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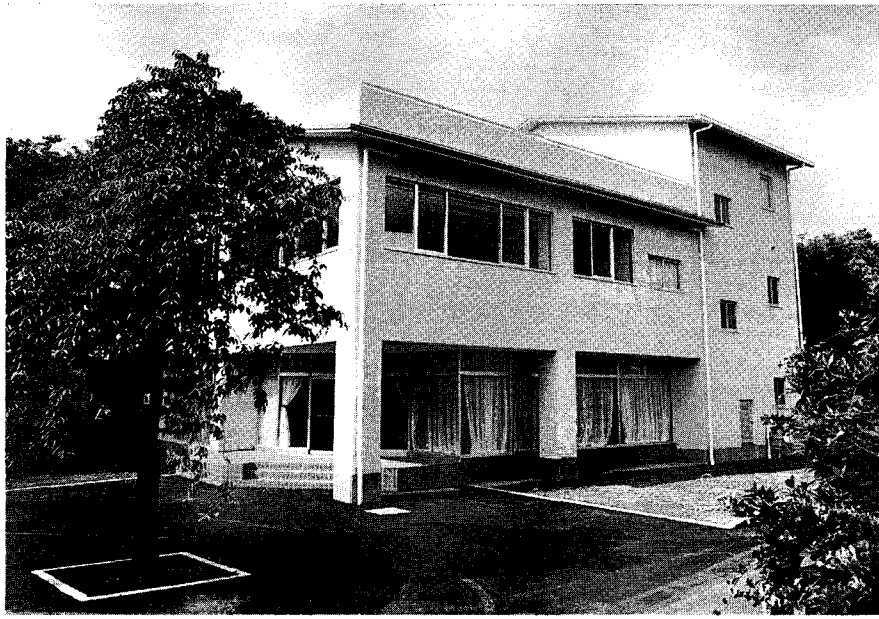

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II. Microbial Genetics

GENERAL STATEMENT

Our institute was established 42 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 more than 20 such students and the number is increasing. I consider it vital for our research activities to have a steady flow of young scientists.

Recent rapid progress in the field of genetics with newly developed approaches has greatly shifted the course of study in biology. I consider it natural that 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. 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. The DNA Data Bank of Japan (DDBJ) is one of the three banks in the world that gather, annotate, store and distribute information on DNA sequences. DDBJ will continue to be active as a regional center for such activities. Our institute also stores and distributes various organisms with genetically characterized traits. Among them services 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. Miyazawa, Dr. K. Nagata and Dr. H. Hayashida were transferred to Gumma University, Tokyo Institute of Technology and Kyoto University, respectively. The Genetic Stock Research Center gained Dr. N. Nakatsuji as professor and Dr. Y. Shirayoshi as research member. Dr. A. Nishimura of the Center was promoted to associate professor. Dr. I. Katsura joined the DNA Research Center as professor, from where Prof. T. Ikemura and Dr. K. Matsumoto were transferred to the Laboratory of Evolutionary Genetics. Dr. S. Horai (Laboratory of Human Genetics) was promoted to the rank of associate professor.

Junichi Tomizawa

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Adviser

MORIWAKI, Daigoro; Honorary member, National Institute of Genetics

PROJECTS OF RESEARCH FOR 1991

1. DEPARTMENT OF MOLECULAR GENETICS

Laboratory of Molecular Genetics

Studies on regulatory mechanisms of gene transcription in prokaryotes (ISHIHAMA, FUJITA and YAMAGISHI)

Studies on molecular architecture of transcription apparatuses in eukaryotes (ISHIHAMA and YAMAGISHI)

Studies on molecular mechanisms of transcription and replication of animal and plant viruses (ISHIHAMA, YAMAGISHI, FUJITA and NAKAMURA)

Laboratory of Mutagenesis

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

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

Radiation sensitivity in mammals (TEZUKA)

Laboratory of Nucleic Acid Chemisry

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

2. DEPARTMENT OF CELL GENETICS

Laboratory of Cytogenetics

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

Structure and function of the mouse MHC high frequency genetic recombination (SHIROISHI and MORIWAKI)

* Genetic stock research center.

Theoretical and experimental bases for karyotype evolution (IMAI)
Study of adrenal function in mouse development by transgene (GOTOH, SHIROISHI and MORIWAKI)

Laboratory of Microbial Genetics

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

Laboratory 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

Laboratory of Developmental Genetics

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

Laboratory of Phenogenetics

Genetic studies on the life history characters in *Bombyx* (MURAKAMI)
Genetic studies on the nerve system characters in *Bombyx* (MURAKAMI)
Studies on development and growth of insect (MINATO)
Studies on the male-specific killing activity of SRO in *Drosophila* (YAMADA)
Genetic studies on maternal effect lethality in higher organisms (YAMADA)

Laboratory of Physiological Genetics

Nerve net formation in *Hydra* (KOIZUMI)

4. DEPARTMENT OF POPULATION GENETICS

Laboratory of Population Genetics

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

Laboratory 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 (GOJOBORI and MORIYAMA)
Studies on molecular evolution of Drosophila genes (MORIYAMA)

Laboratory of Theoretical Genetics

Theoretical studies of population genetics and molecular evolution (KIMURA)
Computer analysis of genetic information (YASUNAGA)

5. DEPARTMENT OF INTEGRATED GENETICS

Laboratory 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)

Laboratory 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)

Laboratory 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)
Genetic and Molecular studies of cell division mechanism in *E. coli.* (NISHIMURA)
Theoretical studies in molecular phylogeny (TATENO)

DNA Research Center

Interaction between proteins and nucleic acids (SHIMAMOTO)
Genetics and molecular genetics of development and behavior of *Caenorhabditis elegans.* (KATSURA)
Computer analysis of DNA sequences (GOJOBORI and UGAWA)
Construction of DNA sequence database (GOJOBORI, Ugawa and TATENO)
Molecular genetics of insect development (UEDA and HIROSE)
Control of gene expression in eukaryotes (HIROSE, HAYASHI and UEDA)
Molecular Biology of *C. elegans* development (KOHARA)
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 1991

I. MOLECULAR GENETICS

The Promoter Selectivity of *Escherichia coli* RNA Polymerase: Two Modes of Protein–Protein Communication between RNA Polymerase and Transcription Factors

Akira ISHIHAMA, Nobuyuki FUJITA, Kazuhiko IGARASHI*, Chao ZOU and Masahiro YAMAGISHI

The promoter selectivity control of RNA polymerase plays a key role in global control of gene transcription in prokaryotes. A number of accessory transcription factors have been identified in *E. coli*, which interact with RNA polymerase and modulate its promoter recognition properties. These factors can be classified into two groups: one group factors form RNA polymerase complexes in the absence of DNA; the other group factors bind to DNA and interact with RNA polymerase only when both are aligned along the same DNA strand (Ishihama, A. (1991) *Molecules and Cells* **1**, 381–384; Ishihama, A. (1992) *Mol. Microbiol.*, in press). Regardless of apparent difference in the order of molecular assembly, ternary complexes are formed between RNA polymerase, transcription factor(s) and DNA signals prior to transcription initiation.

In the course of the molecular anatomy of RNA polymerase subunits, we found that the C-terminal proximal region of α is not necessary for both the RNA polymerase assembly and the catalytic function of RNA synthesis, but is needed for the molecular communication with some class I transcription factors, which mostly bind to their target DNA sites located upstream of the promoters (Igarashi, K. and Ishihama, A. (1991) *Cell* **65**, 1015–1022; Igarashi, K. *et al.* (1991) *Proc. Natl. Acad. Sci. USA* **88**, 8958–8962). Class II factors, however, do not require this contact site I on the α-subunit C-terminus, and instead interact with the still unidentified, putative site II. The class II factors bind to the target DNA signals located near the promoter.

* Present address: Majorie B. Kolver Viral Oncology Lab., University of Chicago.

