported that a null allele of the *ftsZ* gene could not be complemented by a λ transducing phage containing *ftsZ* and the preceding 6 kb region (Dai and Lutkenhaus (1991) *J. Bacteriol.* **173**, 3500–3506), and the authors proposed a possibility of a long transcript being required for full expression of *ftsZ*. Transcription from the promoter identified in the present study at 1.9-kb upstream of *ftsI* might proceed for 18 kb (*i.e.* beyond *ftsZ*) to the only known terminator just downstream of *envA*.

A Chain-Forming Strain, PM61, of *Escherichia coli* Contains, in Addition to the *envC* Mutation, an IS*4* Insertion in the *prc* Gene which Causes Accumulation of the Precursor Form of Penicillin-Binding Protein 3

Yoshihiro YAMAMOTO*, Hiroshi HARA, Doris KARIBIAN**, Kensuke HORIUCHI, James T. PARK*** and Yukinobu NISHIMURA****

Penicillin-binding protein 3 (PBP 3) of *Escherichia coli* is an essential enzyme involved in septum formation. Temperature-sensitive *ftsI* mutants that are defective in PBP 3 are incapable of cell division and grow as filaments at the restrictive temperature. Another class of mutants defective in

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septation form chains of cells rather than filaments: septa are formed but the poles of daughter cells remain fused to each other for a period of time. One such mutant is an *envC* mutant PM61 (Rodolakis *et al.* (1973) *J. Gen. Microbiol.* **75**, 409–416). Since the defect was related to completion of septum formation, we examined penicillin-binding proteins of PM61 and found that it had PBP 3 of larger molecular weight than normal.

By P1 transduction experiments and by transformation with plasmids carrying relevant genes it was shown that the PBP 3 defect was not caused by the *envC* mutation nor by an unidentified mutation in *ftsI*, the structural gene for PBP 3, but by a mutation in the *prc* gene coding for a processing enzyme that cleaves a C-terminal peptide from the precursor form of PBP 3. Southern hybridization experiments showed that an insertion was present in the *prc* gene of PM61. We cloned and sequenced the gene and found an IS*4* sequence in its midst. So the *prc* function is knocked out in PM61, and the large-size PBP 3 in PM61 is an unprocessed precursor form. Since precursor PBP 3 is apparently functional for septum formation (Hara *et al.* (1991) *J. Bacteriol.* **173**, 4779–4813), this alteration must have little to do with the incomplete septation. At the IS*4* insertion point there was a duplication of 12-nucleotide sequence. Comparison of the target sequence for IS*4* insertion into *prc* with previously published ones suggested that considerable variations are allowed for the target sequence.

Osmoregulation of the Fatty Acid Receptor Gene *fadL* through OmpR Protein in *Escherichia coli*

Atsushi HIGASHITANI, Yukinobu NISHIMURA*, Hiroshi HARA, Hirofumi AIBA**, Takeshi MIZUNO** and Kensuke HORIUCHI

The *fadL* gene of *Escherichia coli* codes for an outer membrane protein that is involved in the uptake of long-chain fatty acids. The uptake is regulated by environmental osmolarity, and decreases when the cells are under high osmotic conditions. A temperature-sensitive mutant that requires fatty acid for growth at 42°C was unable to grow at the high temperature even in the presence of fatty acid if the medium contained 10% sucrose. Promoter activity of the *fadL* gene *in vivo* was repressed by high osmolarity in a FadR

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repressor null mutant. Furthermore, *in vitro* transcription of the *fadL* gene was strongly repressed by the addition of OmpR and EnvZ proteins. The results of gel retardation and DNaseI protection experiments indicated that OmpR protein, only after incubation with the protein kinase, EnvZ, in the presence of ATP, specifically binds to at least four sites around the *fadL* promoter, two upstream and two downstream from the transcriptional start site. These results suggest that transcription of the *fadL* gene is osmotically regulated by the OmpR-EnvZ two-component system.

Molecular Mechanism in Determination of Timing of Cell Division in *Escherichia coli* K-12 (I)

Akiko NISHIMURA

To elucidate the molecular mechanism of the timing of cell division in *Escherichia coli* K-12, novel mutants which uncouple DNA replication and cell division were isolated and named *cfcA1*, *cfcB1*, *cfcC1*, and *cfcD1*. The mutations partially suppressed division arrest and lethality induced by inhibition of DNA replication. Under permissive conditions for DNA replication, the division rate of *cfc* mutants was about 1.5 times higher than that of *cfc*$^+$ cells. Complementation and sequencing analysis showed that the cfcA gene encoded the α subunit of glycil–tRNA synthetase. The mutated *cfcA1* gene revealed substitution, 143A by C; this corresponds to the amino acid substitution of Glu48 by Ala. The *cfcB1* mutation was mapped at 1 min ,near *ileS*, on the *E. coli* genetic map. The EcoR1-PvuII DNA fragment (2Kb) which can complement the *cfcB1* mutation was subcloned and is continuing to be se quenced. The *cfcC1* and *cfcD1* mutations were unstable and tend to revert to the *Cfc*$^+$ phenotype with high frequency. These two genes might be involved in an essential process for cell growth. From the genetic and physiologic analysis of cfc mutants and double mutants with relA1, lon, sfiA, or *min B, C, D*, it was concluded that the cfc genes certainly participate in the frequency of cell division. A search to elucidate the global network or the mutual recognition system between processes in the cell cycle such as protein synthesis, DNA replication, cell division, and/or murein synthesis could solve the problem of timing of cell division in the cell cycle.

Molecular Mechanism in Determination of Timing of cell Division in *Escherichia coli* K-12 (II)

Hideki UKAI and Akiko NISHIMURA

We found many multicopy suppressor genes mapped in the *ter* region, which suppressed the temperature sensitive mutation of cell division genes (*fts*) mapped within 10 min from *oriC*. Sequencing analysis of the genes showed primary structure interesting for the cell cycle. The *ftsN1* (Ts) mutation was mapped at 95 min, and was complemented by one of the multicopy suppressor genes, *sunU*. The *ftsN1* mutant showed high ploidy at 41°C. Cell division of the *ftsN1* mutant was arrested gradually at 41°C, but an in crease in viable count immediately ceased. The *ftsN* gene is presumed to be a growth phase-regulated gene because sequence analysis showed that the deduced ftsN gene had a consensus sequence of gear box at the promoter region. The *sunU* gene was predicted to encode a protein composed of 198 amino acids. The C-terminal region of the deduced *SunU* protein showed significant sequence similarity to SulA, a division inhibiter involved in the SOS mechanism. Deletion of 6 amino acids from the C-terminal caused decreased complementation activity for the *ftsN1* mutation and deletion of 19 amino acids caused complete loss of comple mentation activity even when it was overproduced in the cell. Further characterization of these mutants would elucidate the fine control mechanisms of the cell cycle.

III. MAMMALIAN GENETICS

High-Order Chromatin Structure of the Recombinational Hotspot in the Mouse MHC

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

It has been established that reciprocal recombination occurs with high frequency in the mouse major histocompatibility complex (MHC) when some mouse strains are crossed. The breakpoints for recombination in the MHC are clustered at specific sites termed hotspots. The wm7 MHC haplotype derived from Japanese wild mouse enhances the recombination at the hotspot located between the Pb and Ob genes (wm7 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 is yet to be defined. One possibility is that the recombinational hotspot has an open chromatin structure leading to the enhanced accessibility of DNA to the recombination machinery. We attempted to examine this hypothesis with the wm7 hotspot. First, we fractionated testicular germ cells prepared from several different mouse strains by centrifugal elutriation after collagenase and trypsin treatment of testes, and enriched the pachytene cells at which meiotic recombination is thought to occur. In order to analyze the high-order structure of chromatin around the hotspot, we tried to detect DNaseI hypersensitivity sites (DHSS) in the chromosomal region surrounding the hotspot. Clear DHSS was not detected in the 2 kb segment in the vicinity of the hotspot. Now we have extended this analysis to much broader chromosomal segments.

Functional Analysis of the LMP-2 Gene Encoding a Subunit of the Mouse Proteasome

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

Exogenous antigenic proteins, as viruses, 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

termed proteasome, exerts this protein degradation in an energy-dependent manner. In mouse, the LMP-2 gene encodes a subunit of proteasome, which is mapped to a 9 kb distal to the recombinational hotspot which has been identified in the wm7 and cas3 MHC haplotypes. It is still unclear whether the LMP-2 gene plays a role in protein degradation for antigen presentation in immune recognition. We intended to elucidate the function of the LMP-2 gene by destroying this gene through a gene targeting experiment. In addition, we attempted to test the possibility of enhancing the efficiency of homologous recombination using a vector construction which contains the recombinational hotspot. In the beginning of the study, we analyzed the genome structure of the LMP-2 gene and defined the intron-exon organization of this gene. We constructed two different vectors, pLMP2neo-Hot and pLMP2neo-Cold. Both have the Neomycin phosphotransferase (Neo) gene in exon 1 as a positive selection marker and the Herpes simplex virus thymidine kinase (tk) gene at the 3′ end of the LMP-2 gene as a negative selection marker. Only pLMP2neo-Hot has the recombinational hotspot in the 3′ region of the LMP-2 gene. We are planning to transfect embryonic cell lines, F1-1, with these two vector DNAs.

Establishment of Embryonic Stem Cell Lines from B10.H-2 Congenic Strains II

Tomoko SAGAI, Norio NAKATSUJI, Toshihiko SHIROISHI and Kazuo MORIWAKI

We have continued to isolate ES cell lines from B10.H-2 congenic strains, for studying the molecular mechanism of meiotic recombination in the H-2 region. Many ES cell lines have been isolated from various mouse strains, but production of germ-line chimeras has been achieved with only limited strains, for example 129/Sv. As shown in the previous report, we isolated twenty ES cell lines from one B10.H-2 congenic strain, B10.A(R209), which showed high recombination frequency in the H-2 region during meiosis. Thus far, three of them have been tested for chimera formation and germ-line transmission through microinjection into 8 cell embryos of BALB/c, A/J and ICR strains. As a result, we have obtained several chimera mice from them, but no germ-line chimera yet. Several reports suggest that the combination of the genotype of ES cells and host embryo is essential for germ-line transmission of ES cells. Recently Tokunaga and Tsunoda established a new ES cell line,

F1/1, from a (B6xCBA)F1 mouse. They reported that chimeras obtained from F1/1 cells by 8 cell injection produced exclusively ES-derived offsprings (Tokunaga, T. and Tsunoda, Y. (1992) *Develop. Growth* and *Differ.* **34**, 561–566). From the point of view that the two mouse strains, B6 and B10, are genetically very related, it is expected that the (B10.A(R209)×CBA)F1 mouse would yield ES cell lines with a potential for germ-line transmisson as well. We isolated three ES strains from the (B10.A(R209)×CBA)F1 mouse. Now we have obtained several chimera mice from the new ES cell lines and are testing for germ-line transmission of them.

Genetic Control of Male-specific Suppression of Meiotic Recomibnation

Toshihiko SHIROISHI, Tomoko SAGAI and Kazuo MORIWAKI

Sexual difference in the frequency of meiotic recombination is observed in human and mouse chromosomes. The difference is significant in certain chromosomes or particular parts of some chromosomes. We have reported previously that the wm7 MHC haplotype derived from Japanese wild mouse enhances meiotic recombination specifically in females at the recombinational hotspot located in the proximal region of the MHC. Genetic analysis demonstrated that the wm7 haplotype possesses a suppressive factor for recombinations in male meiosis, which is located on the chromosomal segment distal to the hotspot. In the present study, we attempted to map the suppressive factor more precisely. For this purpose, we carried out mating experiments using two intra-MHC recombinants, B10.A(R202) and B10.A (R203), which were generated from the wm7 haplotype. Both recombinants have a genotype of *H-2K*^{a}*-H-2D*^{wm7}. The recombinational breakpoints are located between the Bf and H-2D genes in B10.A(R202), and between the Ea and Cyp21 genes in B10.A(R203). We estimated the recombination frequency in crosses of B10.A(209) and these two recombinants, between the H-2K and H-2D marker loci during male and female meiosis. As a result, crosses involving B10.A(R202) yielded a high recombination frequency both in male and female meiosis. By contrast, crosses involving B10.A(R203) yielded a high recombination frequency only in females. The result clearly showed that the male suppressor was preserved in B10.A(R203), but not in B10.A(R202), indicating that the male suppressor is located between the hotspot and Cyp21 genes, which span about 400 kb in length. The breakpoints of the recombina-

nts obtained in the present experiment were defined by restriction fragment length polymorphism (RFLP) analysis. We found that all breakpoints almost overlapped with the original hotspot identified in the cross between the wm7 and laboratory MHC haplotypes.

Establishment of New Congenic Strain for Insulin-dependent Diabetes Mellitus (IDDM) Susceptibility Gene, *Idd-4*

Shigeharu WAKANA, Toshihiko SHIROISHI and Kazuo MORIWAKI

The NOD (Nonobese diabetic) mouse is widely used as animal model of insulin-dependent diabetes mellitus (IDDM). Genetical analysis in breeding studies between NOD and C57BL/10J mice has suggested that several genes are involved in onset of diabetes and insulitis, a prediabetic condition characterized by lymphocyte infiltration into the islets of Langerhans of the pancreas. At least four susceptibility genes for IDDM have been reported. Among them, the Idd-1 is linked to the MHC on chromosome 17 and *Idd-4* is mapped in the region between the Acrb and Mpo genes on chromosome 11.

In order to investigate the function of the *Idd-4* gene without influence of other susceptibility genes for diabetes, we have tried to produce the congenic mouse strain for the *Idd-4* gene by introducing of the chromosomal segment between the Acrb and Mpo gene from Japanese wild mouse-derived MSM strain into the genetic background of the NOD mice. At present, the backcross has reached to the 7th generations. Histopathological examination revealed that the *Idd-4* congenic mice, NOD.*Idd-4*^{MSM}, did not have insulitis and showed reduced level of glucose content in peripheral blood. These results confirmed the presence of a susceptibility gene for IDDM, which is located between the Acrb and Mpo genes, and usefulness of the NOD.*Idd-4*^{MSM} congenic strain to identify and clone the *Idd-4* gene.

Gene Dosage Effects of Recombinational Hotspots in the Mouse *MHC*

Masayasu YOSHINO, Tomoko SAGAI, Kirsten FISCHER-LINDAHL*,
Toshihiko SHIROISHI and Kazuo MORIWAKI

The meiotic recombinations in the proximal region of the mouse *major*

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histocompatibility complex (*MHC*) are not random, but clustered at specific sites termed hotspots. Some of the *MHC* haplotypes derived from the Asian wild mice, enhance recombination at the hotspots when they are heterozygous with standard laboratory haplotypes. The *wm7* and *cas3* haplotypes enhance recombination at the hotspot located between the *Pb* and *Ob* gene, and the *cas4* at a different hotspot located at a distance of about 100 kb from the *Pb-Ob* hotspot. In order to test the effects of a double dosage of hotspots, we estimated the rate of recombination frequency and determined the localizations of breakpoints in two different crosses, *wm7/cas3* and *wm7/cas4*. In total, we have screened 3570 of the backcross progeny from these two crosses and have obtained 29 of new recombinants between the *H-2K* and *Ab* genes, yielding a frequency of 0.81%. This rate was 40-fold higher than the standard value estimated in crosses between laboratory haplotypes (0.02%), and very similar to those obtained in crosses between wild and laboratory haplotypes. Thus, it appeared that a double dosage of hotspots has no additive effect on meiotic recombination. Molecular mapping by RFLP analysis revealed that the localizations of the recombinational breakpoints were completely identical to those in crosses involving the *wm7*, *cas3* and *cas4*, indicating conservation of site-specificity in crosses between the two haplotypes with the hotspot.