Table 1. Classification of transcription factors

Transcription factor/ Target gene(s) or promoter(s)	Collaborators(s)
CRP (cAMP receptor protein)/ *lac*, *gal*, *uxuAB*, pBR-P4	A. Hanamura/H. Aiba (Nagoya Univ.); S. Busby (Univ. Birmingham); R. Ebright (State Univ. New Jersey); A. Kolb/H. Buc (Pasteur Inst.); M. Thomas/R. E. Glass (Univ. Nottingham)
OmpR/*ompC*, *ompF*	H. Aiba/T. Mizuno (Nagoya Univ.)
PhoB/*pst*	K. Makino/A. Nakata (Osaka Univ.)
Ada/*alk*, *ada*	K. Sakumi/M. Sekiguchi (Kyushu Univ.)
OxyR/*katG*	K. Tao (Univ. Tokyo)
λ repressors/λP_L, λP_{PR}	G. N. Gussin/C. Olson (Univ. Iowa)
MerR/*mer*	A. Z. Ansari/T. V. O'Halloran (Northwestern Univ.)
FadR/*fabA*	C. C. DiRusso (Univ. Tenessee)
Fis/*rrnB*	R. L. Gourse/W. Ross (Univ. Wisconsin)
GalR/*gal*	S. Adhya (NCI NIH)
IHF (integration host factor)/λP_L	A. Oppenheim/H. Giladi (Hebrew Univ.)
MetR/*met*	G. V. Stauffer (Univ. Iowa)
RhaS, RhaR/*rha*	R. Schleif (Johns Hopkins Univ.)
TyrR/*tyr*, *aro*	A. J. Pittard (Univ. Melbourne)
N4 SSB/N4 late genes	L. B. Rothman-Denes (Univ. Chicago)
NtrC, σ^{54}/*glnA*	S. G. Kustu (UC-Berkeley)
NifA, σ^{54}/*nifE*, *nifU*	W. Charlton/M. Buck (Univ. Sussex)

Table 2. Structural studies of α subunit

Subunit assembly	R. S. Hayward (Univ. Edinburgh)
Cross-linking	H. Buc/A. Kolb (Pasteur Inst.)
Fluoresence polarization	R. Ebright (State Univ. New Jersey)
Cys localization	O. Ozoline (Russian Acad. Sci.)
Epitope mapping	K. Sharif/J. S. Krakow (Hunter College), R. R. Burgess (Univ. Wisconsin)
X-ray crystallography	B. C. Wang (Univ. Pittsburgh)
Electron microscopy	A. Ikai (Tokyo Inst. Technology) Y. Fujiyoshi (Prot. Engineer. Res. Inst.)
NMR	Y. Kyogoku (Osaka Univ.)
Module analysis	M. Go (Nagoya Univ.)

A number of known transcription factors, listed in Table 1, are being tested as to their requirement of the contact site I for transcription activation of respective target genes. In parallel, we started structural studies of the contact site I on α subunit (Table 2). These studies are being carried out as

an international collaboration including more than 30 laboratories from 10 different countries.

The Promoter Selectivity of Escherichia coli RNA Polymerase: Fine Mapping of the Contact Site I for cAMP Receptor Protein (CRP)

Chao ZOU, Nobuyuki FUJITA, Kazuhiko IGARASHI* and Akira ISHIHAMA

The α subunit of *E. coli* RNA polymerase plays an essential role in RNA polymerase assembly. The assembly domain is located within N-terminal 235 amino acid resides (intact α is composed of 326 amino acid residues) while the C-terminal proximal region is needed for transcription activation by class-I transcription factors, most of which bind at the respective cis-acting DNA signals located upstream of promoter -35 signal (this contact site is designated as "site I" for RNA polymerase-transcription factor interaction) (Igarashi, K. *et al.* (1991) *J. Mol. Biol.* **218**, 1–6; Hayward, R.S. *et al.* (1991) *J. Mol. Biol.* **221**, 23–29; Igarashi, K. and Ishihama, A. (1991) *Cell* **65**, 1015–1022; Igarashi, K. *et al.* (1991) *Proc. Natl. Acad. Sci. USA* **88**, 8958–8962).

For detailed mapping of the contact site I for CRP (cAMP receptor protein), we introduced random mutations in the contact site I region of α-subunit gene (*rpoA*) using PCR mutagenesis method and, after transformation with this mutant library, screened for Lac$^-$ colonies on lactose-tetrazolium agar plate. We have sequenced the contact site I region for 7 independent Lac$^-$ isolates, 4 of which *rpoA*122, *rpoA*124, *rpoA*125, and *rpoA*129, all carried single mutation at the same nucleotide position which generates mutant α with single amino acid substitution, R265C for α_{122}, α_{124}, and α_{129}, and R265H for α_{125}. Both *rpoA*123 and *rpoA*127 carried single mutation, which led mutant α with N268D (α_{123}) and L270P (α_{127}) substitution. Interestingly, all these 6 mutations are clustered within a short segment, amino acid residue 265 to 270, suggesting that the contact site for CRP is located in this small region. α_{125} had double mutations, one within this CRP-contact site (R265H) and other (K297R) outside of this site, suggesting that R265H is responsible for the mutant phenotype of this particular mutant.

The effects of these mutant α subunits on transcription were examined

* Present address: Majorie B. Kolver Viral Oncology Lab., University of Chicago.

using reconstituted holoenzymes and *lac* and *gal* promoters. CRP-dependent transcription *in vitro* from the *lac*P1 promoter decreased markedly, while CRP-dependent transcription from the *gal*P1 promoter was not affected at all. The results agreed well with the previous finding that the contact site I on α is necessary for transcription activation of *lac*P1 but not *gal*P1 (Igarashi, K. *et al.* (1991) *Proc. Natl. Acad. Sci. USA* **88**, 8958–8962).

The Promoter Selectivity of *Escherichia coli* RNA Polymerase: Functional Mapping of Major Sigma Subunit σ^{70}

Ashok KUMAR*, Nobuyuki FUJITA, Richard S. HAYWARD** and Akira ISHIHAMA

The recognition of nucleotide sequences at promoters by RNA polymerase is crucial to gene transcription and regulation. In *E. coli*, the major form of RNA polymerase ($E\sigma^{70}$) interacts with most promoters by recognizing two hexameric sequence elements, known as −10 (TATAAT) and −35 (TTGACA) signals, located around 10 and 35 bp, respectively, upstream of transcription start site. It is considered that RNA polymerase ($E\sigma^{70}$) makes contacts with the bases in these sequences, and the activity of promoters decreases as the actual sequence deviates from the consensus. Among four conserved amino acid sequences between multiple species of σ subunits from various prokaryotes, the region-2 and region-4 have been suggested to be responsible for recognition of −10 and −35 sequence, respectively.

Over the past few years, however, a number of *E. coli* promoters have been identified, that do not obey these simple rules (Keilty, S. and Rosenberg, M. (1981) *J. Biol. Chem.* **262**, 6389–6395). In these cases, the RNA polymerase $E\sigma^{70}$ recognizes only the −10 sequence (and possibly a sequence upstream of −10 sequence). The basic question we asked was whether the region-4 located at the carboxy terminus of σ^{70} is dispensable for transcription initiation at the "extended −10 promoters" with no significant −35 sequence. We overexpressed the C-terminal truncated σ^{70} (including region-4), purified to homogenity and reconstituted various molecular species of mutant holoenzyme containing the truncated σ^{70} subunits. *In vitro* transcription study indicated that the region-4 is indeed not essential for transcription of the

* On leave of absence from the Institute of Cell and Molecular Biology, University of Edinburgh (supported by the Royal Society and British Council).

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extended −10 promoter. We also found that TGN motif immediately upstream of −10 consensus sequence is important for the function of the extended −10 promoter.

The Promoter Selectivity of *Escherichia coli* RNA Polymerase: Structure and Function of Stationary-phase RNA Polymerase

Miwako OZAKI, Nobuyuki FUJITA and Akira ISHIHAMA

The promoter selectivity control of RNA polymerase plays a major role in global control of gene transcription in *E. coli* during stress response. As an extension of this line study, we analyzed the growth phase-dependent modulation of RNA polymerase.

The overall level of gene transcription in stationary-phase cells decreased to less than 10% the level of exponential growth phase. The synthesis and assembly of RNA polymerase are repressed almost completely (reviewed in Ishihama, A. (1991) In: "Control of Cell Growth and Division", pp. 121–140) and, in addition, the pre-existing RNA polymerase is converted into functionally different forms with altered promoter selectivity (Ozaki, M. *et al.* (1991) *Mol. Gen. Genet.* **230**, 17–23). The stationary-phase RNA polymerase is unable to transcribe *in vitro* promoters associated with genes expressed in exponentially growing cells, such as promoters of the genes for rRNA, ribosomal proteins, tRNA and aminoacyl-tRNA synthetases. Only a limited number of promoters are transcribed, including promoters for the genes encoding D-lactate dehydrogenase, glucose 6-phosphate dehydrogenase, NADH dehydrogenase, catalase hydroperoxidase, ribonuclease H, RecA protein, NusA transcription termination factor, and heat-shock sigma factor σ^{32} (Ozaki, M. *et al.* (1991) *Nucleic Acids Res.* **20**, 257–261).

All the stationary-phase form enzymes contained σ^{70}, the major sigma subunit, and three major core subunits, β', β and α, but were very acidic in net charge. The holoenzyme reconstitution experiments indicated that the functional difference was originated from the core enzyme portion. To our surprise, the stationary-phase form enzymes were converted into the log-phase type enzyme after treatment with nucleotides or pyrophosphate. Adenine nucleotides were most effective in this conversion. One candidate for the phosphorylated factor associated with the stationary-phase RNA polymerase is poly- or oligophosphate. In collaboration with Drs. M. Akiyama and A.

Kornberg (Stanford Univ.), this possibility was indeed found to be the case.