Mutation Rate of Intra-*MHC* Recombinant Mice

Masayasu YOSHINO, TOMOKO SAGAI, Toshihiko SHIROISHI and Kazuo MORIWAKI

In our laboratory, hundreds of intra-*MHC* recombinants have been established from thousands of progeny from 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 rate of recessive and dominant mutations per gamete per locus was roughly estimated to be 2.8×10^{-4} and 7.1×10^{-4}, respectively. These rates were much higher than the standard value, 3.8×10^{-6} for recessive and 3.4×10^{-7} for dominant mutations, which were estimated from laboratory mouse strains. In order to calculate the spontaneous and irradiation-induced mutation rates with the intra-*MHC* recombinants, we have started mutagenesis experiments. We used the PT/7af strain as a tester in genetic crosses with the intra-*MHC* recombinants, as it carries mutated

recessive alleles at seven specific loci. Besides these experiments, the dominant visible mutations were carefully observed as to whether irradiation boosts the mutation rate. In practice, we irradiated the B10.BR.(R228) mouse strain, from which a dominant visible mutation of hairless phenotype (Rim3) was obtained, and mated those mice with testers of the PT strain. As yet, we have detected neither recessive nor dominant mutation in 210 offspring from 5 Gy-irradiated females of B10.BR.(R228). We intend to continue this experiment to screen at least 1000 offspring. The target of this irradiation is the late stage of meiosis and a large lesion in the chromosome is expected. A small lesion in the gene at the early stage of meiosis can be induced by irradiating male mice with two fractions of 5 Gy separated by 24 hours. We plan to set up another mutagenesis experiment using an intra-*MHC* recombinant, B10.(R233), which has spontaneously yielded a recessive visible mutation of hairless phenotype (rim1) and a dominant visible mutation of yellow belly, (Rim5), to estimate the mutation rate after irradiation at the early stage of meiosis in male mice.

Comparison of the DNA Sequence in the D-loop Region of Mouse Mitochondrial DNA. III. Close Relatedness between Japanese Northern Population and the *castaneus* Population in the South-eastern Asia

Hiromichi YONEKAWA, Sumiyo WATANABE, Nobumoto MIYASHITA and Kazuo MORIWAKI

Based on restriction analysis of mitochondrial DNA, we have proposed the hypothesis that Japanese wild mouse "*Mus musculus molossinus*", is originated from a hybrid between ancestral colonies, possibly very small, of *M. m. musculus* and of *M. m. castaneus* (Yonekawa *et al.* (1988) *Mol. Biol. Evol.* **5**, 63). To prove this hypothesis, we analyzed the mtDNA D-loop region from the mice in Japan as well as from those in its neighboring countries. Through this analysis we have previously showed the following lines of evidence: 1) substituted nucleotides in the mtDNA D-loop region, like restriction site variation, can be used as a diagnostic marker for subspecies identification when we construct phyologenic trees using the unweighed pair-grope method with arithmetic mean (UPGMA) or the neighbor-joining (NJ) method. 2) at least four different mtDNA types exist in the Southern population of

Japanese wild mice (Yonekawa *et al.* (1990) *Ann. Rep. Natl. Inst. Genetics, Japan*, **41**, 46–47), although no restriction polymorphisms had been detected in the population (Yonekawa *et al.* (1988) *Mol. Biol. Evol.* **5**, 63), 3) mtDNA of the Japanese southern population was clustered in that of Korean, northern-Chinese and eastern-Russian populations. Thus it is suggested that the southern population was originated from northern Far East and came from there through the Korean peninsula (Yonekawa *et al.* (1991) *Ann. Rep. Natl. Inst. Genetics, Japan*, **42**, 52–54).

Next question is what is the locality where the Japanese northern population with *castaneus* type mtDNA came from. To address this question, we sequenced mtDNA from 27 mice collected from the Southeastern Asia and southern China including 5 samples from Japanese mice. We found that 1) *castaneus* type mtDNA showed at least three distinct clusters in the "*castaneus*" branch of phylogenetic tree, and 2) mtDNA of the Japanese northern population was clustered in that of southeastern Asia, but not in southern China. This suggests that the northern population was originated from the population of southeastern Asia. It is thus concluded that there are two possible routes through which the Japanese mice invade into Japan: one is from northern China through the Korean peninsula and the other is southeastern Asia possibly through islands in southern Pacific.

A New *Pas* Genes Derived from Asian wild Mouse

Nobumoto MIYASHITA, Toshihiko SHIROISHI, Shigeharu WAKANA*,
Hiromichi YONEKAWA** and Kazuo MORIWAKI

Rather classical mating experiments between a resistant strain C57BL and a susceptible strain A have demonstrated the possible presence of a single recessive resistant gene in the susceptibility to urethane-induced pulmonary adenoma in mice (Falconer and Bloom, 1964). This gene was designated "*ptr*" by them, but later "*Pas-1*" (Pulmonary Adenoma Susceptibility-*1*) by Malkinson *et al.*, 1984. Ryan *et al.* (1984) and Gariboldi *et al.* (1993) demonstrated that the major gene for pulmonary adenoma susceptibility was located on chromosome 6, though the relationship between *Pas-1* and *Kras-2* was not necessarily clear, probably because of the additional effect of *H-2*

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complex as shown by us (Miyashita and Moriwaki, 1987). We have achieved mating experiments using A/Wy and B10.A strains which have the same *H-2* haplotype and linkage analysis to make clear whether *Pas-1* is *Kras-2* itself or not. Two recombinants out of 68 N2 progeny were observed, indicating that *Pas-1* is a unique locus independent from *Kras-2* on chromosome 6, but tightly linked to it.

BGR and MSM strains derived from Asian wild mice, *Mus musculus castaneus* and *M. m. molossinus*, have shown a definitely dominant allele(s) in their pulmonary adenoma susceptibility gene(s) which was strikingly different from the resistant strain such as C57BL. F2 progeny from A×BGR cross revealed that this resistant gene(s) is not *Pas-1*. This putative suppression gene is not linked with either *Acrb* (*Trp53*) on chromosome 11 or *D14 Mit7* (*Rb-1*) on chromosome 14. This resistant allele(s) has possibly eliminated during the course of development of the present day's laboratory mouse strains.

IV. DEVELOPMENTAL AND SOMATIC CELL GENETICS

Formation of a New Nerve Network in the Nerve-free Tissue of Epithelial Hydra through Reintroduction of Interstitial Cells

Tsutomu SUGIYAMA, Sumiko MINOBE and Osamu KOIZUMI

Formation of a new nerve network in the nerve-free tissue of epithelial hydra was examined through the reintroduction of interstitial cells into epithelial hydra.

Hydra has a primitive nervous system known as a diffused nerve network. It consists of a large number of nerve cells distributed throughout the tissue. Neuron cell bodies extend one to several long processes into the tissue, and these processes interconnect with each other to form an extensive meshlike network throughout the entire body. Within the network, the neurons are distributed not randomly, but in a highly region-specific manner. For example, the neuron density is about 6 times higher in the head and about 3 times higher in the foot than in the central part of the body column. The neurons are classified into several major types based on morphology, the neuropeptides they contain, or the specific antigens present in them and recognized by various monoclonal antibodies. Distribution of the various types is also highly region-dependent.

To examine the mechanisms responsible for determining the region-specific nerve cell distribution in the network, we employed epithelial hydra in the present study. The tissue of epithelial hydra consists of mostly epithelial cells and is completely free from the interstitial cells and their differentiation products, nerve cells and nematocytes. When the interstitial cells were reintroduced into epithelial hydra, these cells differentiated into nerve cells rapidly, and established a new nerve network in the nerve-free tissue of epithelial hydra within several days.

In the hypostome, the "ganglion" cells first appeared in the whole region at day 1.5 after interstitial cell reintroduction. By day 4, a new network similar to that present in the hypostome of intact hydra was established, with a cluster of "sensory" cells at the hypostome tip and numerous ganglion cells in the adjacent tissue. In the tentacles, nerve cells first appeared at day 1.5–2.5. However, their distribution was initially restricted only to the base of the

tentacles. The area of distribution then gradually expanded, reaching the tentacle tip in about 7 days. The rate of this expansion was about the same as the speed of the epithelial tissue displacement from the base to the tip of the tentacles.

These observations show that a new nerve network closely resembling that in normal hydra can be established relatively quickly after reintroduction of the interstitial cells into the nerve-free tissue of epithelial hydra. This suggests the the network formation is controlled primarily by the epithelial tissue environment.

A Simplified Method for Detecting Cell Type Specific cDNA Clones from Hydra

Takeshi OHKAWA*, Koichiro SHIOKAWA* and Toshitaka FUJISAWA

The cell type specific expression of a given cDNA is normally achieved through *in situ* hybridization, which involves rather tedious procedures. For a large scale screening of cDNA clones for any particular cell type, it is desirable to have a more simplified and convenient method. For this purpose, we established an efficient cell fractionation method utilizing elutriation. About 1,000 hydra (10^8 cells) were treated with pronase (8 mg/ml of hypertonic cell dissociation medium) at room temperature for 1–2 h. By the treatment, cell–cell adhesion molecules are digested and tissue is easily dissociated into single cells. The resultant cell suspension was subjected to elutriation (constant flow rate of 15 ml/min and varying centrifugal speed from 800 to 3,800 rpm) and 7 fractions were collected. An examination of each fraction under Nomarsky optics revealed that 7 basic cell types which make up hydra tissue were nicely separated according to their sizes.

Total RNA was extracted from each fraction, fractionated by agarose gel electrophoresis and blotted onto nylon membranes. Using clones whose expression with respect to cell types is already known, northern hybridization was carried out. Signals were detected in fractions exactly as expected. These results indicate that we have a significantly simplified method at hand for sorting cDNA clones according to their specific cell type expression.

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Protease Sensitivities of Hydra Cell–Cell Adhesion

Masayuki HATTA and Tsutomu SUGIYAMA

Morphogenesis of multicellular animals are mediated by harmony of multiple cellular movements such as folding, invagination or evagination of epithelial cell sheets, transition of cells between epithelial and mesenchymal states and segregation of different type of cells. In these cellular activities, cell–cell adhesion mechanisms have been shown to play crucial roles. Hydra is an animal which has a simple body plan and a strong regenerative capacity, and morphogenesis during regeneration of Hydra has been investigated for many years. However cellular behavior or molecular mechanisms of the regeneration have been little investigated. We have started a project to examine cellular and molecular aspects of Hydra regeneration processes, focusing on cell–cell adhesion systems.

First, we developed solutions for protease treatment of Hydra tissue to obtain viable dissociated single cells and for reaggregation of the dissociated cells. Three proteases were then used for digestion of cell surface molecules: collagenase which has a high specificity on collagens, trypsin which has a moderate substrate specificity and pronase which has a wide spectrum of substrate specificity. About 0.2 g wet weight of Hydra were treated with each of the three proteases at 0.1% concentration in dissociation solution for 30 minutes at 18°C, rotating at 80 rpm and pipetting every 10 minutes. Cells were collected by centrifugation at 5000 rpm for 1 minute and resuspended in reaggregation solution. After removal of tissue fragments by decantation, the suspension of cells were subjected to reaggegation assay in 24-wells multiplates with rotation at 80 rpm for 30 minutes at 18°C. Cells treated with collagenase and trypsin formed cell aggregates, but cells treated with pronase did not. Most of the aggregates consisted of either ectodermal or endodermal cells, resulting segregation of cell-type specific aggregates. Repeated experiments yielded reproducible results, except for trypsin treated cells which sometimes failed to form aggregates.

These results indicate that Hydra cells have specific cell–cell adhesion systems for ectoderm–ectoderm and endoderm–endoderm adhesion, and that they are mediated by adhesion molecules which are resistant to collagenase, partially sensitive to trypsin and sensitive to pronase.

A project to identify and isolate these adhesion molecules is under way presently.

DNA Supercoiling Facilitates the Assembly of Transcriptionally Active Chromatin on the Adenovirus Major Late Promoter

Reiko OHBA, Hisahiro TABUCHI and Susumu HIROSE

Assembly of nucleosomes on the adenovirus major late promoter blocked initiation of transcription by RNA polymerase II. However, the formation of transcription preinitiation complexes prevented subsequent assembly of promoter sequences into nucleosomes and allowed transcription on the chromatin templates. When the formation of preinitiation complexes was in competition with nucleosome assembly, transcription on linear or relaxed closed circular DNA was inactivated by nucleosome assembly over the promoter region. However, transcription on partially supercoiled DNA (mean superhelical density of -0.036) remained active because the rapid formation of preinitiation complexes prevented subsequent assembly of promoter sequences into nucleosomes. For details, see *Biochem. Biophys. Res. Commun.* **186**, 963–969, 1992.

Embryonal Long Terminal Repeat-Binding Protein Is a Murine Homolog of FTZ-F1, a Member of the Steroid Receptor Superfamily

Toshio TSUKIYAMA*, Hitoshi UEDA, Susumu HIROSE and Ohtsura NIWA*

The embryonal long terminal repeat-binding protein, ELP, is present in undifferentiated mouse embryonal carcinoma cells. It binds to and suppresses transcription of the Moloney leukemia virus long terminal repeat in undifferentiated murine embryonal carcinoma cells. We found that ELP is a mouse homolog of *Drosophila* FTZ-F1, which positively regulates transcription of the *fushi tarazu* gene in blastoderm-stage embryos of the fly. As members of the steroid receptor superfamily, ELP and FTZ-F1 have both DNA binding and putative ligand binding domains which are well conserved between the two. ELP and FTZ-F1 function in the extremely early stage of development. A high degree of conservation between the two transcription factors during the evolution of these species indicates the importance of their functions in early-stage embryogenesis. In addition, the sequence elements they recognize do not contain repeat units, in contrast to other steroid

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receptors, which usually bind to either palindromic or direct repeat sequences. For details, see *Mol. Cell. Biol.* **12**, 1286–1291, 1992.