Regulation of the *Escherichia coli rmf* Gene Encoding Ribosome Modulation Factor

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

Ribosome modulation factor (RMF) is a small protein of 55 amino acid residues associated with 100S ribosome dimers which appear in stationary phase cells of *Escherichia coli* (Wada, A. *et al.* (1990) *Proc. Natl. Acad. Sci. USA* **87**, 2657–2661). The structural gene, *rmf*, has been cloned from Kohara's phage library of the entire *E. coli* genome, sequenced and mapped at 21.8 min of the *E. coli* chromosome. To examine the expression pattern of *rmf* under various growth conditions, we carried out Northern blot hybridization and β-galactosidase assay for a *rmf-lacZ* fusion gene. In rapidly growing cells in nutrient-rich media, the expression of *rmf-lacZ* was hardly detected but during the growth transition from exponential to stationary phase, both the level of *rmf-lacZ* RNA and the activity of β-galactosidase rapidly increased in a parallel way. Thus, the *rmf* gene seems to be transcribed only in stationary phase.

Next, we examined the effect of growth rate on *rmf* expression. When cells were grown at slow rates in poor media, *rmf-lacZ* was expressed even in exponential growing phase. The level of β-galactosidase activity was inversely correlated to growth rate. The nucleotide sequence of the promoter region deduced from the position of transcription start site deviates from the consensus sequence recognized by all the known RNA polymerase, *i.e.*, holoenzymes containing not only the major σ^{70} subunit but also other minor sigma subunits. In fact, *rmf* was not transcribed *in vitro* by purified RNA polymerase containing σ^{70} subunit. When *rmf* on the chromosome was disrupted, the resultant mutants lost viability during stationary phase, suggesting that *rmf* is one of the essential genes for survival in stationary phase. In the *rmf* mutant, 100S ribosomes were not detected even in stationary phase. Analysis of the function(s) of RMF and the activity of 100S ribosomes

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dimers as well as search for *cis*-acting element(s) and *trans*-acting factor(s) required for the expression of *rmf* will be our future projects.

Structure and Function of RNA Polymerase II from *Schizosaccharomyces pombe*: Cloning and Sequence Determination of the Second Largest Subunit Gene

Makiko KAWAGISHI, Masahiro YAMAGISHI and Akira ISHIHAMA

In spite of increasing knowledge of transcription factors, relatively little is known on the structure and function of RNA polymerase in eukaryotes. We then started cloning and DNA sequencing of the *Schizosaccharomyces pombe* genes encoding the putative subunits of RNA polymerase II. Last year, we cloned the gene, *rpb1*, coding for the largest subunit and determined the DNA sequence (Azuma, Y. *et al.* (1991) *Nucleic Acids Res.* **19**, 461–468). This year, the gene, *rpb2*, encoding the second largest subunit of RNA polymerase II has been cloned from *Schizosaccharomyces pombe* using the corresponding gene, *RPB2*, of *Saccharomyces cerevisiae* as a probe for cross-hybridization. We have determined the complete nucleotide sequence of *rpb2*, and parts of PCR-amplified *rpb2* cDNA. The predicted coding sequence, interrupted by a short intron, is able to code for a polypeptide of 1210 amino acid residues with a calculated molecular weight of 138 kilodaltons. Deduced amino acid sequence of *rpb2* was nearly 70% identical with that of *S. cerevisiae RPB2.* Detailed Southern analysis of genomic DNA digested with various restriction enzymes showed that *rpb2* is present as a single copy in the *S. pombe* genome. Northern analysis showed that the transcript of *rpb2* is about 4 kb in length.

Recently it has been reported that a local sequence similarity exists between the second largest subunit of RNA polymerase II and bacterial RNases (Shirai, T. and Go, M. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 9056–9060). We expressed the "RNase-like domain" of the second largest subunit of *S. pombe* RNA polymerase II in *E. coli.* The product exhibited RNA binding activity in a preliminary experiment.

Structure and Function of Influenza Virus RNA Polymerase: Reconstitution of Functional RNA Polymerase from Three Subunits Expressed Using a Recombinant Baculovirus System

Makoto KOBAYASHI, Kotaro TUCHIYA*, Kyosuke NAGATA** and Akira ISHIHAMA

Influenza virus RNA polymerase catalyzes multiple step reactions in both 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). Genetic and biochemical studies suggested that PB1 is involved in polymerization of nucleotides to nascent RNA chains while PB2 is involved in recognition of host cell capped mRNA. The role of PA protein is not known yet, but temperature-sensitive mutations in the PA gene affect only vRNA synthesis, but not mRNA synthesis.

For detailed analysis of the role of each P protein and the functional domains on each P polypeptide, we expressed individual P proteins in cultured insect cells after infection with recombinant baculovirses (Kobayashi, M. *et al.* (1992) *Virus Res.* **22**, 235–245). For this purpose, we inserted the cDNA for each P gene into a baculovirus vector as to express under the control of the polyhedrin promoter which is extremely active at the late stage of baculovirus infection. Using these recombinant viruses, we produced large amounts of each P protein in insect cells. PB1 and PB2 accumulated in cell nuclei whereas PA stayed in cytoplasm. Both the PB1 and PB2 proteins were purified from aggregates in the respective nuclear extract, and the PA was partially purified from the cytoplasm. RNA polymerase was reconstituted by mixing the three P proteins in a urea solution and then dialyzing against a reconstitution buffer. The reconstituted enzyme was able to transcribe model RNA templates in the presence of ApG as a primer. Minus-sense RNA was a better template than plus-sense RNA.

Inhibition Mechanism of Influenza Virus Growth by Lignin

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Previously we found that lignin, an abundant component in plant, inhibits

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the growth of influenza virus and the activity of virion-associated RNA polymerase (Nagata, K. *et al.* (1990) *Antiviral Res.* **13**, 11–22; Sakagami, M. *et al.* (1990) *Biochem. Biophys. Res. Commun.* **172**, 1267–1272). The polymerized phenolic structure of lignified materials is responsible for this anti-influenza virus activity (Harada, H. *et al.* (1991) *Antiviral Res.* **15**, 41–50). For detailed understanding of the virus growth inhibition, we attempted to identify the target of lignin action by isolating lignin-resistant virus mutants. After four passages of WSN strain (H1N1) in MDCK cells in the presence of lignin, we isolated two mutants which were resistant to lignin up to 200 μg/ml. The growth of wild-type parental virus was completely inhibited at this concentration.

After mixed infection of the resistant mutants with lignin-sensitive Aichi strain (H3N2) in MDCK cells, lignin-resistant reassortants were isolated in the presence of anti-H1 antibodies. Although the growth of Aichi strain is retarded in MDCK cells, all the lignin-resistant reassortants were found to carry RNA segment 7 from WSN strain. The results suggest that either M1 or M2 encoded by WSN RNA segment 7 plays an essential role in growth of influenza virus in MDCK cells, and that such fast growers are apparently resistant to lignin.

Inhibition Mechanism of Influenza Virus Growth by Mx1 Protein: Self-assembly of Mx1 and GTP-dependent Conformational Change

Manabu NAKAYAMA, Kazumori YAZAKI*, Akira KUSANO**, Kyosuke NAGATA***, Nobuo HANAI** and Akira ISHIAHAMA

Murine Mx1 protein, a host factor involved in resistance against influenza virus intection, is an inferferon-induced nuclear protein. Last year, we purified the Mx1 protein from extracts of *E. coli* cells expressing Mx1 cDNA, and found that the Mx1 protein is a GTP-binding protein with GTPase activity (Nakayama, M. *et al.* (1991) *J. Biol. Chem.* **266**, 21404–21408; Nakayama, M. *et al.* (1992) *Virus Res.* **22**, 227–234). To confirm the conclusion, we purified the native Mx1 protein from poly(I): poly(C)-treated A2G mouse liver. The native Mx1 exhibited GTPase activity, and both the

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K_m and V_{max} are essentially identical with those of Mx1 purified from cDNA-expressing *E. coli.*

As judged from elution profile from gel filtration columns, the Mx1 protein was indicated to form self-assembled polymers. By negative staining electron microscopy, both preparations of the Mx1 protein were observed as big polymers, forming "horseshoe"-like structure (Nakayama, M. *et al.*, submitted for publication). By making a set of deletion derivatives, the motif for self-assembly was mapped between amino acid residues 51–99. This motif is highly conserved in not only Mx family proteins but also Mx-related proteins.

Microscopic analysis of immunostained mouse liver revealed that the Mx1 protein exists as giant complexes in nuclei. These observations altogether suggest that the self-assembly of Mx1 protein is essential for its biological activities including antiviral function.