A Novel DNA-Binding Motif Abuts the Zinc Finger Domain of Insect Nuclear Hormone Receptor FTZ-F1 and Mouse Embryonal Long Terminal Repeat-Binding Protein

Hitoshi UEDA, Guan-Cheng SUN, Takehide MURATA and Susumu HIROSE

Fruit fly FTZ-F1, silkworm BmFTZ-F1, and mouse embryonal long terminal repeat-binding protein are members of the nuclear hormone receptor superfamily, which recognizes the same sequence, 5′PyCAAGG PyCPu-3′. Among these proteins, a 30-amino-acid basic region abutting the C-terminal end of the zinc finger motif, designated the FTZ-F1 box, is conserved. Gel mobility shift competition by various mutant peptides of the DNA-binding region revealed that the FTZ-F1 box as well as the zinc finger motif is involved in the high-affinity binding of FTZ-F1 to its target site. Using a gel mobility shift matrix competition assay, we demonstrated that the FTZ-F1 box governs the recognition of the first three bases, while the zinc finger region recognizes the remaining part of the binding sequence. We also demonstrated that the DNA-binding region of FTZ-F1 recognizes and binds to DNA as a monomer. The occurrence of the FTZ-F1 box sequence in other members of the nuclear hormone receptor superfamily raises the possibility that these receptors constitute a unique subfamily which binds to DNA as a monomer. For details, see *Mol. Cell. Biol.* **12**, 5667–5672, 1992.

Production of Antibodies Specific for Double Stranded Antigen DNA Cloned from Immune Complexes in Plasma of a SLE Patient

Kunihiko TERADA*, Susumu HIROSE and Eiji OKUHARA*

The antigenicity of antigen DNA isolated from immune complexes in the plasma of an SLE patient was examined. DDY mice were immunized with the cloned antigen DNA carrying a sequence homologous with a part of bacteriophage f1 (KS8 DNA) by the coupling method, and the antibody response was estimated by radioimmuno assay. Antibodies specific to double stranded DNA were elicited. Moreover, the antibodies showed preferential

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binding to KS8 DNA than other DNA derived from *Escherichia coli.* These results suggest that KS8 DNA has a significant antigenicity in mice. For details, see *Biochem. Biophys. Res. Commun.* **183**, 797–802, 1992

Permeability of Dechorionated Eggs and Developmental Anomalies in *Drosophila*

Kiyoshi MINATO

In previous experiments, the freshly laid and dechorionated eggs of *Drosophila melanogaster* developed abnormally when incubated in aqueous solutions such as distilled water and 0.9% NaCl solution, whereas they developed normally when incubated in a non-aqueous solution such as liquid paraffin. Because this phenomenon was not found when the incubated eggs were at more developed stages, it appeared to be due to the permeability of the vitelline membrane of the eggs to water or water-soluble substances, which is thought to remain for a while after the fertilization of eggs (Yoon and Fox 1965, Limboug and Zalokar 1973). When incubated in distilled water, most eggs swelled gradually without developing any structure, while, when incubated in 0.9% NaCl solution, most eggs showed, interestingly, blastoderm formation only in the posterior-half and resulting thereafter in embryos with an amorphous bag-like structure herniated at the anterior half.

This time, the limiting time or the time, incubated after which, eggs can develop normally was determined precisely. Consequently it proved to be about 30 to 60 minutes after fertilization (at 25°C) during which nuclei are still dividing actively in the inside embryos. This is much earlier than the time of two hours, roughly determined previously, the time at which nuclei had migrated to the surface of embryos to become syncythial blastoderm.

Control of Imaginal Cell Development by the *escargot* Gene of *Drosophila*

Shigeo HAYASHI, Susumu HIROSE, Tony METCALFE and Allan SHIRRAS

During the larval period, larval cells undergo multiple rounds of DNA replication without cell division and become giant polyploid cells. In contrast, imaginal cells remain diploid and retain the ability to divide. Mutations in the *escargot* (*esg*) locus cause a variety of defects in adult structures

derived from imaginal discs and histoblasts, such as loss of the abdominal cuticule and malformation of the wings and legs. The *esg* locus is more than 70 kb long and contains a small transcription unit of about 2.7 kb which potentially encodes a zinc finger protein. *esg* RNA is expressed in wing, haltare, leg and genital imaginal discs and in abdominal histoblast nests in the embryo. Expression in imaginal tissues is also found in third instar larvae. In *esg* mutant larvae, normally diploid abdominal histoblasts replicate DNA without cell division and become similar in appearance to the polyploid larval epidermal cells. *esg* mutations cause an endo-replication in abdominal histoblasts during the larval period, and an increase in nuclear size and DNA content. These apparently polyploid mutant histoblasts fail to proliferate during pupariation. This leads to the observed deletion in the adult abdomen. In contrast to the severe abdominal defects which are essentially due to deletion of histoblast-derived structures, the leg and wing defects in esg mutants are limited to malformation. This difference in sensitivity to *esg* mutations may reflect a different temporal pattern of cell division in imaginal discs and abdominal histoblasts. While imaginal disc cells are actively dividing during the larval stages, abdominal histoblasts is arrested in G2 interphase. The histoblasts enter mitosis only after pupariation under the influence of a change in hormonal environment. Cell cycle arrest of the abdominal histoblasts during the larval stage in addition to the reduction of *esg* function may be a necessary condition for becoming polyploid. A similar esg mutant phenotype was also found in some imaginal discs whose cell division was blocked by the *raf* mutation. These results suggest that one of the normal functions of *esg* is the maintenance of diploidy of imaginal cells. Details of this work will be published (Development, in press).

Role of the *escargot* Gene during *Drosophila* Development

Naoyuki FUSE, Susumu HIROSE and Shigeo HAYASHI

To further investigate the role of the *esg* gene during embryonic and imaginal development, we studied the effects of ectopic *esg* expression. Fly strains containing the *esg* gene linked to a heat shock gene promoter have been constructed. The strains transiently expressed abundant *esg* protein with heat shock. However the animals tolerated this treatment and developed to normal adults albeit with reduced fertility in some cases. As an alternative

method of ectopic expression, strains carrying the *esg* gene placed downstream of the binding site for a yeast transcriptional activator GAL4 were constructed. When these strains were crossed to a strain carrying the GAL 4 gene under the control of salivary gland specific enhancer, the *esg* gene was ectopically expressed in the salivary gland and great reduction of viability was observed. In such larvae, endoreplication of salivary gland cells was severely inhibited and the size of salivary gland cells was reduced. Taken together, these results suggest that, *Drosophila* can tolerate transient ectopic *esg* expression but continuous expression is inhibitory to larval growth. These results support our previous hypothesis that *esg* may play a positive role in distinguishing larval versus imaginal cell identity (S. Hayashi, S. Hirose, T. Metcalfe, A. Shirras, Development in press).

Regulation of *Drosophila* Neural Development by a Putative Secreted Protein

Hideyuki OKANO, Shigeo HAYASHI, Tei-ichi TANIMURA, Kazunobu SAWAMOTO, Shingo YOSHIKAWA, Jiro WATANABE, Masayuki IWASAKI, Susumu HIROSE, Katsuhiko MIKOSHIBA and Craig MONOTELL

Drosophila strawberry (*sty*) was isolated as a novel visual system mutant displaying a rough eye phenotype. Analyses of mutant phenotypes and the expression pattern of the gene suggested that the *sty* gene has pleiotropic functions. Mutations in the *sty* gene affect eye development, leading to irregular spacing of ommatidia, an increase in the number of photoreceptor cells, and abnormal axonal projection and the disruption optic lobe structure in the adult fly. In addition to affecting the visual system, they cause abnormal head involution, a change in the number of sensilla in the antennomaxillary complex in the embryonic stage, and abnormal morphogenesis of maxillary palp and wings in later stages. We examined the expression of the *sty* gene during development in terms of *lacZ* expression from enhancer trap elements inserted within the *sty* gene. During embryogenesis, expression of *lacZ* showed a segmental pattern in the ectoderm and in the nervous system. In the eye imaginal discs, *lacZ* began to be expressed in photoreceptor cells, a few rows posterior to the morphogenetic furrow. It was also expressed in the wing disc. In the adult, it was expressed in the retina and lamina. We cloned the *sty* gene by P-element tagging. On the basis of the phenotype of loss of function, its expression pattern, and the predicted structure of its

product, a secreted peptide with a putative epidermal growth factor (EGF) motif, it appears that *sty* encodes a diffusible protein with pleiotropic functions acting as a signal involved in lateral inhibition within the developing nervous system and also as a factor involved in axonal guidance. Details of this work have been published (Okano *et al.*, *Differentiation* **52**, 1–11, 1992).

Genetics of Fluoride-resistance of 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 interesting regulatory mechanisms, because it depletes Ca^{++}, inhibits phosphatases, and activates G-proteins. We are trying to discover and elucidate such regulatory mechanisms through the isolation and characterization of *C. elegans* mutants resistant to NaF.

We have isolated 13 recessive fluoride-resistant mutants that map in 5 new genes, *flr-1X*, *flr-2 V*, *flr-3 IV*, *flr-4 X* and *flr-5 V*. They are grouped into two categories, class 1 and class 2. Class 1 mutants (*flr-1*, *flr-3*, and *flr-4*) are resistant to 10 mM NaF, but they grow twice as slowly as wild type worms and have a small brood size even in the absence of NaF. In contrast, class 2 mutants (*flr-2* and *flr-5*) are not completely resistant to 10 mM NaF. 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. Namely, class 2 mutations are epistatic to class 1 mutations concerning growth rate and brood size, but hypostatic concerning the degree of fluoride-resistance. We interpret this relationship by assuming that the *flr* gene products form a metabolic, transport or signal transduction pathway that confers fluoride sensitivity on *C. elegans*. Class 1 and class 2 gene products form the lower and upper part of the pathway, respectively, and between the two parts there is an intermediate that delays the growth of *C. elegans* when accumulated. Mutations in class 1 genes cause fluoride-resistance by blocking the pathway. Moreover, they cause accumulation of the intermediate and decrease of the growth rate. Class 2 mutations prevent the accumulation of the intermediate and restore the growth rate. There is probably a narrow bypass to the intermediate, because class 2 mutants are only weakly resistant to fluoride

ion.

To understand the biochemical reactions involved in fluoride-sensitivity we cloned some of the *flr* genes by the transposon-tagging method. For the *flr-1* gene we first cloned a 2.8 kb BamHI genomic DNA fragment containing transposon Tc1 (1.6 kb) from a Tc1-insertion mutant *flr-1(ut11)* and then a 3.7 kb HindIII fragment from wild-type worms, using the DNA fragment flanking the Tc1 as the probe. For the *flr-3* gene we cloned a 3.6 kb EcoRI fragment containing Tc1 from a Tc1-insertion mutant *flr-3(ut9)*. In addition, we have isolated cDNA clones of *flr-1* and *flr-3* using their genomic DNA fragments as the probes. Sequencing of the genomic and cDNA clones is in progress.

Caenorhabditis elegans Larval Lethal Mutations That Cause Gross Morphological Changes

Isao KATSURA, Ryuichi HISHIDA and Takeshi ISHIHARA

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 (See the Annual Report of 1991 for details). 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 to 8 genes. Three of them map in known genes that are thought to act in signal transduction: *let-23* (receptor tyrosine kinase that acts in vulval induction), *let-341* (another gene that acts in vulval induction), and *clr-1* (lethal mutation in this gene is suppressed by mutations in *sem-5*, which codes for a growth factor receptor binding protein). We therefore expect that most of the *clr-1*-like mutations are related to signal transduction that acts in an unidentified function essential to larvae as well as in vulval induction or sex myoblast migration in some cases. Of the other seven mutants that we have isolated, two were sent to J. Kimble's laboratory (University of Wisconsin) and found to contain mutations deleting the *lag-2* gene, which acts in various cell-cell interactions. The remaining five mutants seem to define four new genes, which we are trying to clone. In addition to the mutants mentioned above we have isolated and are analyzing four new mutants with the *clr-1*-like

phenotype.

C. elegans Mutants Abnormal in Movement or Shape of the Head

Takeshi ISHIHARA and Isao KATSURA

In relation to the studies mentioned above we isolated seven *clr-1*-like mutants of low penetrance, i.e., mutants of which some worms die as larvae that have a space between the intestine and the body wall, while others grow to adulthood and multiply. The adult worms are abnormal in the shape of the head in two of the mutants, and in the movement of the head in four. These results are important in the following aspects: first it shows that the *clr-1*-like phenotype may be related to defects in formation of an unidentified organ/tissue in the head, and second it provides a method for selecting for mutants in head movement, which are difficult to recognize. We are now mapping those mutations and analyzing their phenotypes. We are also isolating mutants in head movement by direct inspection, using those mutants as positive controls.

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

Takeshi ISHIHARA and Isao KATSURA

In the head of *C. elegans*, there are circuits of many interneurons, which process information from the head sensory organs and control motor neurons. To elucidate the mechanisms of formation and function of the neural circuits, we are genetically analyzing the nervous system of the head in *C. elegans*. As a first step we are making markers for various nerve cells in the head using the "promoter trapping" method (Hope I., 1991, *Development* **113**, 399–408). Namely, we ligate random genomic DNA fragments of *C. elegans* to the upstream of a cloned *lacZ* gene, transfer the DNA constructs into *C. elegans* by microinjection and look for worms in which a defined set of nerve cells are stained by Xgal. We expect that such markers will be useful in analyzing structural changes of the neural system in various mutants such as those mentioned above and in isolating mutants abnormal in neural morphology.

Systematic Analysis of *C. elegans* cDNA

Yuji KOHARA, Hironobu MITSUKI, Akiko NISHIGAKI, Tomoko MOTOHASHI and Akiko SUGIMOTO

Aiming to ultimately understand the network of gene expression in development of the worm, we are trying to identify and characterize all of its cDNA species.

(1) cDNA libraries were made from mRNA of a mixed-stage population using λZAPII as a vector. The cDNA were size-fractionated on agarose gel before cloning. Firstly, 8,000 independent plaques were picked up from a library of cDNA longer than 1.5 Kb. Abundant cDNA clones were removed by hybridization screening to establish a set of about 4,600 less abundant or rare cDNA clones.

(2) cDNA inserts of individual clones were PCR-amplified directly from phage suspension using vector primers, purified on S-400 micro spin column of the 96 well format, analyzed for their size and yield on agarose gel, and subjected to the following analyses.

(a) Tag sequencing: by single pass sequencing from 5′-end (using a vector primer) and 3′-poly-A end (using anchored oligo (dT) primer) on a ABI auto-sequencer. The tags were subjected to DNA and protein database search. The 3′-tags, as ID sequences of individual genes, were also compared with each other for classification.

(b) Mapping on the genome: by hybridization to the YAC polytene filters using the DIG-based non-RI system.

(c) Analysis of expression pattern: by hybridization to the membrane strips on which all cDNA species from single embryos at various stages have been dot-blotted.

Thus far, about 900 clones have been processed for (a) and (b). Briefly, 4% of them matches *C. elegans* gene or cosmid sequences, 8% the Chris Martin's cDNA clones, and 23% shows significant similarity in the database search. To speed the tag sequencing, the clones will be shared by the genomic sequencing consortium. After assessing the extent of saturation with respect to cDNA species in the clone set, we are going to make a set of rarer clones.

Tc1 Insertional Mutant Bank of *C. elegans*

Yoshiki ANDACHI and Yuji KOHARA

In order to device efficient ways to disrupt any genes of interest, we have made a transposon Tc1 insertional mutant bank and have developed a strategy to retrieve mutant strains.

A mutator strain RW7097 (*mut-6*) gives visible mutants by Tc1 insertion of a given gene spontaneously at the frequency of $10^{-4}\sim10^{-5}$, meaning that one can expect to get one insertional mutant of a given gene out of $10^4\sim10^5$ independent worms of this strain. Thus, pools of the mutant strain RW7097, each containing about 100 larvae (L1s), were fed in 6 cm NGM-agarose plates to produce F1 progeny. Duplicate frozen stocks were made from a part of the F1 worms and genomic DNA were extracted from the another part. About 100 pools have been processed at one time in this way, and we have prepared 800 pools (altogether 80,000 independent worms) thus far.

Methods to retrieve insertional mutants are as follows. Aliquots of the DNA prepared from 12 individual pools (corresponding to 1200 independent worms) were mixed and subjected to PCR analysis using one primer from Tc-1 and another from a target gene. Since a lot of non-specific PCR products were generated in the reaction, we have developed the following nested PCR strategy: For the first PCR, a target-gene specific primer was biotinylated at the 5′-end to purify the product amplified from the target region by using streptavidin-conjugated magnetic beads. The product bound to the beads were subjected to second PCR using a set of nested primers and subjected to agarose gel electrophoresis. "Hot-start" of PCR by simply putting a reaction plate on the block of a PCR apparatus pre-heated at 94°C also turned out to contribute to reduction of the non-specific products. When a mix of 12 pools gave a positive band, the DNA from individual pools were analyzed as above to select a positive pool. From the frozen stock of the positive pool, about 200 worms were grown separately and the same PCR analysis as above was performed to finally identify a mutant worm.

Insertion mutants have been searched for several known genes to test the quality of the bank and insertion sites have been analyzed. Although the problem of false positive bands in our PCR assay are still open, we have successfully isolated at least one insertional mutant for *unc-22* gene out of 200 pools and each one for *unc-54* and *lin-12* genes out of 600 pools. Positions of the Tc1 insertion were determined by direct sequencing of positive PCR

bands obtained in the mutant screening. In the *unc-22* mutant, Tc1 turned out to be inserted in the fifth exon at 554 bp upstream the splice junction. This mutant showed a typical *unc-22* phenotype (twitching) and we have confirmed co-segregation between the insertion and appearance of the phenotype by crossing with wild type N2 strain. The *unc-54* mutant did not show any clear phenotype and we found that the insertion was in the first intron. As to the *lin-12* mutant, we have located the insertion in the ninth exon at 62 bp downstream the splice junction. Some defects of gonad and analysis of its phenotype is underway.

Search for Localized Maternal mRNA in Early Embryos of *C. elegans*

Hiroaki TABARA and Yuji KOHARA

A fertilized egg of *C. elegans* asymmetrically divides to give rise to the somatic founder cell AB and the germ line founder cell P1. The P1 blastomere divides 3 times in the stem cell fashion, producing 3 somatic founder cells EMS, C, D and the germ line precursor cell P4. Although the fates of the blastomeres are thought to be determined by maternal elements (cytoplasmic determinants) as well as cell–cell interaction, the substances of the cytoplasmic determinants are largely unknown. Candidates for the determinants could be some maternal mRNA and such determinants are highly expected to be asymmetrically segregated during early cleavage. Thus, we have began to search for localized mRNAs in early embryos.

Embryos were obtained by alkaline bleach treatment of adults and egg shells were lysed by chitinase. An embryo at 2 cell stage was picked up, and the blastomeres were separated physically using siliconized glass needles attached to a micro-manipulator. Nucleic acids were extracted from the individual blastomeres and 3′-proximal region of whole cDNA species were amplified by a method developed in this laboratory. The amplified cDNA were labeled and used for differential screening of a cDNA library made from early embryos (mostly earlier than the stage of gastrulation) using λZapII as a vector. As a first trial, we screened 5×10^4 plaques and obtained 8 positive clones. These clones were classified into three AB specific and one P1 specific clones. Analysis of these clones are in progress.

Analsis of Morphogenesis in Postimplantation 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. Analysis of molecular and cellular mechanisms of this period has advanced through the use of various experimental methods including whole embryo culture and culture of embryonic tissues and cells (Nakatsuji, N. (1992) *Devel. Growth Differ.* **34**, 489–499). In our studies, particular attention is paid to the formation of the mesodermal cell layer at the primitive streak, the appearance and migration of the primordial germ cells, and differentiation of the central nervous system.

In collaboration with another laboratory (Dr. I. Nagata at Tokyo Metropolitan Institute for Neuroscience), we are also 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 on an artificially fabricated microstructure on the quartz surface (Nagata, I. and Nakatsuji, N. (1993) *Development* **117**, 401–408). We are now analyzing the roles of the perpendicular contact guidance in the actual morphogenesis of brain cortices.

Manipulation of Embryogenesis Using Mammalian Embryonic Cells

Norio NAKATSUJI and Yasuaki SHIRAYOSHI

Isolation and manipulation of embryonic cells in culture and reincorporation of such manipulated cells into embryos would facilitate many experimental strategies in basic research on embryogenesis and also in more applied research in the medical and agricultural sciences.

We used embryonic stem (ES) cell lines established from mouse blastocysts for an analysis of embryogenesis. For example, we isolated a cell line which expresses β-galactosidase, and analyzed chimaeric tissues using these labeled cells. Also, we are trying to expand such strategy into other mammalian species (Kashiwazaki, N. *et al.* (1992) *Vet. Rec.* **130**, 186–187).

We have been studying the primordial germ cells (PGCs) which may be

used for manipulation of the mouse germ line. As a first step, we isolated germ cells and somatic cells from fetal ovaries (12.5–14.5 days post coitum), cultured them as dispersed cells, and made reaggregates. Such reaggregates (reconstituted fetal ovaries) were transplanted into ovary capsules of adult female mice. Then, they gave birth to offspring from transplanted germ cells (oogonia or oocytes) (Hashimoto, K. *et al.* (1992) *Devel. Growth Differ.* **34**, 233–238). We are now developing a new in vitro experimental system through the culture of PGCs for analysis of proliferation and differentiation of germ cells (Kawase, E. *et al.* (1993), *Devel. Biol.*, in press).

Molecular Analysis of Cell Differentiation in Postimplantation Mouse Embryos

Yasuaki SHIRAYOSHI and Norio NAKATSUJI

Determination of cell fate and cell differentiation are crucial events in early embryogenesis. In our laboratory, we have started experiments to approach such problems from their molecular aspects. One area of experimentation involves identifying important genes in 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 the neural plate and neural tube formation is initiated, and screened for important genes relating to CNS development.

V. POPULATION GENETICS

The Meaning of Natural Selection Revisited at the Molecular Level

Tomoko OHTA

Various molecular interaction mechanisms cause biased transmission of genes. Meiotic drive is an example of strong bias, and compartmentalization of mammalian chromosomes reflects weak bias. Such biases are the results of interaction between DNA and proteins, and should be distinguished from natural selection. Separating the effects of molecular interaction from those of natural selection, however, is often very difficult. Natural selection and molecular mechanisms interact, and our understanding of how selection works requires revision. For details, see *Trends in Ecology and Evolution* 7, 311–312.

Theoretical Study of Near Neutrality. II. Effect of Subdivided Population Structure With Local Extinction and Recolonization

Tomoko OHTA

There are several unsolved problems concerning the model of nearly neutral mutations. One is the interaction of subdivided population structure and weak selection that spatially fluctuates. The model of nearly neutral mutations whose selection coefficient spatially fluctuates has been studied by adopting the island model with periodic extinction-recolonization. Both the number of colonies and the migration rate play significant roles in determining mutants' behavior, and selection is ineffective when the extinction-recolonization is frequent with low migration rate. In summary, the number of mutant substitutions decreases and the polymorphism increases by increasing the total population size, and/or decreasing the extinction-recolonization rate. However, by increasing the total size of the population, the mutant substitution rate does not become as low when compared with that in panmictic populations, because of the extinction-recolonization, especially when the migration rate is limited. It is also found that the model satisfactorily explains the contrasting patterns of molecular polymorphisms observed

in sibling species of *Drosophila*, including heterozygosity, proportion of polymorphism and fixation index. For details, see *Genetics* **130**, 917–923.

Simulation Study of a Multigene Family, With Special Reference to the Evolution of Compensatory Advantageous Mutations

Christopher J. BASTEN and Tomoko OHTA

We investigate the evolution of a multigene family incorporating the forces of drift, mutation, gene conversion, unequal crossing over and selection. The use of simulation studies is required due to the complexity of the model. Selection is modeled in two modes: positive selection as a function of the number of different beneficial alleles and negative selection against deleterious alleles. We assume that gene conversion is unbiased, and that all mutations are initially deleterious. Compensation between mutants creates beneficial and neutral alleles, and allowances are made for compensatory mutations either within or between the members of a multigene family. We find that gene conversion can enhance the rate of acquisition of compensatory advantageous mutations when genes are redundant. For details, see *Genetics* **132**, 247–252.

Gene Conversion Generates Hypervariability at the Variable Regions of Kallikreins and Their Inhibitors

Tomoko OHTA and Christopher J. BASTEN

The two mechanisms for generating hypervariability at the reactive center of serine proteases and their inhibitors are gene conversion followed by natural selection and natural selection for point mutation. One way to clarify the effects of these two mechanisms is to calculate separately the number of nonsynonymous substitutions and that of synonymous substitutions at the variable regions and at the conserved regions. Our data analysis shows that not only the number of nonsynonymous substitutions but also the number of synonymous substitutions at the variable regions exceed the corresponding numbers at the conserved regions. Thus gene conversion has provided needed variability at the variable regions of serine proteases and their inhibitors. Natural selection has helped perpetuate such variability. For details, see *Molecular Phylogenetics and Evolution* **1**, 87–90.

Statistical Method for Estimating the Effective Population Size in Pacific Salmon

Fumio TAJIMA

The effective population size is one of the most important parameters that determine the genetic variation in a population. Recently, a statistical method for estimating the effective breeding size of Pacific salmon (*Oncorhynchus* spp.) which have unusual life history features, i.e., semelparity with overlapping year classes, has been developed (Waples (1990) *J. Hered.* **81**, 277–289). In this method, however, computer simulations are required. I have developed a simple algorithm for estimating the effective population size without computer simulation. This algorithm can be applied as long as the distribution of age at which a spawner returns is known. For details, see *J. Hered.* **83**, 309–311.

Statistical Method for Estimating the Standard Errors of Branch Lengths in a Phylogenetic Tree Reconstructed without Assuming Equal Rates of Nucleotide Substitution among Different Lineages

Fumio TAJIMA

A statistical method was developed for estimating the standard errors of branch lengths in a phylogenetic tree reconstructed without assuming equal rates of nucleotide substitution among different lineages. This method can be easily used for testing whether the length of an interior branch in a reconstructed tree is positive or not, i.e., whether the topology of the tree is correct or not. Computer simulations indicated that this method is appropriate for a statistical test. As an example, this method was applied to phylogenetic trees reconstructed for the four hominoid species: human, chimpanzee, gorilla, and orangutan. The results obtained show that the present method provides a powerful statistical test. For details, see *Mol. Biol. Evol.* **9**, 168–181.

Evolutionary Genetics of Human Paleo-populations

Naoyuki TAKAHATA

Genetic variation revealed at the DNA level provides useful information about not only the evolutionary mechanisms involved but also the history of

organisms. As to the former, we have learned, at least in principle, how and what kind of genetic variation is generated at the molecular level and how it is shaped by the mechanisms acting at the population level. An important task is to clarify outcomes of simultaneously interacting molecular and population mechanisms and to assess their relative importance in actual evolutionary processes. Another exciting finding emerging in current evolutionary biology is the possibility that genetic variation, together with appropriate theories, may shed new light on the population history of organisms and population dynamics including speciation processes. In this pursuit, it is important to delimit the time scale in which genetic variants have persisted and therefore are useful as a document of population histories. Equally important, to interpret these data, is development of appropriate theories. Focussing on these two points, I present some theoretical results and apply them to the major histocompatibility complex loci in humans. I argue that the human lineage has never experienced severe bottlenecks and that the extent of panmixia of the population might have been low before humans migrated out of Africa but later became high. The implication is related to the origin of modern humans, subsequent racial differentiation and the founder principle, a popular mechanism often invoked in speciation. See Population Paleo-Genetics, pp. 1–21, N. Takahata (ed.), Japan Scientific Society Press, 1992 for details.

Some Comments on Calibration of Molecular Evolutionary Rates

Naoyuki TAKAHATA and Yoko SATTA

Molecular evolutionary studies rely much on the existence of a molecular clock, which allows one to date molecular events on the geological timescale. Any method of calibrating the rate of molecular evolution begins with aligning two homologous DNA sequences that have been separated for a known time period (t) and counting the number of nucleotide differences (D) in a specified region. The proportion of nucleotide differences per site p is obtained by dividing D by the total number (n) of nucleotide sites compared. The value of p is used to estimate the actual number of substitutions (K) that have occurred in both sequences during t and the evolutionary rate which is given by $K/(2t)$. This final step requires a stochastic model which describes the total number of substitutions per site per unit time and substitution

patterns among the four different nucleotides. Two basic assumptions are commonly made. First, the sites in a specified region of DNA sequences change stochastically according to a common rule and the change follows the Poisson process. These assumptions imply that there is no heterogeneity in intrinsic substitution rates within the region and that accumulation of nucleotide changes can be described by a stationary Markov process. Elsewhere, Takahata questioned the validity of the second assumption for nucleotide substitutions in the antigen recognition site (ARS) of the major histocompatibility complex (*Mhc*) genes. In this communication, we focus on the first assumption, although our discussion is intimately related to the second. The same problem was discussed in relation to amino acid sequence data, but it seems more important for DNA sequence data because the number of possible states treated in a model is only four rather than 20. See *Immunogenetics* **36**, 126–129, 1992 for details.

Trans-species Polymorphism of the Major Histocompatibility Complex (*Mhc*) Loci

Naoyuki TAKAHATA, Yoko SATTA and Jan KLEIN

The functional major histocompatibility complex (*Mhc*) genes are highly polymorphic. The *Mhc* polymorphism is unusual in many respects, among which the trans-species mode of evolution is the most remarkable. It has convincingly been shown that a large number of allelic lineages at functional *Mhc* loci have persisted for tens of millions of years in various lines of species that include humans. Because of the long persistence time, some human *Mhc* (*HLA*) allelic lineages are shared not only be African apes, but also by Old World monkeys, and the divergence of alleles often predates species divergences to a great extent. This finding has raised many challenging problems for evolutionary biologists. In this paper we present our analysis of the nucleotide substitution rates as well as the mode and intensity of natural selection occurring at the primate functional *Mhc* genes. The implication of the *HLA* polymorphism for human evolution is discussed. See Progress in Immunology VIII (Proceedings of the 8th International Congress of Immunology Budapest), pp. 153–158, J. Gergely *et al.* (eds.), Springer Hungarica, 1992 for details.