Molecular Architecture of Rice Stripe Virus: Genome Structure and Role of RNA Polymerase

Akira ISHIHAMA, Chika HAMAMATSU, Ikuo NAKAMURA, Pascale BARBIER*, Mami TAKAHASHI** and Shigemitsu TORIYAMA**

Rice stripe virus (RSV), the prototype of the tenuiviruses, is characterized by its unique genome composition, consisting of four segment each of both single-strand and double-strand RNA. Viral particles are composed of only two viral proteins, 35 kDa coat protein (CP) and a minor polypeptide with an estimated size of 230 kDa, but contain RNA polymerase activity. Terminal sequences of both 5′- and 3′-proximal ends are conserved among four RNA segments, and are complementary to each other (Takahashi, M. *et al.* (1990) *J. Gen. Virol.* **71**, 2817–2821), indicating that RSV is a member of either negative- or ambi-sense RNA viruses. Analysis of the complete sequence of two small RNA segments indicated that both segment 3 and 4 are ambisence, containing an open reading frame at the 5′-terminal region of both vRNA and its complementary RNA (cRNA). This year, we sequenced RNA segment 2 and the results suggest that it is also ambisence RNA (Takahashi, M. *et al.*, submitted for publication).

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As an attempt to reveal the molecular mechanism of virus multiplication, we attempted to purify RNA polymerase and to analyze the mechanism of RNA synthesis using purified RNA polymerase. The RNA polymerase was dissociated from viral RNA (vRNA) by CsCl centrifugation. The solubilized RNA–free RNA polymerase transcribed a model RNA template of 50 nucleotides in length, still carrying the 5′- and 3′-terminal conserved sequences of all four RNA segments (Barbier, P. *et al.* (1992) *J. Virol.* **66**, 6171–6174). A 3′-terminal half molecule of the model template was also active as a template. Hence, we proposed that the 3′-terminal conserved sequence serves as a promoter for the RNA polymerase.

Intron 1 as a Determinant of the Cell Cycle-Dependent Expression of Human Thymidylate Synthase

Atsushi TAKAYANAGI, Sumiko KANEDA, Dai AYASAWA and Takeshi SENO

Thymidylate synthase (TS) catalyzes the only pathway for de novo synthesis of dTMP and plays a key role in balancing the four nucleotide precursors for DNA replication. Impairment of this enzyme causes various biological and genetic abnormalities. TS activity and mRNA content are associated with the DNA synthesis phase in the cell cycle: they increase sharply 20- to 40-fold at the G1-S border during serum-induced transition from the resting (G0) phase to the S phase in cultured mammalian cells.

We cloned biologically active human TS cDNA and the genomic DNA clones covering the entire human TS gene of about 16 kb which is divided by six introns. Determination of their structures enabled us to investigate the region of the human TS gene responsible for S-phase-dependent expression through the construction of various chimeric minigenes for TS and the examination of their mode of expression during the cell cycle. For this, we introduced each construct into a TS-negative mutant of rat 3Y1 cells. Through serum-restricted synchronization of the transformant cells, we examined the expression of the introduced gene during the transition from the G0 phase to the late S phase by measuring TS enzyme activity, mRNA level and transcription rate. We found that a minigene possessing the genomic 5′-flanking region and intron 1 without other introns was sufficient for S-phase specific expression at the normal extent and pattern at both mRNA and enzymatic activity levels. A TS cDNA clone driven by an SV40-

based expression vector showed constitutive expression. Insertion of intron 1 into the cDNA clone in the normal location converted the constitutive expression to a S-phase dependent one, although the conversion was incomplete, that is, a considerable level of enzyme activity and mRNA were still detected in G0 phase cells and also their levels in the S phase were lower than normal. Replacement of the viral 5′-promoter region of the cDNA clone with the genomic 5′-flanking sequence also gave rise to the same change. To obtain the fully cell cycle-dependent expression equivalent to that of the *bona fide* TS gene, however, the coexistence of the two regions was required. Results obtained by a nuclear run-on assay suggested that posttranscriptional controls are also involved in this type of expression.

Regulatory Sequences in the Human Thymidylate Synthase Gene are Clustered at the 5′-End of the First Intron

Sumiko KANEDA, Nobuyuki HORIE, Keiichi TAKEISHI, Atsushi TAKAYANAGI, Dai AYUSAWA and Takeshi SENO

A human thymidylate synthase (TS) minigene containing 5′- and 3′-flanking sequences 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 a much lower frequency. Introduction of intron 1 into the above intronless or intron 2-minigene restored transforming activities regardless of position and orientation. However, introduction of intron 1 into TS cDNA on an SV40-based vector did not stimulate transformation frequency. 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 indicate that the multiple regulatory sequences clustered in intron 1 stimulate TS gene expression in concert with the homologous promoter.

Complementation by a Cloned Human Ubiquitin-Activating Enzyme E1 of the S-Phase-Arrested Mouse FM3A Cell Mutant with Thermolabile E1

Sumiko KANEDA, Takeshi SENO, Dai AYUSAWA, Hideyo YASUDA and Yasuko MURAKAMI

Duringe the genetic characterization of temperature-sensitive mutants of mouse FM3A cells by the cell–cell hybridization test and DNA-mediated gene transfer, we unexpectedly found that an unusually high proportion of these ts mutants, although showing different phenotypes, were of a single complementation group to which the widely used FM3A cell mutant ts85 with a thermolabile ubiquitin-activating enzyme E1 also belonged.

We identified one of the above mutants designated tsFS20, as a mutant with thermolabile E1 through biochemical analysis and transfection with a full-length cDNA encoding the human E1 enzyme. Upon a shift-up of temperature, asynchronously growing tsFS20 cells showed multiple points of cell-cycle arrest as revealed with flow cytofluorometry. When the mutant cells that had been synchronized at the G1-S phase were allowed to traverse at a nonpermissive temperature, the cells accumulated in the mid-S phase. The specificity of the accumulation was further evidenced by the absence of G2-specific cdc2 kinase activity in the arrested cells. In contrast, ts85 cells under the same conditions reached the G2-phase as reported previously and showed an increase of cdc2 kinase activity. Thus, the E1 mutation seems to produce effects differentially, in S-phase progression and possibly in the G2-phase, depending on the type of mutation occurring in the coding region. It should be mentioned in this context that the E1 enzyme is a large protein of 1,058 amino acid residues and could be divided into multiple functional domains including those for interaction with a family of the ubiquitin carrier proteins E2. We also demonstrated genetically that E1 is involved in the degradation of abnormal protein in the cells: degradation of short-lived abnormal proteins in tsFS20 cells decreased to about 50% at the nonpermissive temperature, while the block was fully restored to the wild-type level in transformant cells. The unusually high incidence of the ts E1 mutation could be explained by the fact that we mapped the E1 gene to human X chromosome (Xp11.3–p11.23) and by the recent report that the E1 is a determinant of heat tolerance in cells.

DNA Degradation during Thymidylate Stress is Coupled with DNA Synthesis in Mammalian Cells

Fumiaki YAMAO, Yukiko NAGAI, Sei YOSHIDA, Sumiko KANEDA and Takeshi SENO

Thymidylate stress is known to cause rapid cell death, so called thymineless death. In mammalian cells chromosomal DNA degradation occurs during this stress, resulting in DNA fragments ranging from 50 to 200 kb in length. Previous studies suggest that the DNA cleavage is coupled with the S phase of cell cycle. In order to investigate the molecular basis of the DNA degradation, temperature sensitive growth mutants of the mouse mammary carcinoma cell line FM3A were selected as conditional thymineless death resistants at high temperature(39.5°C). Among the ts mutants were those whose chromosomal DNA is not degradable under thymidylate stress even at a permissive temperature(33.5°C). Consequently, twenty-four mutants were established and clarified into two complementing groups with cell fusion analysis. The mutated gene in one group was identified as that for ubiquitin activating enzyme E1 since mutants were not complemented with ts85, an authentic E1 mutant of FM3A. Simultaneously, another complementing group was found to consist of mutants of the DNA polymerase alpha subunit. A previous analysis of E1 mutants of mouse cell lines suggested that ubiquitination is somehow involved in DNA synthesis and the cell cycle progression of the S/G2 phase. Thus, thymineless death resistance selected mutants deficient in DNA synthesis in mammalian cells, which is in good contrast to mutants of protein synthesis as predominantly selected by the same method in *E. coli*. This indicates that the anomaly of DNA synthesis caused by mutational alteration of the replication machinery suppressed DNA degradation under thymidylate stress. This is in accordance with previous observations that DNA degradation occurs during the S phase of the cell cycle. The results also imply a substantial involvement of ubiquitin in DNA synthesis of mammalian cells.

Structural Analysis of Wild Type and Mutant cDNAs for Mouse Ubiquitin Activating Enzyme E1.

Nobuyuki IMAI, Sumiko KANEDA, Yukiko NAGAI, Takeshi SENO and Fumiaki YAMAO

The ubiquitin system appears essential for cell growth because conditional lethal mutants of enzymes involved in the conjugation pathway have been isolated in mammalian somatic cell lines and yeast. In mouse cell lines, many temperature sensitive growth mutants have been ascribed to a defect in the E 1 enzyme in spite of multifarious phenotypes in terms of arresting points in the cell cycle.

As a first step in clarifying the pleiotrophic effects of E1 mutations on the cell cycle, we isolated functional mouse E1 cDNA from the mouse mammary carcinoma cell line FM3A. The cDNA was screened by hybridization with a cDNA probe specific for human E1. The complete sequence was found to consist of 91 bp of 5′flanking sequence, a complete protein coding region of 1058 amino acids(117.8 kDa), and a 230 bp 3′UTR with poly(A) at the end. Amino acid sequences of mouse and human E1 proteins were identical in their length and shared 95% homology as a whole. The homology of the mouse E1 amino acid sequence to that of yeast and wheat was 53% and 44%, respectively. The cDNA fragment was re-cloned to pSG5, a mammalian expression vector linked to the SV40 early promoter, and transfected to temperature sensitive E1 mutants of FM3A. Although transfection efficiency was rather variable among the mutant lines, most of the growth in the mutant lines was rescued at restrictive temperature. Thus, the cDNA functions well in the cell. Mutations in seven FM3A mutant cell lines were identified in the sequence, all of which were found to be localized in the C terminal half of the E1 sequence.

Kinetic Study on Transcription by Immobilized Operons: Requirement for β,γ-phosphodiester Bond of ATP in Iinitiation by Prokaryotic RNA Polymerases

Tomoko KUBORI, Hiroyuki TERADA* and Nobuo SHIMAMOTO

The requirements for the β,γ-phosphodiester bond of ATP in transcription

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initiation by prokaryotic RNA polymerases has long been questioned. A method we devised for determining these requirements consists of immobilizing template DNA and separating the transcription complex from released products.

A template DNA fragment was fixed to acrylamide beads at its ends with tight binding between avidin and biotin. The immobilized DNA was a template as good as free DNA for *E. coli*, bacteriophage T7, T3, and SP6 RNA polymerases. Transcription was started by adding a set of substrates, and then interrupted by rapidly diluting substrates to 0.1 μM. Elongation was resumed by adding the second set of substrates. Using either ATP or an ATP analogues as one of four substrates, we determined that the β,γ-pyrophosphate bond of ATP increased the yield of long transcripts in the early stage of elongation, but did not affect it much in the late stage. Moreover, abortive initiation was activated with such an ATP analogue. The requirement for intact ATP in productive initiation was found in the all transcription system examined. Thus ATP is likely to be used to keep the elongation complex in productive form in the early period of initiation.

Single-Molecule Dynamics of Transcription by Immobilized Operons: Sliding of RNA polymerase on DNA

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 a critical 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 its contribution to promoter binding is now being evaluated.

Morphological Pathway of Flagellar Assembly in *Salmonella typhimurium*

Tomoko KUBORI, Nobuo SHIMAMOTO, Shigeru YAMAGUCHI*, Keiichi NAMBA**
and Shin-Ichi AIZAWA***

The process of flagellar assembly was investigated in *Salmonella typhimurium*. Seven types of flagellar precursors produced by various flagellar mutants were purified by CsCl density gradient protocol. They were characterized morphologically by electron microscopy, and biochemically by two-dimensional gel electrophoresis. The MS ring is formed in the absence of any other flagellar components, including the switch complex and the putative export apparatus. Four proteins previously identified as rod components, FlgB, FlgC, FlgF, and FlgG, plus another protein, FliE, assemble cooperatively into a stable structure. Hook is formed in two distinct steps: formation of proximal part and elongation. Proximal part formation occurs, but elongation does not occur, in the absence of the LP ring. FlgD is necessary for hook formation, but not for LP-ring formation. A revised pathway of flagellar assembly was proposed based on these and other results.

Structural Study on *E. coli* Single-Stranded DNA Binding Protein

Katsuhiko KAMATA****, Taketomi TSUKIHARA*****, and Nobuo SHIMAMOTO

E. coli Single-Stranded DNA Binding Protein (SSB) is a 19kda 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 by exchanging SSB between replication forks and mRNAs.

As the first step for understanding the mechanism of these characteristic bindings, the protein was over-produced and purified to homogeneity. Crystals of the protein were formed with a salting out procedure and a larger

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crystal suitable for X-ray crystallography was obtained. A diffraction pattern of 3.5 angstrom is now being analyzed. The protein seems to have two structural domains although it is a small peptide.

Negative Supercoiling of DNA Facilitates an Interaction between Transcription Factor IID and the Fibroin Gene Promoter

Mitsuko MIZUTANI, Tsutomu OHTA, Hajime WATANABE*, Hiroshi HANDA* and Susumu HIROSE

Transcription of the fibroin gene can be reconstituted with partially purified components from HeLa cells. Transcription factors IIB, IID, and IIE and RNA polymerase II are required for accurate initiation of transcription. Linear and relaxed closed circular DNA show a similar level of template activity.

However, transcription of closed circular DNA is stimulated when negative supercoils are introduced by the addition of DNA topoisomerase II and a supercoiling factor purified from the posterior silk gland of *Bombyx mori*. Dissection of transcription into pre- and postinitiation steps by the use of Sarkosyl reveals that DNA supercoiling promotes formation of a preinitiation complex. Furthermore, order of addition experiments suggest that DNA supercoiling facilitates a functional binding of transcription factor IID to the promoter. For details, see *Proc. Natl. Acad. Sci. USA* **88**, 718–722, 1991.

DNA Superhelicity Affects the Formation of Transcription Preinitiation Complex on Eukaryotic Genes Differently

Mitsuko MIZUTANI, Kiyoe URA and Susumu HIROSE

In vitro transcription was reconstituted with HeLa cell transcription factors and RNA polymerase II, which were essentially free from DNA topoisomerase activities. DNA templates with defined negative superhelical densities were tested for transcription activity. Transcription of the *Bombyx mori* fibroin gene increases and plateaus from templates of increasing superhelicity, and transcription from the adenovirus 2 major late promoter rises and then falls, while transcription of the *Drosophila hsp70* gene remains unchanged. Dissection of transcription into pre- and postinitiation steps by

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the use of Sarkosyl reveals that formation of a preinitiation complex on the fibroin gene or the adenovirus 2 major late promoter is slow on relaxed DNA and accelerated by DNA superhelicity. On the contrary, the preinitiation complex assembles rapidly on the *hsp70* gene irrespective of DNA topology. As is the case with the fibroin gene promoter, DNA superhelicity appears to facilitate the interaction of transcription factor IID to the adenovirus 2 major late promoter. For details, see *Nucleic Acids Res.* **19**, 2907–2911, 1991.

Possible Role of DNA Topoisomerase II on Transcription of the Homeobox Gene *Hox-2.1* in F9 Embryonal Carcinoma Cells

Kiyoe URA and Susumu HIROSE

The *Hox-2.1* gene is one of the homeobox-containing genes located in the *Hox-2* cluster on mouse chromosome 11. In this study, we examined transcription of the *Hox-2.1* gene during differentiation of F9 embryonal carcinoma cells induced by treatment with retinoic acid. The level of *Hox-2.1* mRNA increases rapidly after induction of differentiation and then falls. Nuclear run-on experiments demonstrate that the rate of transcription for the *Hox-2.1* gene also increases upon differentiation. Treatment of F9 cells with a DNA topoisomerase II inhibitor etoposide (VP-16) during differentiation blocks the accumulation of *Hox-2.1* mRNA. Nuclear run-on analyses revealed that etoposide inhibits transcription of the *Hox-2.1* gene during F9 cell differentiation. Measurements of the level of *Hox-2.1* mRNA after blocking transcription by actinomycin D show that etoposide does not affect the stability of the mRNA. These observations indicate that DNA topoisomerase II is involved in the control of *Hox-2.1* gene transcription. For details, see *Nucleic Acids Res.* **19**, 6087–6092, 1991.

Defining a Sequence Recognized with BmFTZ-F1, a Sequence Specific DNA Binding Factor in the Silkworm, *Bombyx mori*, as Revealed by Direct Sequencing of Bound Oligonucleotides and Gel Mobility Shift Competition Analysis

Hitoshi UEDA and Susumu HIROSE

BmFTZ-F1 is a *Bombyx mori* homologue of FTZ-F1, a positive regulator of the *fushi tarazu* gene of *Drosophila melanogaster*. In order to determine the

recognition sequence of this factor, we made three sets of oligonucleotide mixture which contained 4 possible nucleotides at different positions within the previously proposed 12-bp binding consensus sequence. Oligonucleotides which bound to purified BmFTZ-F1 were separated by a gel mobility shift procedure and a binding sequence was determined by direct sequencing through the Maxam–Gilbert method. With this analysis, 5 positions showed clear sequence preference, 4 positions showed partial sequence preference and 3 positions showed no sequence preference. The importance of each nucleotide at each position was confirmed by a gel mobility shift competition analysis and the results were presented as a quantitative difference in binding affinity. From these analyses, we concluded that the best binding sequence of BmFTZ-F1 is 5′-PyCAAGGPyCPu-3′. This method may be useful for the determination of a binding sequence in other sequence specific DNA binding factors. For details, see *Nucleic Acids Res.* **19**, 3689–3693, 1991.