Polymorphism and Balancing Selection at Major Histocompatibility Complex Loci

Naoyuki TAKAHATA, Yoko SATTA and Jan KLEIN

Amino acid replacements in the peptide-binding region (PBR) of the functional major histocompatibility complex (*Mhc*) genes appear to be driven by balancing selection. Of the various types of balancing selection, we have examined a model equivalent to overdominance that confers heterozygote advantage. As discussed by A. Robertson, overdominance selection tends to maintain alleles that have more or less the same degree of heterozygote advantage. Because of this symmetry, the model makes various testable predictions about the genealogical relationships among different alleles and provides ways of analyzing DNA sequences of *Mhc* alleles. In this paper, we analyze DNA sequences of 85 alleles at the *HLA-A*, *-B*, *-C*, *-DRB1* and *-DQB1* loci with respect to the number of alleles and extent of nucleotide differences at the PBR, as well as at the synonymous (presumably neutral) sites. Theory suggests that the number of alleles that differ at the sites targeted by selection (presumably the nonsynonymous sites in the PBR) should be equal to the mean number of nucleotide substitutions among pairs of alleles. We also demonstrate that the nucleotide substitution rate at the targeted sites relative to that of neutral sites may be much larger than 1. The predictions of the presented model are in surprisingly good agreement with the actual data and thus provide means for inferring certain population parameters. For overdominance selection in a finite population at equilibrium, the product of selection intensity (s) against homozygotes and the effective population size (N) is estimated to be 350–3000, being largest at the *B* locus and smallest at the C locus. We argue that N is of the order of 10^5 and s is several percent at most, if the mutation rate per site per generation is 10^{-8}. See *Genetics* **130**, 925–938, 1992 for details.

Evolution of the Mouse *t* Haplotype: Recent and Worldwide Introgression to *Mus Musculus*

Takashi MORITA, Hiroshi KUBOTA, Keiko MURATA, Masami NOZAKI, Christiane DELARBRE, Keith WILLISON, Yoko SATTA, Mitsuru SAKAIZUMI, Naoyuki TAKAHATA, Gabriel GACHELIN and Aizo MATSUSHIRO

Mouse *t* haplotypes are variants of chromosome 17, consisting of four inversions. Despite the homozygous lethality and pleiotropic effect on embryonic development, sperm production, and recombination, they have widely spread in natural populations of the house mouse (10–40% in frequency) because of the meiotic drive advantage. We sequenced 14 *Tcp*-1 (*t*-complex polypeptide 1) genes from four *t* haplotypes, nine wild mice, and a rat as a reference. From a comparison of intron sequences of 610 base pairs, we dated the origin of *t* haplotypes to 2.9 ± 0.7 million years ago, which predates the splitting of *Mus musculus* subspecies (≈ 1 million years ago). However, the *Tcp*-1 intron sequences of *t* haplotypes from different *M. musculus* subspecies from various parts of the world show no divergence, indicating the recent introgression (no earlier than 0.8 million years ago) of a single ancestral type. Nucleotide changes in coding regions are also consistent with this conclusion. Hence, polymorphisms among *t* haplotypes including lethality factors have accumulated during this short time period independently in each *M. musculus* subspecies. See *Proc. Natl. Acad. Sci. USA.* **89**, 6851–6855, 1992 for details.

Evolution of the Primate DRB Region

Jan KLEIN, Yoko SATTA, Colm O'hUIGIN, Werner E. MAYER and Naoyuki TAKAHATA

There are eight DRB loci in the human population, DRB1 through DRB8, of which four at the most are present on any one chromosome; some human chromosomes carry only one, two, or three DRB loci. We discuss the evolutionary origin of the individual loci, taking into account all the available information on the DRB region not only of humans, but of other primates as well. This information includes exon 2 sequences of over 200 genes, entire coding sequences of some 60 genes, exon and intron sequences of 10 genes and pseudogenes, sequences of 11 Alu repeats inserted into DRB (pseudo)

genes, and the worked-out organization of five different human, two gorilla, one chimpanzee, and one orang-utan DRB haplotypes. Our discussion is based on the consideration of evolutionary trees constructed by both distance and maximum parsimony methods from nucleotide sequence data, of diagnostic substitutions (i.e. nucleotide or amino acid substitutions characterizing a particular group of genes and absent in all or almost all other genes), of the presence or absence of particular inserts in the genes, and of the overall organization of the DRB haplotypes. See HLA 1991, pp. 45–56, T. Kimiyoshi, M. Aizawa and T. Sasazuki (ed.), Oxford University Press, Oxford, 1992 for details.

VI. EVOLUTIONARY GENETICS

Long-range G+C% Mosaic Structures of the Human Genome and a Border of the Mosaic Domains; New Genes Found in the MHC Class III Region

SUGAYA, K.*, WADA, K.**, MATSUMOTO, K. and IKEMURA, T.

The genome of warm-blooded vertebrate is known to be a mosaic of compartments differing in G+C content, designated "isochores", which seemed to relate to staining chromosomal bands. One approach to investigating the evolutional process in producing the mosaic structure and understanding its biological significance, is thought to be in the characterization of the border of the mosaic domains on the sequence level. After surveying human gene sequences in DNA databases, which were linked within several hundred kb, two possible borders of long-range G+C% mosaics were previously assigned; one between X-linked G6PD and F8C, and the other between HLA-DRA (in MHC Class 2) and CYP21 (in MHC Class 3) on 6p21.3. Recently we obtained cosmids and YAC contiguous clones, which completely cover the latter border in the MHC locus. Near (or at) the border, a characteristic genome portion (at least 50 kb) that is mainly composed of Alu repetitive sequences was found. In its class III-side neighboring region, we found four new genes, Notch-like gene (homolog of mouse mammary tumor int-3 gene), PBX2 gene (a homeobox gene), RAGE (receptor for advanced glycosylation end products of proteins) gene, and extracellular matrix tenascin-like gene, in order from centromere to telomere. For details, see "*HLA 1991*" (Tsuji *et al.*, eds.), Oxford Univ. Press, 2, 125–128 and 185–190., and *Immunogenetics*, 36, 400–404, 1992.

Analyses on Codon Usage

T. IKEMURA

The cellular tRNA content of *E. coli*, *Salmonella typhimurium*, and *Saccharomyces cerevisiae* was measured about ten years ago by our group, and

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organism-specific codon choice patterns of these microorganisms were attributed to the availability of tRNA isoacceptors within a cell. In those studies, the following four deductions were made, and this year, based on a vast amout of codon usage data accumulated after publication, these were reexamined and confirmed.

1) There is a close correlation between tRNA content and codon usage in most protein genes sequenced for these organisms. The exceptional cases are largely confined to genes with low expression. 2) There are clear similarities in codon choice among different genes of one microorganism, regardless of gene function. We called this organism specific codon-choice the "codon dialect" of the organism. The codon dialect is related to the specific tRNA isoacceptor population of an organism. 3) The extent of bias in codon choice is related to the protein production level of each gene. Codon usage in genes for abundant protein molecules is always more dependent on tRNA content than that in moderately or poorly expressed genes. 4) Foreign-type genes such as those of transposons, plasmids, and viruses often have quite different codon patterns from those of host organisms, and thus the above deductions are not necessarily applicable to them.

Regarding codon-choice patterns, the distinction between unicellular microorganisms and multicellular higher eukaryotes, rather than the distinction between prokaryotes and eukaryotes,was emphasized. In unicellular organisms, most, if not all, genes are expressed in individual cells. To maintain efficiency of translation processes (e.g., to save GTP energy used in the proof-reading process), these genes have an analogous codon-choice pattern (codon dialect) that fits the tRNA population. In the case of higher eukaryotes, each body is composed of an enormous number of cells. Many of the cells are highly differentiated, and a restricted spectrum of genes is expressed in individual cells. The target of Darwinian selection is a whole body instead of individual cells. Thus, fitness change caused by a synonymous change in an ordinary gene should be much smaller than that for unicellular organisms. A synonymous change to a codon with higher tRNA availability may save a certain amount of GTP energy and effective working time of ribosomes in a restricted portion of cells. However, the contribution of this change in overall fitness of a body should be extremely small, so it would presumably be counted as a neutral mutation. This contrasts with the case of unicellular organisms where the cell itself is the direct target of selection. Codon usage in ordinary genes of multicellular organisms is

therefore thought to be less stringently constrained by tRNA availability than that in genes of unicellular organisms. When some constraints from factors other than translation efficiency exist (e.g., long-range G+C% mosaic structures of the genome), their codon choices presumably follow the constraints. The evident diversity of the third codon G+C% and correlation of this diversity with the G+C% mosaic structures may reflect the weaker constraint imposed by tRNA content. For details, see CODON USAGE, in "*Plant Molecular Biology LABFAX*" (Broun, T. A., ed.), Blacwell Scientific Publications, in press, and CORRELATION BETWEEN CODON USAGE AND tRNA CONTENT IN MICROORGANISMS, in "*Transfer RNA in Protein Synthesis*" (Hatfield, D. L. *et al.*, ed.) CRC Press (1992).

Evolutionary Rate of Insertions and Deletions in Nucleotide Sequences

Naruya SAITOU

Gaps created through alignment are routinely eliminated when we compare nucleotide sequences, and nucleotide substitutions have been extensively studied. However, insertions and deletions that are responsible for gaps in aligned sequences seem to occur rather frequently, especially in non-coding regions. We studied the rate of insertions and deletions in pseudogene sequences by using (1) Tajima and Nei's evolutionary distance due to insertions and deletions and (2) the maximum parsimony principle to locate insertion/deletion events on a phylogenetic tree. The rate of insertion/deletion events was found to be rather constant. For details, see *Molecular Paleo-Population Biology: Proceedings of the Seventeenth Taniguchi Symposium* (N. Takahata ed.), pp. 335–343, Japan Science Society Press, Tokyo, 1992.

Concerted Evolution of the Primate Immunoglobulin Alpha Gene through Gene Conversion

Shohji KAWAMURA*, Naruya SAITOU and Shintaroh UEDA*

We determined four nucleotide sequences of the hominoid immunoglobulin alpha (Cα) genes (chimpanzee Cα2, gorilla Cα2, and gibbon Cα1 and Cα2 genes), and these new data made it possible to examine gene

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conversions of all the hominoid Cα genes. The following three methods were used to detect gene conversions: 1) phenetic tree construction, 2) detection of the DNA segment which has extremely low variability between duplicated Cα genes under the assumption of random distribution of varied and unvaried sites, and 3) site-by-site search of shared nucleotide changes between duplicated Cα genes by comparing sequences among species on the basis of a parsimony principle. Results by using method 1 suggest the occurrence of gene conversion at least in the human, chimpanzee, and gorilla lineages, while those by using method 2 do in the human, gorilla, and gibbon Cα genes. Lack of a long segment of unvaried sites between the chimpanzee Cα1 and Cα2 genes might be because gene conversions were so old that mutations had accumulated, or because detectably long conversions did not occur. By using method 3 we identified clusters of shared nucleotide changes between duplicated Cα genes in any of human, chimpanzee, gorilla, gibbon, and their hypothetical ancestors. These results indicate that gene conversions occurred rather frequently between duplicated Cα genes during hominoid evolution. For details, see *J. Biol. Chem.* **267**, 7359–7367, 1992.

Genetic Affinities of Human Populations

Naruya SAITOU, Katsushi TOKUNAGA* and Keiichi OMOTO*

Differentiation of human populations does not necessarily follow a simple model of population fission, and gene migrations may occur after a relatively long isolation. Thus for describing genetic relationships among populations, a network (unrooted tree) of genetic affinity seems to be more appropriate than a dendrogram (rooted tree), which is expected to estimate the phylogenetic tree of populations. The neighbor-joining method, in which the principle of minimum evolution is used for constructing networks, may be suitable for this purpose. We applied this method to three sets of genetic distance data and compared the resulting affinity networks with those obtained by other methods. It was found that the genetic affinity networks generally reflect the geographical isolation and migration of human populations. For details, see *Society for the Study of Human Biology Symposium Series 33: Isolation, Migration, and Health* (R. F. Roberts, N. Fujiki and K. Torizuka eds.), pp. 118–129, Cambridge University Press, Cambridge, 1992.

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Construction of the MHC Database and its Utilization to Anthropological Studies

Takashi GOJOBORI

With help of many researchers, we successfully compiled all the data that were sent to the Central Data Analysis Committee by the participants of the Eleventh International Histocompatibility Workshop. The data compiled were stored into the relational database called "MHC database." This database was created by use of a commercial relational database management software (RDBMS). Specifically, we used Informix-SQL version 2.10. This database contains the HLA typing data for a total of 27,765 individuals sampled from various ethnic groups in the world.

Using the MHC database, anthropological studies were performed by obtaining the following data: (1) frequencies of HLA antigens for each ethnic group, (2) haplotype frequencies and linkage disequilibria among the HLA genes in each ethnic group, (3) genetic distances among the various ethnic groups, (4) a phylogenetic tree based on the genetic distances, and (5) geographical distributions of each HLA allele. From these data, we evaluated the degrees of genetic variation at the HLA loci for each ethnic group. For details, see HLA 1991 (Oxford University Press) 1: 65, 66–67, 67–76, 623–626, and 627–632.

Rates of Synonymous Substitution and Base Composition for Nuclear Genes in Drosophila

Etsuko N. MORIYAMA and Takashi GOJOBORI

We compared the rates of synonymous (silent) substitution among various genes in a number of species of Drosophila. First, we found that even for a particular gene, the rate of synonymous substitution varied considerably with Drosophila lineages. Second, we showed a large amount of variation in synonymous substitution rates among nuclear genes in Drosophila. These rates of synonymous substitution were correlated negatively with C content and positively with A content at the third codon positions. Nucleotide sequences were also compared between pseudogenes and their functional homologs. The C content of the pseudogenes was lower than that of the functional genes and the A content of the former was higher than that of the

latter. Because the synonymous substitution for functional genes and the nucleotide substitution for pseudogenes are exempted from any selective constraint at the protein level, these observations could be explained by a biased pattern of mutation in the Drosophila nuclear genome. Such a bias in the mutation pattern may affect the molecular clock (local clock) of each nuclear gene of each species. Finally, we obtained the average rates of synonymous substitution for three gene groups in Drosophila; 11.0×10^{-9}, 17.5×10^{-9} and 27.1×10^{-9}/site/year.

For details, see Genetics 130: 855–864.

Patterns of Nucleotide Substitution Inferred from the Phylogenies of the Class I Major Histocompatibility Complex Genes

Tadashi IMANISHI and Takashi GOJOBORI

Patterns of nucleotide substitutions in human major histocompatibility complex (MHC) class I genes were estimated by using phylogenetic tress in the form of a set of 12 parameters, each of which represents the relative frequency of substitutions from a particular nucleotide to another. The pattern at the antigen recognition sites (ARS) in functional MHC genes was remarkably different from that at the remaining coding region (non-ARS). In particular, the proportion of transitions (P_s) among all the nucleotide substitutions was extremely low at the third codon positions of ARS. In the HLA-A genes, P_s at the third codon positions was only 6% in ARS, whereas it was 69% in non-ARS. In HLA-B, the corresponding values were 30% in ARS and 80% in non-ARS, respectively. On the other hand, P_s in a class I pseudogene (HLA-H) was 57%, which was in good agreement with P_s in other pseudogenes. Because pseudogenes are selectively neutral, the pattern in pseudogenes is regarded as the pattern of spontaneous substitution mutations. In general, the pattern in functional genes that are subject to selective forces deviates from the pattern in pseudogenes. At the third codon positions in coding regions, transitions scarcely cause amino acid replacements, whereas about half of transversions do cause replacements. Accordingly, P_s at the third codon positions decreases if amino acid replacements are accelerated by natural selection but increases if amino acids are conserved by functional constraint. Our observations imply that the ARS region is subject to natural selection favoring amino acid replacement, whereas the non-ARS region is

subject to functional constraint.