Search for Target Genes of *Drosophila* Homeotic Genes

Shigeo HAYASHI, Naoyuki FUSE and Susumu HIROSE

Homeotic genes of *Drosophila* play a central role in the specification of body segments. Genes that are controlled by the homeotic genes, the target genes, are not well understood. We are using a genetic and molecular approach to identify such genes. Formation of the anterior spiracle, which is part of the tracheal system, is controlled by homeotic genes in the bithorax complex. The study of genes required for the formation of the anterior spiracle should lead to identification of the target genes. Using an "enhancer trap" screen, we have identified lines which express β-galactosidase specifically in the precursor of the anterior spiracle. Using those lines, genetic and molecular characterization of genes expressed and required in the anterior spiracle are in progress.

Molecular Cloning of *escargot*, a Gene Controlling Segmentation of Larva and Adult.

Shigeo HAYASHI, Allan SHIRRAS and Susumu HIROSE

Mutations in the *escargot* (formally called fleabag) locus cause a variety of defects in adult structures such as malformation of wing and leg and loss of

abdominal cuticular structures. The *escargot* locus is more than 70 kb long but contains a small transcription unit of about 2.7 kb which potentially encodes a zinc finger protein highly homologous to the *Drosophila snail* protein. Abdominal histblasts in *escargot* mutant larva become polyploid, suggesting the normal function of *escargot* is maintainance of ploidy so that histblasts can enter the mitotic cycle during metamorphosis.

FTZ-F1, a Steroid Hormone Receptor-like Protein Implicated in the Activation *of fushi tarazu.*

Giovanni LAVORGNA, Hitoshi UEDA, Joachim CLOS and Carl WU

The *Drosophila* homeobox segmentation gene *fushi tarazu.* (*ftz*) is expressed in a seven-stripe pattern during early embryogenesis. This characteristic pattern is largely specified by the zebra element located immediately upstream of the (*ftz*) transcriptional start site. The FTZ-F1 protein, one of multiple DNA binding factors that interact with the zebra element, is implicated in the activation of *ftz* transcription, especially in stripes 1, 2, 3, and 6. An FTZ-F1 complementary DNA has been cloned by recognition site screening of a *Drosophila* expression library. The identity of the *FTZ-F1* complementary DNA clone was confirmed by immunological cross-reaction with antibodies to FTZ-F1 and by sequence analysis of peptides from purified FTZ-F1 protein. The predicted amino acid sequence of FTZ-F1 revealed that the protein is a member of the nuclear hormone receptor superfamily. This finding raised the possibility that a hormonal ligand affects the expression of a homeobox segmentation gene in early embryonic development. For details, see *Science* **252**, 848–851, 1991.

Accumulation of Wx Protein and Amylose in Rice Seeds Increase in Response to Cool Temperatures

Hiro-Yuki HIRANO and Yoshio SANO

When rice plants are exposed to cool temperatures (less than 20°C) during seed development, the amylose content, relative to total starch, in the endosperm increase causing a lower quality of rice when cooked. We examined this response to cool temperatures with respect to gene expression at the *wx* locus, which controls amylose synthesis in the endosperm.

Rice plants were grown during seed maturation at two different temperatures, 28°C (normal) and 18°C (cool), after anthesis. The amounts of the gene product (Wx protein) of the *wx* locus were estimated by Western blotting analysis. The results indicated that not only amylose content but also the amount of Wx protein increased in seeds matured at 18°C compared with those at 28°C. Then, plants were grown at 18°C for different lengths of time during the period, in which Wx protein accumulated linearly, that is, for 1, 2, 4, 8, or 12 days from the 7th day after anthesis, and then grown at normal temperatures until seed maturation. The longer plants were exposed to a temperature of 18°C, the higher the level of amylose and Wx protein accumulated in mature seeds. The extent of increase in the amylose content was linearly correlated with that in the Wx protein level. These results clearly indicates that the increase of amylose content at cool temperatures is brought about by an elevated level of Wx protein, which catalyzes amylose synthesis.

The regulation responding to a slight difference of temperatures may be very important for the growth and survival of plants. The study on the response of the Wx protein accumulation responding to a cool temperature may provide us one of the best clues for investigating this regulation.

Some Trans-acting Loci (*du*) that Regulate the Expression of the *wx*$^{+}$ Gene in Rice

Hiro-Yuki HIRANO, Haruo SARUYAMA* and Yoshio SANO

The *wx* locus controls amylose synthesis in the endosperm and pollen of rice. Amylose content, relative to total starch, greatly affects seed phenotype; seeds of the wild type look transparent but seeds of *wx* mutants, which contain no amylose, appear non-transparent when they are observed under penetrating light. We found several mutants whose phenotypes were intermediate to the wild type and the null *wx* mutant by screening the mutants induced with EMS.

These mutants were shown to have reduced levels of amylose content (3%–8%), compared with an original wild strain, Norin 8 (16%). Genetic analysis indicated six mutants, designated *dull* (*du*), were independent of the *wx* locus, suggesting that the *du* loci are responsible for regulating *wx*$^{+}$ gene expression or modulating the enzymatic activity of the *wx*$^{+}$ gene product

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(Wx protein). Western blot analysis with anti-serum for Wx protein indicated that the amount of Wx protein was reduced in all mutants although the extent of reduction is variable. This strongly supports the former possibility, namely, the reduced levels of the amylose content are caused by the lower amount of Wx protein in *du* mutants. Northern blot analysis showed that this reduction of the Wx protein level was controlled at the transcriptional level. The *du* loci may encode specific trans-acting factors that activate the transcription of the wx^+ gene.

We analyzed the total protein in mature seeds by two dimensional gel electrophoresis and found that not only Wx protein but also a few additional proteins decreased in *du* mutants. In addition, a few proteins were increased in *du* mutants. These results suggest that the *du* loci may control the synthesis of a few specific proteins including Wx protein through their transcription and may have both the function for activating and for repressing the genes that encode them. The function, which may be positive regulation or negative, may depend on the genes affected. It is of great interest what kind of genes are controlled by the *du* loci and what mechanisms underlie this regulation.

The Promoter of the Rice wx^+ Gene is Functional for Tissue Specific Expression in Pollen in the Transgenic Petunia

Hiro-Yuki HIRANO, Yoshibumi KOMEDA* and Yoshio SANO

Gene expression at the *wx* locus of rice is regulated 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). Of great interest to us is the molecular mechanism of tissue-specific gene regulation in two distinctly different and differentiated tissues. In order to examine whether this mechanism for tissue specificity is functional in a dicotyledonous plant, we introduced recombinant DNA consisting of the promoter region of the rice wx^+ gene and the coding region for bacterial β-glucronidase (GUS) and assayed the GUS activity for monitoring the wx^+ promoter function. The GUS activities in the seed and leaf in all transgenic petunia were as low as that in non-transformed plants. In the pollen, on the other hand, the GUS activities were two to ten fold higher than those of a non-transformant. This result

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indicates that the wx^+ gene promoter in rice is functional in the pollen of the dicotyledonous plant but not in the endosperm, reflecting the difference in the development of endosperm between dicotyledonous and monocotyledonous plants. The molecular mechanism that switches on the wx^+ gene in the pollen and off in the leaf may be common to both monocotyledonous and dicotyledonous plants.

II. MICROBIAL GENETICS

Sequencing Analysis of Primer RNA for Minus Stand Synthesis of Bacteriophage f1

Nahoko HIGASHITANI, Atsushi HIGASHITANI and Kensuke HORIUCHI

The F-specific filamentous bacteriophage f1 possesses a circular, single-stranded genome. Upon infection, the viral single-stranded DNA (plus strand) is converted to a double-stranded replicative form (RF) through synthesis of the complementary (minus) strand. Synthesis of the minus strand is initiated at a unique site (Geider *et al.* (1978) *Proc. Natl. Acad. Sci. USA.* **75**, 645–649) on the single-stranded DNA by synthesis of an RNA primer, which is catalyzed by the host RNA polymerase. The region required for the function of the minus strand origin includes two inverted repeats, which form two possible hairpin structures named B and C.

In order to determine which sequence(s) is essential for the origin function, we constructed a number of deletion and insertion mutants. The results of functional analyses of the mutant origins suggested that deletion of a region in the 3′ end of hairpin C, which contains the previously reported initiation point of the primer RNA (nucleotide 5756 of f1 DNA) (Geider *et al.*, 1978), did not seriously affect the origin function (Higashitani *et al.* (1990) *Annual Rrep.* **41**, 31–32). R411 is such a mutant which carries a deletion ranging from nucleotide 5755 to 5766. Thus, the R411 deletion should include nucleotides that correspond to the first 2 nucleotides of the primer RNA. Yet, R411 showed normal plaque morphology, and grew normally.