For details, see J. Mol. Evol., 35 : 196–204 and HLA 1991 (Oxford University Press), 1: 357–359.

Evolutionary Origin of a Kunitz-Type Trypsin Inhibitor Domain Inserted in the Amyloid β Precursor Protein of Alzheimer's Disease

Kazuho IKEO, Kei TAKAHASHI, and Takashi GOJOBORI

The Kunitz-type protease inhibitor is one of the serine protease inhibitors. It is found in blood, saliva, and all tissues in mammals. Recently, a Kunitz-type sequence was found in the protein sequence of the amyloid β precursor protein (βAAP). It is known that βAAP accumulates in the neuritic plaques and cerebrovascular deposits of patients with Alzheimer's disease. Collagen type VI in chicken also has an insertion of a Kunitz-type sequence. To elucidate the evolutionary origin of these insertion sequences, we constructed a phylogenetic tree by use of all the available sequences of Kunitz-type inhibitors. The tree shows that the ancestral gene of the Kunitz-type inhibitor appeared about 500 million years ago. Thereafter, this gene duplicated itself many times, and some of the duplicates were inserted into other protein-coding genes. During this process, the Kunitz-type sequence in the present βAPP gene diverged from its ancestral gene about 270 million years ago and was inserted into the gene soon after duplication. Although the function of the insertion sequences is unknown, our molecular evolutionary analysis shows that these insertion sequences in βAAP have an evolutionarily close relationship with the inter-α-trypsin inhibitor or trypstatin, which inhibits the activity of tryptase, a novel membranebound serine protease in human T4+ lymphocytes.

For details, see J. Mol. Evol. 34: 536–543.

Allele and Haplotype Frequencies for HLA and Complement Loci in Various Ethnic Groups

Tadashi IMANISHI, Tatsuya AKAZA, Akinori KIMURA,
Katsushi TOKUNAGA, and Takashi GOJOBORI

Frequencies of alleles and haplotypes for HLA and complement loci were estimated from the typing data in the MHC database compiled at the

Eleventh International Histocompatibility Workshop. All the frequencies were estimated by the maximum likelihood method assuming the Hardy-Weinberg law. We used the data only for the ethnic groups whose number of sampled individuals exceeded 50. As the results, we found that there were vast amounts of heterogeneities not only within a single ethnic group but also among different ethnic groups. This confirms that the HLA loci are genetically very heterogeneous.

We also computed the values of linkage disequilibria for two, three, four, and five loci. Our results show that some loci are strongly linked.

For details, see HLA 1991 (Oxford University Press) 1: 76–79 and 1065–1220.

Six Strains of Human Immunodeficiency Virus Type-1 Isolated in Japan and their Molecular Phylogeny

Nobuaki SHIMIZU, Yasuhito TAKEUCHI, Takaji NARUSE, Minoru INAGAKI, Etsuko N. MORIYAMA, Takashi GOJOBORI, and Hiroo HOSHINO

Five strains of human immunodeficiency virus type 1 (HIV-1) were isolated from five Japanese hemophilia patients. Two isolates, HIV-1 [GUN-1] and HIV-1 [GUN-2], were from brother patients with hemophilia B. The other three isolates, HIV-1 [GUN-3], HIV-1 [GUN-4], and HIV-1 [GUN-5], were from homophilia A patients. Another HIV-1 strain, HIV-1 [GUN-6], was isolated from a Canadian male homosexual with AIDS. The restriction endonuclease cleavage maps of the proviral genomes of these six HIV-1 strains revealed that they were apparently different from each other. The phylogenetic trees constructed using restriction maps and nucleotide sequences were quite similar, indicating that phylogenetic analyses of Japanese HIV-1 isolates can be done using restriction maps of the proviruses. Phylogenetic analyses showed that they were more closely related to HIV-1s which had been reported to be isolated from homosexual patients in the United States than those isolated from African patients. In particular, GUN-1 and GUN-2 isolates were on the branch of a San Francisco isolate, ARV2, while GUN-5 and GUN-6 isolates were on the branch of HTLV-IIIB-related isolates from New York.

For details, see J. Mol. Evol. 35: 329–336.

Complement Studies of the Eleventh International Histocompatibility Workshop

Katsushi TOKUNAGA, Hidenori TANAKA, Gottfried MAUFF, Koichi SUZUKI, Hiroaki NISHIMUKAI, Georges HAUPTMANN, Julie FINLAY, Tadashi IMANISHI, Takashi GOJOBORI, Tatsuya AKAZA, Takeo JUJI, and Roger L. DAWKINS

In this study, we had three main purposes: (1) to see the associations between complement and HLA alleles in various ethnic groups, (2) to identify common MHC haplotypes in different ethnic groups, and (3) to examine the association of complement alleles with certain diseases. In order to achieve these purposes, we used complement data from as many individuals as possible.

We successfully identified a number of 'new' variants when a large number of samples from various ethnic groups were examined. In particular, two new and until-now-not-described variants of C2 were isolated from European Caucasoids, and at least 10 new variants of C4 were observed in various ethnic groups.

A total of 51 ethnic groups with approximately 4500 individuals were examined for the complement allotypes. All gene frequencies of Bf, C4A, and C4B, and haplotype frequencies of Bf-C4A-C4B, were estimated for each ethnic group. Five-locus complement-HLA haplotype frequencies with HLA-A and HLA-B alleles or with HLA-B and HLA-DR alleles were also calculated. We found that there were apparently two major types of MHC haplotype patterns. First, many of MHC haplotypes showed a rather restricted distribution of geography. Thus, these multilocus haplotypes should be excellent ancestral markers for the characterization of various human populations. Second, some MHC haplotypes showed a very wide distribution beyond ethnic boundaries. At present, we are still examining the association of complement alleles with certain diseases.

For details, see HLA 1991 (Oxford University Press) 1: 947–954.

VII. HUMAN GENETICS

A Simple Method for Generating Sequence Tagged Sites Adjacent to the Human Telomere Associated Repeat Sequence

Takashi IMAMURA, Hitoshi NAKASHIMA, Masako SAKAI and Rie INABA

Tandemly repetitive DNA sequences are thought to have the potential to express the locus-specific organization of their folding structure in the nucleus. Because of the inherent possibilities of rapid evolutionary sequence alterations, including a tolerance for mutation events, repetitive sequence units are predestined to develop and balance locus-specific repetitve higher order structures. This potential may create a specific chromatin folding structure whenever there is selection force at the position of this repetitive DNA sequence in the genome. Human tandemly repetitive sequences are of interest for two reasons. Firstly, they define the boundaries or the signposts of the physical and genetic maps of human chromosomes and thus are particularly important in the mapping of the human genome. Secondly, some of them are essential for human chromosome function.

This study attempted to explore the principal organization of human telomere-associated (or proterminal) DNA at 48 distinct telomeres. We devised a conventional ligation mediated PCR protocol employing magnetic bead- immobilized primer to create relatively short restriction enzyme fragments from human telomere-associated regions. The general scheme to producing proterminal sequences involves, as a first step, PCR with one telomere repeat primer biotinylated. Genomic DNAs were restriction enzyme digested and were ligated to a unidirectional linker primer, or a pUC19 polylinker site which was cleaved and dephosphorylated. The PCR amplified products were immobilized on streptavidin coated magnetic beads, which were substrates for the second round PCR. Using three different kinds of enzymes, the 48 telomere-associated regions have been cloned. The sequence studies will yield new STS markers. The primer extension technique provides a powerful tool for preferentially cloning sequences adjacent to blocks of the telomeric repeat. The cloning strategy is dependent on a given restriction site present within reach of the PCR amplification. The small fragment sizes of the insert might contribute to their stability in the plasmid

vector and facilitate their rapid sequencing. This way long, the specific telomeric sequence can be coupled to a solid matrix that might be useful in affinity isolation of overlapping sequences for walking towards centromere. The cloning of the subtelomeric sequences devoid of the terminal repeats also facilitates molecular analysis of specific chromosomal localization by hybridization *in situ* to metaphase chromosomes. This work is in progress.

The preliminary sequencing results suggest that subtelomeric regions are organized as a string of distinct short sequence elements several kilobases throughout their length, comprising several multiple, different, but related tandem motifs. The chromosomal distribution of these sequences could be highly polymorphic, which might constitute an apparently new repeat sequence family. The data thus identify a new source of human genetic variation on the human chromosome ends.

Mapping of 60 Cosmid Clones Isolated from a Human 18 Chromosome Library by Fluorescent *in situ* Hybridization

Hitoshi NAKASHIMA, Masako SAKAI, Rie INABA and Takashi IMAMURA

Sixty cosmids have been mapped to metaphase chromosomes by fluorescent *in situ* hybridization under conditions that suppress signals from repetitive DNA sequences. The cosmid clones were isolated from a human chromosome 18 specific library prepared from the several human-mouse hybrid cell lines, each containing, as the only human chromosome component, either one whole chromosome 18 or its short arm and the centromere portion. The long arm of chromosome 18 appeared to be deleted entirely in the latter cells. These hybrid cell lines were established by fusion of the human-mouse hybrid cells (NA11010) that retained human chromosomes 9 and 18 (ATCC, Rockville, MD) with the rodent cells (THY-M), kindly provided by Dr. S. Kaneda, National Institute of Genetics, which were selected in culture by the thymidylate synthetase activity. The human chromosome 18-specific hybrid cell lines, 126-16 and 126-21, etc. were finally established by these procedures in this laboratory. The cosmid library, prepared using pSuperCos1 vector (Strategene, Inc., La Jolla, CA), was plated at low density on LB agar plates containing ampicillin and grown overnight. Single colonies were picked up and inoculated individually in 96-well microtiter plates containing LB/amp medium. Colonies from 16 each of the 96-well microtiter plate were gridded

onto a nylon membrane using a 96-well replicator. These membranes were hybridized with total human DNA radiolabeled by nick-translation. Colony selection was performed in duplicate and only those colonies that gave intense autoradiographic signals were grown overnight. Pure cosmid DNA were prepared by plasmid SELECT-MP Miniprep DNA purification system (3′–5′, Inc., Boulder, CO). Cosmid contaning large inserts (30–45 kb) were identified by restiriction digestion analysis.

To map single-copy genes in the human genome we have used fluorescent *in situ* hybridization (FISH) of nonisotopically labeled probes to human metaphase chromosomes by a modification of a published procedure (Pinkel, 1986). The fluorescent images were recorded using the CCD camera and microscopic image prosessing system (Astromed Ltd., UK). Of the 100 cosmids thus far analysed, 60 were mapped on the specific chromosome band region of chromosome 18, which include both telomeric and pericentromeric regions of the short and long arms. None of the cosmids was mapped fortuitously to the other autosomes, suggesting that the library contained human DNA fragments only from chromosome 18. For construction of the reference map by *in situ* hybridization, 200 cosmid clones were randomly chosen from the library of short arm, and 400 clones from the library of whole chromosome 18, which may be comprising about 80% (or 20%) as much DNA of the short arm (or whole regions) of human chromosome 18.

The resources of human chromosome cosmid libraries have recently been available for mapping, but the localization *in situ* of a large number of cosmids to this chromosome has not been reported. The technique of FISH has proven to be a very efficient and accurate methodology for gene mapping and has been used to verify mapping data generated by somatic hybrid panels. Cosmid localization to specific bands of chromosome 18 in our studies will contribute to the physical and genetic mapping of this chromosome in general and may have some potential for their application in clinical cytogenetics as well. It is especially interesting to reveal that initially, the majority of cosmid DNA markers mapped to human chromosome 18 appeared to be on the R-banded negative regions. Such a nonrandom distribution pattern, caused by either cloning or mapping techniques as suggested by previous reports, has not been obvious. We selected recently a number of cosmids that gave a positive fluorochrome signals on the supposedly R-band positive regions of DAPI stained chromosomes, but not on the R-banded metaphase chromosome preparations. It is likely that these clones contain DNA derived from

the R-band positive regions. Further characterization of these clones may provide more understanding of the structure and organization of GC- or AT-rich sequences, and the possible function of the human chromosome relative to the G- or R-banding patterns.

Human Chromosome 18-specific Microsatellite Polymorphic Markers Spatially Resolvable on the Map by Fluorescent *in situ* Hybridization

Hitoshi NAKASHIMA, Masako SAKAI, Rie INABE and Takashi IMAMURA

An area of increasing importance in human genetics research is the strategy of reverse genetics or positional cloning, whereby a gene associated with a genetic disease is isolated on the basis of its approximate chromosomal position. Genetic analyses of the type require polymorphic markers that are informative, i.e. heterozygous, in the relevant individuals. In this study, we have isolated 12 cosmid clones mapped to the R-banded positive or negative region of the short arm of chromosome 18, which contained (CA)n dinucleotide repeat sequence. We also isolated several other clones positive for (CA) n repeat sequence out of 50 clones mapped to specific band region of the long arm of chromosome 18. Since polymorphisms of this type are generally detected by PCR amplification using primers homologous to unique seqences flanking the repeats, and could be effective single locus probes, we are analysing the sequences of subclones containing microsatellite and the flanking region. These markers isolated in this study will be useful for the genetic linkage analysis.

The past decade has seen great advances in the field of human gene mapping. In particular, it has become possible to isolate genes mutated in certain human diseases. The first step towards isolation of a disease gene in this way is its localization to a specific chromosome region. Except for disorders associated with cytognetically detectabe chromosomal rearrangements, chromosomal localization of the gene involved will usually rely on linkage mapping. This process has been revolutionalized by the ability to detect variation between individuals at the DNA level, in the form of restriction fragement length polymorphisms (RFLPs). Probes that detect such variation, and are of known chromosomal localization, can be used to study cosegregation of an RFLP with the disease phenotypes in affected

families. More recently, polymorphisms in the length of tandemly repeated simple sequences such as (CA)n (microsatellites) have proven to be a source of easily detectable highly polymorphic markers for genetic analyses. The high frequency of simple repeats and their apparently random distribution throughtout the genome means that markers from virtually any genomic region can be made informative for genetic analysis.

Man's Place in Hominoidea Revealed by Mitochondrial DNA Genealogy

Satoshi HORAI, Yoko SATTA, Kenji HAYASAKA, Rumi KONDO, Tadashi INOUE, Takafumi ISHIDA, Seiji HAYASHI and Naoyuki TAKAHATA

Molecular biology has resurrected C. Darwin and T. H. Huxley's question about the origin of man, but the precise branching pattern and dating remain controversial. To settle this issue, a large amount of sequence information is required. We determined mitochondrial (mt) DNA sequences for five hominoids; pygmy and common chimpanzees, gorilla, orangutan, and siamang. The common region compared with the known human sequence is 4759 base pairs (bp) long, encompassing genes for 11 transfer RNAs and 6 proteins. Because of the high substitution rates in mammalian mtDNA and an unprecedentedly large region compared, the sequence differences clearly indicate that the closest relatives to human are chimpanzees rather than gorilla. For dating the divergences of human, chimpanzee and gorilla, we used only unsaturated parts of sequence differences in which the mtDNA genealogy is not obscured by multiple substitutions. The result suggests that gorilla branched off 7.7 ± 0.7 million years (Myr) ago (0.59 ± 0.05 relative to the divergence time between orangutan and the African apes) and human did 4.7 ± 0.5 Myr ago (0.36 ± 0.04), the time difference between these divergences being as long as 3 Myr (0.23). For details, see *J. Mol. Evol.* **35**, 32–43 (1992).

Mitochondrial DNA Mutation and Leigh's Syndrome

Ryoichi SAKUTA, Yu-ichi GOTO, Satoshi HORAI, Tatsuya OGINO, Harumi YOSHINAGA, Shunsuke OHTAHARA and Ikuya NONAKA

Leigh's syndrome, a progressive neurodegenerative disorder with preferential involvement of the basal ganglia, is a group of heterogeneous metabolic disorders.

After Tatuch and coworkers reported mtDNA mutation from T to G at position 8993 within the ATPase 6 gene in a female infant with Leigh's syndrome and her mother, we examined mtDNA in muscle biopsy speciments from 23 patients with the clinical characteristics of Leigh's syndrome to determine whether this mutation is a common abnormality in this disorder.

MtDNA in the region from positions 8838 to 9017 within the ATPase 6 gene was amplified by polymerase chain reaction and digested with the restriction enzyme HpaII. Since the 8993 mutation created a HpaII recognition site (CCTG to CCGG). With this procedure, we detected the mutation in 1 of 23 patients in heteroplasmic fashion. In a previous study on mtDNA restriction analysis, there was no recognition site of HpaII at position 8993 in 116 normal Japanese subjects.

This point mutation leads to an amino acid change from a highly conserved leucine to arginine in ATPase subunit 6, thus probably causing failure in ATP synthesis. Because the mutation is detectable in mtDNA from blood samples by using a simple PCR method, more patients with identical mtDNA mutation may be identified in Leigh's syndrome and other neurological disorders. For details, see *Ann. Neurol.* **32**, 597–598 (1992).

Is Parkinson's Disease a Mitochondrial Disorder?

Yuko NAKAGAWA-HATTORI, Hiroya YOSHINO, Tomoyoshi KONDO, Yoshikuni MIZUNO and Satoshi HORAI

Parkinson's disease (PD) is a common degenerative disease, but its etiology is still unknown. However, since the discovery of MPTP, many investigators have been interested in the mitochondrial function in PD. We investigated mitochondrial functions in PD patients using the methods which have successfully been applied to mitochondrial myopathies (MM), i.e. assay of lactate and pyruvate, measurement of muscle mitochondrial respiratory enzyme activities and Southern blot analysis of muscle mitochondrial DNA. Parkinson's disease patients did not differ from controls in the mean blood and CSF (cerebrospinal fluid) lactate and pyruvate levels at the basal resting state or during an aerobic exercise. But mitochondrial complex I activity of the skeletal muscle was significantly decreased in PD. In the Southern blot analysis, we could not find major deletions or insertions of mitochondrial DNA in PD. Our studies disclosed a differential mitochondrial impairment

between PD and MM. We discuss the implication of our observation. For details, see *J. Neurol. Sci.* **107**, 29–33 (1992).

Mitochondrial Myopathy, Encephalopathy, Lactic Acidosis, and Stroke-like Episodes (MELAS): A Correlative Study of the Clinical Features and Mitochondrial DNA Mutation

Yu-ichi GOTO, Satoshi HORAI, Taro MATSUOKA, Yasutomi KOGA, Kenji NIHEI and Ikuya NONAKA

We studied 40 MELAS patients (21 male and 19 female) to characterize the clinical features and biochemical and muscle biopsy findings related to the mtDNA mutation at the nucleotide position of 3,243, the most common genetic defect in MELAS. The most frequent symptom was episodic sudden headache with vomiting and convulsions, which commonly affected patients aged 5 to 15 years (80%). Biochemical defects in the muscle were variable; 13 patients had complex I, seven complex IV, and four complexes I+IV deficiencies. In four muscle biopsies without ragged-red fibers or any enzyme defect, we based the diagnosis on the identification of strongly SDH-reactive blood vessels, which occurred in 87.5% of the biopsies. The mtDNA mutation was present in 32 of 40 patients (80%). We conclude that there are no clinical and pathologic differences between the patients with and without this mtDNA mutation. For details, see *Neurology* **42**, 545–550 (1992).

A Novel Point Mutation in the Mitochondrial $tRNA^{Leu(UUR)}$ Gene in a Family with Mitochondrial Myopathy

Yu-ichi GOTO, Megumu TOJO, Jun TOHYAMA, Satoshi HORAI and Ikuya NONAKA

A T-to-C transition mutation at nucleotide position 3,250 in the mitochondrial $tRNA^{Leu(UUR)}$ gene was present in a family with mitochondrial myopathy. Two of three muscle biopsies examined had complex I (NADH-ubiquinone oxidoreductase) deficiency. Heteroplasmy of wild and mutant mitochondrial DNA was detected by Nae I digestion of the polymerase chain reaction products with a modified primer. This was found in blood or muscle samples or both from all seven members examined. Similar to the 3,243 mutation in most patients with MELAS (mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes), the new mutation site

was located in the dihydrouridine loop and embedded in the binding region of mitochondrial transcription termination factor. Elucidation of the effects of this mutation may help clarify the role of mitochondrial tRNAs and transcription termination. For details, see *Ann. Neurol.* **31**, 672–675 (1992).

VIII. APPLIED GENETICS

Polymorphism of Bacterial Blight Resistance in Natural Populations of Wild and Cultivated Rice

Hiroko MORISHIMA and Toshie MIYABAYASHI

This study was initiated to investigate the evolutionary dynamics of a plant-pathogen system in natural and agricultural ecosystems. We have already demonstrated that land-races of rice and their wild progenitors are polymorphic within populations in resistance to a bacterial blight disease caused by *Xanthomonous oryzae* pv. *oryzae* (*Xoo*). The following is our recent experimental result which suggests possible mechanisms for maintaining resistance polymorphism in natural populations. Bacterial blight resistance in rice plants was assessed by innoculation of four representative *Xoo* races using the clipping method.

(1) The effect of habitat on resistance variations

In wild rice, perennial ecotypes adapted to deepwater conditions consist mostly of resistant plants, while annual ecotypes adapted to drier sites tend to be polymorphic, preserving a high frequency of susceptible plants. A similar tendency was found in Bangladesh cultivars: floating rice grown in a deepwater area were generally resistant, while cultivars in non-deepwater areas and those in the dry season were either resistant or susceptible. It was inferred from the above results that deepwater condition which favours the growth and development of pathogen *Xoo* has produces selection pressure resulting in a high frequency of resistant plants. On the other hand, in dry condition which is unfavorable to *Xoo*, selection for resistance must have been relaxed, resulting in polymorphism.

(2) Resistance variations on a fine scale in local populations

Two wild rice populations of the annual type in a suburb of Bangkok, which were located 1 km apart from each other (NE3 and NE4), and samples taken along a transect line connecting the above two populations were examined regarding the frequency of resistant plants.

NE4 showed a higher frequency of resistant plants (resistant to all four races: 58%) than NE3 (6%). This tendency was consistently observed in the samples collected in different years. Populations sampled on a transect line

showed a cline of decreasing frequency from NE4 to NE3 sites. Similar geographical clines between these two populations were found for some phenotypic characteristics such as plant height and flowering time, but no such trend was found in the allelic frequency of polymorphic isozyme loci.

The habitat of NE3 is a roadside grass land which is completely parched during the dry season; typical of an annual type habitat, while, NE4 is an exceptional annual population which inhabits a swampy place adjacent to a pond which retains water throughout the year. This geographical cline in bacterial blight resistance observed on a local scale could be explained by the differences in water conditions between NE3 and NE4. Namely, selection for resistant plants must have operated more strongly in NE4 in which the environment favours the pathogen than in NE3.

(3) Is there a resistance cost?

Frequency dependent selection between genetically heterogeneous pathogens and different host genotypes is one possible mechanism for maintaining resistance polymorphism. The cost of resistance is often argued as another mechanism for resistance polymorphism. We found a negative correlation between the degree of resistance and fitness character in a disease-free environment among lines derived from NE4. If this is the case, resistant plants are less fit than susceptible ones in a disease-free environment, and polymorphism can occur due to a balance of opposing selective forces.

The birth of *japonica* Rice in East China

Yo-Ichiro SATO, S. X. TANG*, L. U. YANG** and L. H. TANG***

To inquire into the geographical origin of cultivated rice, spikelets excavated from archaeological sites at Homedu (as old as 7,000 years), were examined to determine whether they contained spikelets of wild rice. Out of eighty-one spikelets examined, five showed characteristics of wild rice under a scanning electron microscope (SEM), although the previous observation had not revealed their existence. Other spikelets showed characteristics lying between those of wild and cultivated rice, suggesting the occurrence of natural hybridization between wild and cultivated rice, or the existence of an

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intermediate type. This indicates that a primitive stage of domestication had developed there 7,000 years ago. Recent archaeological data suggests the existence of an older site with proof of rice cultivation in the middle basin of the Yangtze River. Molecular genetic analysis indicated that the progenitors of *indica* and *japonica* inhabited the tropics and China, respectively. It may be inferred that the middle and lower basins of the Yangtze river including the Homedu area are the places of origin of *Japonica* rice.

Sampling Strategy in Constructing a Core Collection from on Entire Collection of Germplasms

Katsuei YONEZAWA, Tetsuro NOMURA* and Hiroko MORISHIMA

A core collection is composed of representative sample entries taken from an entire germplasm collection. Sample size and stratification strategy for sampling core entries from a structured germplasm collection were investigated, based on a theoretical model which takes account of both the amount of genetic diversity to be retained and its maintenance. It was derived that the optimum sample fraction could be defined but not be uniquely specified. It depends on various genetic and resource parameters, primarily on the degree of genetic redundancy in the entire collection, and the amount of resources available for maintenance (quantified by the number of plants treated for rejuvenation of core entries). The optimum sample fraction is larger with a lower redundancy among accessions, and/or with a lower allelic diversity within accessions, indicating that the sample fraction should be larger in species with a higher selfing rate. A larger sample fraction will be optimum with a smaller total amount of available resources and/or with a longer duration of maintenance of the core collection (defined by the number of generations for the maintenance). A sample fraction in the range of 20 to 30 percent may be taken as appropriate in a situation where accessions in the entire collection are neither very heavily nor very lightly redundant ($0.90 > Dr > 0.2$, in terms of the degree of redundancy as defined by the authors), and the core entries are maintained for ten to twenty cycles of rejuvenation with plants of the order of 10^3 being treated for one cycle of rejuvenation.

Five stratified sampling strategies were compared by analyzing fourteen

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hypothetical and four real germplasm collections composed of a number of groups. It was concluded that a proportional strategy, where the sample size is stratified in proportion to the number of accessions in each group, is the most reliable, while, of course, a strategy where the stratification is made in proportion to the range of genetic diversity is the best when the genetic diversity is known in advance. Sampling procedures for germplasm collections where accessions are hierarchically structured from the level of groups or single accessions were discussed. In the aim of drawing both the pattern and range of genetic diversity in the germplasm collection, procedures using the uniqueness value of Crozier (1992) were developed.

Recognition of the Water Surface by a Deepwater-Tolerance Gene (dw_3) in Rice

Mitsugu EIGUCHI, Hiro-Yuki HIRANO, Hiroko MORISHIMA and Yoshio SANO

Phenotypic plasticity which is characterized by changes in the morphology and physiology of a plant associated with changes in the environmental conditions is particularly important in plants due to their sessile life style. Deepwater and floating rice (*Oryza sativa*) in which internodal elongation starts with an increase in water depth in order to overcome the stress associated with submergence and the lack of internodal elongation in air-grown deepwater rice or in non-deepwater rice are examples of plant plasticity. In previous studies the physiological basis for tolerance to deepwater was ascribed to the enhancement of internodal elongation resulting from the action of the plant hormones, ethylene and gibberellin. Recently, we have observed that the gene dw_3 harboured by a floating type of wild rice (*Oryza rufipogon*) induces internodal elongation in response to submergence and is responsible for the survival of the plant at a depth of 1 m (Eiguchi *et al.* (1993) *J. Heredity* **84**, 201–205). In addition, the response induced by dw_3 was markedly attenuated after floral initiation, indicating that the genic expression was regulated during the development of the plant. This observation prompted us to try to determine whether dw_3 was responsible only for the timing of induction of internodal elongation under varying water regimes. We report here that in rice plants harbouring the dw_3 gene, stem elongation and heading time undergo considerable changes in response to variations in water depth.

To analyse the genic expression of dw_3, which is responsible for the induction of internodal elongation in response to submergence, phenotypic changes were examined under varying water regimes (water depths ranging from 0 m to 1.5 m) by using a rice cultivar (T65) susceptible to submergence in deepwater and its near-isogenic line carrying the gene dw_3 (T65dw_3). T65 dw_3 survived after being submerged in water at various depths while T65 died when submerged in water at 1.5 m- and 1.0 m-depths. The plant height of T65dw_3 markedly changed depending on the water depth. An increase in the number of elongated internodes and a delay in heading time were manifested after submergence in deepwater. These observations indicate that T65dw_3 recognized the water surface and adjusted the number of elongated internodes to complete its life cycle.

Deepwater rice cultivars are mostly photoperiod sensitive. Practically, photoperiod sensitivity may be essential for deepwater rice when flood hazards occur over a long period of time and the optimum harvest date is predicted for safety. Information on the plasticity of floral induction affected by deepwater is limited. The present study confirmed that the timing of floral induction in T65dw_3 changed substantially in response to variations in the water depth in spite of the fact that is almost neutral in regards to photoperiod. For details see *Jpn. J. Breed.* **43**, 135–139, 1993.

Genetic Comparisons of Chromosome 6 between Wild and Cultivated Rice

Yoshio SANO

The objective of this study was to detect genetic differentiation between wild and cultivated rice. Recent works on RFLP mapping encourage identification of agronomically important genes which have not yet been mapped on chromosomes. This implies that conventional techniques are not sufficient for identification of a complex nature of genes. Genomic differentiation is so complicated that it is hard to choose chromosomal regions to be studied. I have chosen chromosome 6 in this experiment because of the following points; 1) in rice there are lots of conventional markers for genetic analysis on it, 2) accumulated evidence suggests significant genetic divergences between and within species. To study the genetic differentiation on chromosome 6 between wild and cultivated rice, recombinant inbred lines

were made in the present experiment. The construction of recombinant inbred lines was confirmed to be a powerful method for detecting genes otherwise difficult to detect.