These results prompted us to re-examine the start site of the primer RNA synthesis. We determined the sequence of the primer RNA by synthesizing it in the presence of chain-terminating ribonucleotide triphosphate analogues (3′-deoxy NTP's). The results indicated that the primer RNA of R411 had a sequence identical to the wild-type primer: pppAGGGCGAUGGCCCA-CUACGU. The size of the primer RNA was 20 nucleotides and the sequence was complementary to the f1 DNA sequence from nucleotide 5736 to 5717. This meant that the primer synthesis started at a site 20 bases downstream from the site previously reported. Therefore, R411 does not lack the normal

initiation point of the primer RNA.

We also sequenced the primer RNA of an N-specific filamentous phage IKe. The DNA sequence of IKe has a high degree of homology to f1, particularly in hairpins B and C. This suggests that the initiation of complementary strand synthesis in IKe is accomplished by the same RNA polymerase-dependent mechanism (Peeters, B. *et al.* (1985) *J. Mol. Biol.* **181**, 27–39). However, this homology ends at position 5751 of f1, and as a consequence the sequences around the previously reported initiation site are different between f1 and IKe. Our results showed that the sequence of the IKe primer was pppAAGGCGAUGGCCCACUACGU, complementary to the region from the nucleotide 6441 to 6422 of IKe DNA. A high degree of homology between the f1 and IKe primer RNAs is obvious.

In summary, our results indicate that the start site of the RNA primer is located 20 bases downstream from the site previously reported by Geider *et al.* (1978).

A Single Amino Acid Substitution Reduces the Superhelicity Requirement of a Replication Initiator Protein

Atsushi HIGASHITANI and Kensuke HORIUCHI

The origin of rolling circle replication in filamentous coliphage consists of a core origin that is absolutely required and an adjacent replication enhancer sequence that increases in vivo replication 30 to 100-fold. The core origin binds the initiator protein (gpII) which either nicks or relaxes the negatively superhelical replicative form DNA (RFI). Nicking at the origin, but not relaxation, leads to initiation of DNA replication. Our results indicate that the ratio of nicking to relaxation (nicking-closing) *in vitro* depends on the superhelical density of the substrate (Fig. 1). We studied the effect of single amino acid substitutions in gpII, which allow wild-type levels of replication in the absence of the enhancer, on origin nicking and binding. These mutations map to three positions within the N-terminal portion (codon 40, 41, and 73) of gene II (410 codons). The mutation M40I (Met40→Ile; previously called mp-1) has been shown to increase the co-operativity with which the protein binds the origin to form a functional complex for the nicking reaction (Greenstein. D & Horiuchi. K. (1990) *J. Mol. Biol.* **211**, 91–101).

We further characterized the enhancer-independent mutants, and the

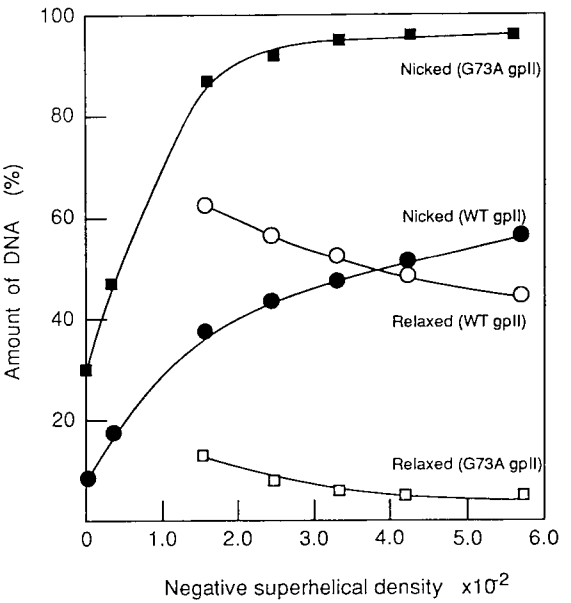

Fig. 1.

nicking activity of an enhancer-independent G73A (Gly73→Ala) mutant gpII. The G73A mutation yielded more nicking and less relaxation of RFI, compared to the wild-type protein. The mutant gpII also showed a reduced requirement for superhelicity of the substrate in the nicking reaction (Fig.1). We propose that the relaxation activity of gpII negatively regulates replication initiation, and that both the increase in the negative superhelicity of the substrate and the action of the replication enhancer may antagonize the relaxation activity.

DnaK Protein Is Involved in Replication of *Escherichia coli* Chromosome by Protecting the Initiation Protein DnaA from Heat Inactivation

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

The DnaK protein is one of the major heat shock proteins of *E. coli* and participates in various cellular functions including phage and chromosomal DNA replication. Although the role of the DnaK protein in λ phage DNA replication is well established, it is not clear how it participates in chromosomal DNA replication. Since a mutant of the *dnaK* gene defective in replication initiation has been isolated, it is probable that the DnaK protein is involved in the initiation of DNA replication. On the other hand, there is an increasing number of evidences which shows that the DnaK protein, acting as a 'chaperone', can protect and reactivate various proteins from heat inactivation. From these facts we postulated that the DnaK protein may be involved in chromosomal DNA replication by protecting the initiation protein DnaA from heat. We examined this possibility by *in vivo* and *in vitro* experiments, and the results obtained are in support of this hypothesis.

In vitro Protein Phosphorylation in *Escherichia coli* Cell Cycle Mutants

Atsushi HIGASHITANI, Seiichi YASUDA, Yukinobu NISHIMURA** and Kensuke HORIUCHI

Protein phosphorylation and dephosphorylation play important roles in the regulation of physiological functions of various proteins in both eukaryotes and prokaryotes. Bacterial phosphorylation systems can be classified into three different types, the classical protein kinase systems, the two-component systems in adaptive responses, and the sugar phosphotransfer systems. In several eukaryotes, *cdc*2 kinase has been identified as a key enzyme commonly involved in the regulation of the cell cycle. In prokaryotes, however, *cdc*2-like kinase has not been defined and kinase systems for cell cycle regulation have not been identified. In order to search for cell cycle-dependent protein

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phosphorylation systems in *E. coli*, we carried out SDS-PAGE analyses of proteins that are phosphorylated *in vitro* in crude cell extracts. The extracts were prepared from logarithmic phase and stationary phase cells, and from cells carrying a temperature-sensitive (ts) mutation in various cell cycle-related genes (*dnaA*, *dnaB*, *dnaC*, *ftsI*, *ftsZ*, unidentified *fts* mutations, and unidentified *par* mutations).

In the wild type cell extracts, we detected about 20 phosphorylated proteins after incubation with γ-^{32}P ATP *in vitro*. Two of them, 18 kd and 10 kd phosphorylated proteins, were identified as phosphotransferase enzyme II (IIIglc) and histidine phosphotransferase (HPr), respectively, by sequencing the amino-terminal amino acids followed by computer analysis. 100 kd and 40 kd phosphorylated proteins were specifically detected only in extracts from the stationary phase. Phosphorylation of three proteins, 29 kd, 12 kd, and 10 kd proteins, also increased in stationary cell extracts as compared with logarithmic phase extracts. The 29 kd phosphorylated protein was identified as the α-subunit of succinyl-CoA synthetase from its amino-terminal sequence.

Extracts from a temperature-sensitive partition mutant *par*530 specifically lacked a 62 kd signal of protein phosphorylation, while its temperature-resistant revertant regained the 62 kd spot. In crude extracts of *dnaA* mutant cells grown at a nonpermissive temperature (42°C), phosphorylation of 58 kd, 34 kd, and 17 kd proteins decreased. The three proteins did not show any changes in the degree of phosphorylation in extracts of the wild type cells or other mutants such as *dnaB*, ftsI, and *ftsZ* cultured at 42°C. The phosphorylation of the 34 kd protein increased upon addition of purified DnaK protein to the *in vitro* reaction with wild-type cell extracts. The DnaK protein is a phosphorylated protein by itself. We are in the process of identifying the phosphorylated proteins described above and their physiological roles.

Osmotic Regulation of Uptake of Long-Chain Fatty Acids in *Escherichia coli.*: *In vitro* Interaction of OmpR Protein with a Fatty Acid Receptor Gene *fadL*

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

Thermosensitive *fabB* mutant of *E. coli* can grow at 42°C when supplemented with oleate. We found that this particular growth was drastically depressed in a medium of high osmolarity. The growth response was apparently dependent on the function of OmpR, which is known to osmotically regulate the biosynthesis of major outer membrane proteins, OmpF and OmpC. The *fadL* gene of *E. coli* codes for an outer membrane protein involved in the uptake of long-chain fatty acids. We investigated whether the uptake of long-chain fatty acids is regulated by environmental osmolarity, and if the *fadL* gene is controlled by the OmpR protein.