A large chromosomal segment of chromosome 6 was introduced into a genetic tester from the wild progenitor (*Oryza rufipogon*) by successive backcrossing, which was readily carried out because of the presence of a gamete eliminator S_6 on the segment. After introducing the segment, recombinants with various lengths of the segment were obtained. This made it possible to identify genetic factors by comparing the various segments in the same genetic background. One of the genes detected is an enhancer for photoperiod-sensitivity whose cooperative interaction with Se_1 leads to sensitivity as strong as that in wild rice. The other is a low crossability gene which produces unidirectional incompatibility. It was surprising that the three genes contribute to sexual affinity, resulting in a cluster of genes for pre- and post-fertilization barriers. Efforts are now under way to locate other genes for plant height, plant and seed morphologies, and heading date on the segment.

Comparisons of the same region of chromosome 6 between the two cultivated rice species revealed that another gamete eliminator, its modifiers and recombination restriction occur on it. Similar abnormalities have been reported in fruit fly and mice in relation to meiotic drive, although they have been found to occur within a population. It is interesting to note that similar systems appear in the mechanism of reproductive isolation among rice taxa.

Hopefully, further studies will be readily extended by using various molecular markers in the near future, after elucidating the complex nature of the chromosomal segment. The importance of efforts to detect unidentified genes with biological significance must be recognized. Most genes are waiting to be analyzed in various ways. For details see *Jpn. J. Breed.* **42**, 561–572, 1992.

Is an Egg-Killer Present in Rice?

Yoshio SANO

Hybrid sterility due to allelic interaction was first reported in *Nicotiana.* An alien gene (pollen killer) in *Nicotiana plumbaginifolia* caused the degeneration of pollen not carrying it, when the chromosome on which it occurred was added to the *Nicotiana tabacum* complement. The genetic basis for

hybrid sterility between species seems to be complex. Accumulated evidence, however, suggests that such genes causing gametic abortion are of wide spread between and within plant species. Most of such genes detected so far were of the gametophytic type and they were classified into three types, gamete eliminator, pollen-killer and egg-killer, depending on the gametes affected. The former two were frequently detected in plants. The only example of an egg-killer was reported in a maize-*Tripsacum* hybrid. An extra chromosome derived from *Tripsacum* was transmitted to a high proportion of the progeny through the egg. This was explained by assuming the existance of an egg-killer on the extra chromosome, which induces abortion of megaspores not carrying it. Data however were limited. The present experiment was carried out to confirm the presence of an egg-killer recently proposed as the genetic mechanism of hybrid sterility observed in Asian rice cultivars (*Oryza sativa*).

Hybrid sterility due to allelic interaction at a single locus was also reported in some varietal crosses within *O. sativa.* Recent progress included the detection of the wide compatibility gene, S_5^n, which was recognized as a gene that greatly reduces seed infertility in the Japonica-Indica hybrid. It was assumed that there are two additional alleles at the S_5 locus and that an S_5^i allele from the Indica parent induces abortion of megaspores carrying the other allele (S_5^j from Japonica parent) in the heterozygote (S_5^i/S_5^j), suggesting that S_5^i may act as an egg-killer against S_5^j. The locus attracted rice breeders since the hybrid sterility gene might prevail among commonly used breeding materials.

The present study was carried out in order to confirm that the proposed S_5^i allele acts as an egg-killer against the opposite S_5^j allele in the Indica-Japonica hybrid. A conspicuous feature of an egg-killer is a high rate of its transmission into the progeny through the egg. Backcrossing experiments were made by using the Indica-Japonica hybrid in which S_5^i and S_5^j had been assumed to be involved. Although an egg-killer was easily identified through backcross experiments, the present study failed to detect any egg-killer in the Indica-Japonica hybrid, providing evidence counter to the proposed genetic mechanism for hybrid sterility in Asian rice. This study will appear in *Theor. Appl. Genet. 1993.*

IX. DATABASE

DNA Database Release 11 from DNA Data Bank of Japan

Yoshihiro UGAWA, Kazuho IKEO, Yukiko YAMAZAKI, Motono HORIE, Masako IWASE, Mari SAITO, Yumiko SATO, Shigeko SUZUKI, Yuko HAWAGAWA, Asako HASEGAWA, Yoshie HATTORI, Mary SHIMOYAMA, Rikiko SUZUKI, Reiko UCHIDA, Yoko SHIDAHARA, Yoko MATSUSHIMA, Akino WATANABE, Yoko UEDA, Tatsuko KAWAMOTO, Hajime KITAKAMI, Naruya SAITOU, Takashi GOJOBORI and Yoshio TATENO

The DNA database Release 11 includes 65,693 entries which correspond to 84,839,075 bases. This release contains the newest data prepared by DDBJ, GenBank and the EMBL Data Library as of June 1993. Thanks to international collaboration between the three data banks, the International Nucleotide Sequence Database has been organized as a unified database.

All the entries designated by the accession numbers with "D's" have been collected and processed by DDBJ, and the rest have been prepared by GenBank and the EMBL Data Library. Because the release contains unified data, all the entries have been annotated with common feature keys. The present release was prepared by processing the data on the relational data base management system (Sybase). It does not include amino acid sequence data, because the genetic code system is known to be no longer uniform among the species and organella, and DDBJ is not yet prepared for this.

Integration and Search System for a Large-Scale DNA Database

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A flat-file system is inadequate for building, integrating and searching a large-scale DNA database whose entries are continuously increasing in an explosive manner. GenBank helped DDBJ to convert flat-file data constructed by DDBJ to a relational format and trained DDBJ staff to use the Annotator's Workbench, AWB, which is a tool for building the DNA database based on the relational database management system, SyBase.

However, integration and search functions are still performed utilizing the flat-file system at DDBJ. We are developing a new intelligent system, Alife

(*A l*arge-scale database *i*ntegration *f*acility with *e*-mail server) to integrate and search the DNA Database. The Alife system includes some functions developed by GenBank for implementing an integrated international database on SyBase. We developed a relational schema, named ddbj-schema, as well as an online and *e*-mail service system for database users. It is necessary to convert the DNA database built with the GenBank schema using AWB, into a relational format using the ddbj-schema. A conversion system was developed using DDBJ's virtual tables as a component of the Alife system.

The ddbj-schema will be utilized to integrate three databanks DDBJ, NCBI including GenBank, and the EMBL Data Library. In addition, the ddbj-schema will be utilized to update the ESTs (Expressed Sequence Tags)/TSFs (Transcribed Sequence Fragments) database submitted by the Japanese human cDNA project at Prof. Matsubara's Laboratory, Osaka University and other such projects.

We are investigating, with Prof. Makinouchi of Kyushu University, the possibility of rewriting the Alife system implementing the relational database using an object-oriented database system.

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ABSTRACTS OF DIARY FOR 1992

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357th–Feb. 3	Coat protein mediated resistance against plant virus in transgenic plants (R. N. Beachy)
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Mishima Geneticists' Club

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Mar. 28, 1991–	Engelbert Hobmayer, University of Munich, Germany
Jan. 3–Mar. 31, 1992	Jianying Luo, Hunter College of CUNY, U.S.A.
Jan. 28–30	Gerald P. Holmquist, City of Hope Medical Center, U.S.A.
Feb. 3–4	Roger N. Beachy, The Scripps Research Institute, U.S.A.
Mar. 5–29	William Provine, Cornell University, U.S.A.
Mar. 21	Masahiro Akiyama, Stanford University, U.S.A.
Apr. 7	Pierre Boursot, University Monpellier, France.
Apr. 10	Angus C. Nairn, The Rockefeller University, U.S.A.
Apr. 13–June 30	Ashok Kumar, University of Edinburgh, U.K.
Apr. 22–23	Noboru Sueoka, University of Colorado at Boulder, U.S.A.
Apr. 22–23	Tamiko Sueoka, University of Colorado at Boulder, U.S.A.
May 25	Dieter Gallwitz, Max-Planck-Institute for Biophysical Chemistry, Germany
June 5	Stefan Jentsch, Friedrich-Miescher-Laboratorium der Max-Planck-Gesellschaft, Germany
June 7–9	Martin F. Gellert, National Institutes of Health, U.S.A.
June 9	Maria Jasin, Memorial Sloan-Kettering Cancer Center, U.S.A.
June 15–20	Ok-Soon Heo, National Institute of Safety Research, Ministry of Health and Social Affairs, Korea
June 15–20	Eui-Sik Han, National Institute of Health, Ministry of Health and Social Affairs, Korea
June 28–30	Menachem Rahat, The Hebrew University of Jerusalem, Israel
July 2–5	Vanda Reich, The Hebrew University of Jerusalem, Israel
July 3–4	Michael Ashburner, University of Cambridge, U.K.
July 4–Aug. 10	Flemming Hansen, Technical University of Denmark,

	Denmark
July 4–Sept. 22	Richard S. Hayward, University of Edinburgh, U.K.
July 9	Jaap Goudsmit, University of Amsterdam, The Netherlands
July 21–22	You-di Liao, Institute of Biomedical Science, Academia Sinica, Taiwan, China
July 21–22	Soo-Chen Cheng, Institute of Molecular Biology, Taiwan, China
July 21–25	Bei-Chang Yang, National Cheng-Kung University, Korea
July 24–25	Younghoon Lee, Korea Advanced Institute of Science and Technology, Korea
July 25–27	Changwon Kang, Korea Advanced Institute of Science and Technology, Korea
July 25–28	Diopankar Chatterji, Centre for Cellular and Molecular Biology, India
July 24–26	Samit Adhya, Indian Institute of Chemical Biology, India
July 25–Aug. 4	Tove Atlung, Technical University of Denmark, Denmark
Aug. 15–21	Lobov V. Frisman, Institute of Biology and Pedology Far East Branch, Russian Academy, Russia
Aug. 15–21	Vladimir P. Korablev, Institute of Biology and Pedology Far East Branch, Russian Academy, Russia
Aug. 15–21	Galina M. Chelomina, Institute of Biology and Pedology Far East Branch, Russian Academy, Russia
Aug. 15–21	Lydumila Yakimenko, Institute of Biology and Pedology Far East Branch, Russian Academy, Russia
Aug. 15–21	Alexei P. Kryukov, Institute of Biology and Pedology Far East Branch, Russian Academy, Russia
Aug. 15–21	Irina V. Kartavtseva, Institute of Biology and Pedology Far East Branch, Russian Academy, Russia
Aug. 24–27	Kenneth K. Kidd, Yale University, U.S.A.
Sept. 10–12	Karin Petersen, University of Edinburgh, U.K.
Sept. 27–30	O. G. Ward, Los Alamos National Laboratory, U.S.A.
Sept. 29–Oct. 30	Chitrakon Songkran, Rice Research Institute, Thailand
Sept. 16–Dec. 14	Toshie Kawano, LEZEP Instituto Butantan, Brazil

Nov. 9–16	Michael Griffiths, Royal Perth Hospital, Australia
Nov. 10	Robert H. Waterston, Washington University School of Medicine, U.S.A.
Nov. 19	Susan W. Serjeantson, The Australian National University, Australia
Nov. 20–22	Klaus Scherrer, Institut Jacques Monod,CNRS, France
Nov. 30–Dec. 1	Keiko Udaka, Max-Planck-Institute for Biology, Germany
Dec. 3	Faith Keenan, Hearst Newspapers, Washington Bureau, U.S.A.
Dec. 3–5	Peter J. Bryant, University of California, U.S.A.
Dec. 11	James Ostell, National Center for Biotechnology Information, U.S.A.
Dec. 11	Maynard Olson, University of Washington, U.S.A.

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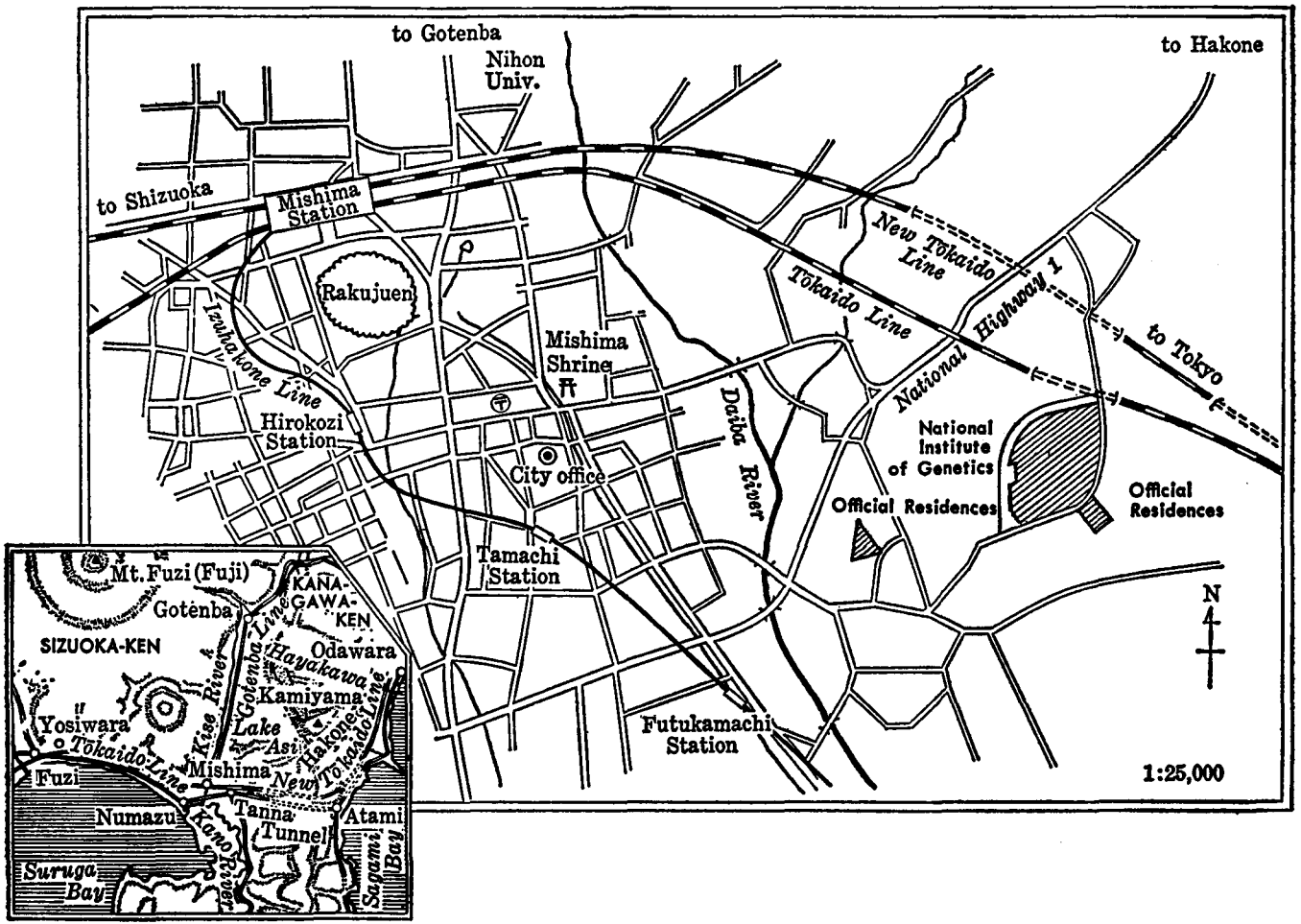
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