The uptake of long-chain fatty acids in *E. coli* decreased when the cells

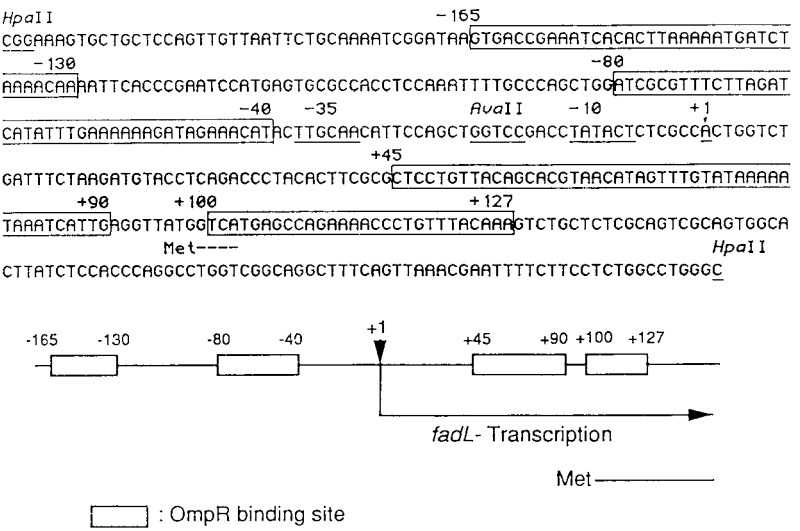

Fig. 2.

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were cultured under high osmotic conditions. Gel retardation assays and DNase I protection experiments showed that phosphorylated OmpR specifically bound to four DNA regions, two upstream and two downstream from the *fadL*-transcriptional start site (Fig. 2). These results suggest that the expression of the *fadL* gene may be both positively and negatively controlled by OmpR-EnvZ proteins in the absence of the FadR repressor.

Correlation of a Subset of the pLC Plasmids to the Physical Map of *Escherichia coli* K12

Akiko NISHIMURA, Kiyotaka AKIYAMA, Yuji KOHARA and Kensuke HORIUCHI

We determined map positions of the *Escheichia coli* K12 portions of a subset of the hybrid *E. coli*-ColE1 plasmids constructed by Clarke and Carbon. The probe DNA of pLC plasmids was labeled with digoxigenine-dUTP, hybridized to the 476 phage clones of the *E. coli* ordered clones bank miniset, which was adsorbed on a strip of nylon membrane filters, and detected by enzyme-linked immunoassay and a subsequent enzyme-catalyzed color reaction. The total number of Clarke-Carbon plasmids we analyzed was 518, for which chromosomal locations of 297 clones were newly determined in the present study. Another 180 plasmids gave results that agreed with those reported previously, and the remaining 41 plasmids gave map positions different from those described in the previous report. A chromosome map of *E. coli* which shows the locations of 518 pLC plasmids on it was presented, as well as a table which correlates the pLC plasmids with the clones of the *E. coli* ordered clone bank miniset on the basis of the hubridization data. We estimate that approximately one-half of the entire genome of *E. coli* was covered by the pLC plasmids used in this study. For details, see *Microbiol. Rev.* **56**, 137–151, 1992.

III. MAMMALIAN GENETICS

Genetic Status of *Mus musculus* Subspecies in Far East Russia

Kazuo MORIWAKI, Hitoshi SUZUKI*, Tsuyoshi KAWASHIMA, Kimiyuki TSUCHIYA**, Hiromichi YONEKAWA***, Ludmila V. YAKIMENKO****, Vladimir P. KORABLEV**** and Alexei P. KRYUKOV****

Our previous survey demonstrated that the Chang Jiang River is a good geographical barrier between the two subspecies of *Mus musculus*, *castaneus* and *musculus*. The former inhabits areas south of the river and the latter areas north of it. In the north western areas such as Xinjian Uygur province, however,the mtDNA RELPs of wild mice are often of the *castaneus* type. This finding suggests the possibility that the *castanceus* subspecies had widely inhabited east Asia including the northern areas in the past. To accumulate more data supporting this notion, the present study attempted to find out whether the wild mice inhabiting Far East Russia are related to the *castaneus* subspecies or not. As summarized in Table 1, the wild mice collected from the Primorye region including Rudnaya and Vladivostok, revealed a definite relationship to the *castaneus* subspecies in mtDNA, rDNA and HbbDNA, though none of them was a complete *Mus musculus castaneus*. The occurrence of *castaneus* genomes in the wild mice collected from Primorye and also those from northern localities in China likely supports the above idea. But reservation is that the Primorye mice could have been transferred from Japan or southern China in the relatively recent past. The molecular comparison of the mtDNA D-loop between wild mice from Primorye and those from Japan is now underway.

In Novii, Teli and Krementi which adjoin northern China, a new allele of Hbb, wl, which was first found in northwestern China (Kawashima *et al.*, 1991, *Jpn. J. Genet* **66**, 491–500), is frequently observed. This may suggest that a similar type of mouse as those in northwestern China in habits these regions of Russia. In those regions, both the mtDNA and rDNA are wholely

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Table 1. RFLPs of mtDNA, rDNA and HbbDNA in house mice collected from Far East Russia

Place of collection	No. of sample	mtDNA			rDNA		HbbDNA			
		cas	dom	mus	mus	mus/cas-dom	d/d	d/p	d/w1	?
North caucasus	1			1	1		1			
Novii	3			3	3		1		2	
Teli	5			5	5		2	1	2	
Krementi	3			3	3		1		2	
Birakan	4			4	4		4			
Rudnaya	2	2			2	4	2			
Gorny	4		2	2	1	6	4			
Vladivostok	7	3		4			6		1	1
Total	29	5	2	22	19	10	21	1	7	1

the *musculus* type. Nevertheless their HBB frequently shows a d-type which could have originated from the *castaneus* subspecies. This seems to again support the above hypothesis.

Male-Specific Suppressor of Meiotic Recombination in the MHC Region

Toshihiko SIROISHI, Tomoko SAGAI and Kazuo MORIWAKI

The wm7 MHC haplotype enhance meiotic recombination at the specific sites, the so called "hotspot" in the proximal region of the MHC. A unique characteristic of this enhancement is sex-specificity in that high rate of recombination frequency is observed only in female meiosis but not in male meiosis. Recombinants generated from the cross involved by the wm7 haplotype retain the ability to enhance the recombination at the hotspot in the same region when their chromosomal segments proximal to the hotspot are derived from the wm7 haplotype. On the other hand, recombinants to carry the wm7-derived chromosomal segment in the distal region to the hotspot lost the ability to enhance the recombination. Taking these facts into account, we concluded that enhancer activity of the wm7 haplotype resides in the region proximal to the hotspot, while suppressor activity is located in the distal region (see *EMBO J.* **10**, 681–686, 1991). In the present study, we attempted to examine whether the recombination suppressor of the wm7 haplotype acts

only in the *cis*-position relative to the enhancer. In order to address this question, we estimaed the recombination frequency of the cross between B10. A(R209) and B10.A(R201) strain. The former mouse retained the activity to enhance recombination but lost the suppressor. The latter mouse has preserved the wm7-derived chromosome in the region distal to the hotspot, in which the suppressor is expected to be present. The result from this mating experiment clerarly indicated that the rate of recombination frequency at the hotspot in female meiosis is as high as the cross between the wm7 and inbred strain. On the contrary, there was no recombination in male meiosis. Thus, it appeared that male-specific suppressor of recombination act both in *cis* and *trans* position relative to the recombination enhancer.

Genetic Organization of the Recombinational Hotsopt within the MHC: A Tight Linkage of the Hotspot to the LMP-2, HAM1 and HAM2 Genes

Tsuyoshi KOIDE*, Toshihiko SHIROISHI and Kazuo MORIWAKI

It is known that homologous recombination during meiosis occurs preferentially at the hotspot between Pb and Ob loci within the MHC region of mouse. Two sequence elements characteristic to the hotspot, MT-consensus and repeat of TCTG tetramer, have been identified at the hotspot. There is a cluster of genes encoding for the MHC class II molecules in this region as well as Pb and Ob. Recent reports have shown that there are several functional genes, such as LMP-2, HAM1 and HAM2 genes, in the same region. Our preliminary southern hybridization analysis of a cosmid DNA including the hotspot indicated that the LMP-2 gene and a part of HAM1 gene are located to the same cosmid clone. Sequencing analysis of this clone demonstrated that the TCTG repeat is adjacent to the LMP-2 gene. It is located to 1.9 kb downstream from polyadenylation signal of LMP-2 gene. Other groups have reported that the HAM1 gene is located to the upstream of the LMP-2 gene in head to head manner. Furthermore HAM2 gene, which is homologous to the HAM1 gene, is located to the just downstream of the HAM1 gene in the same orientation. The LMP-2 gene is considered to be one of the subunits of proteasome having multiple proteolytic activities in

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cells. The HAM1 and HAM2 genes are thought to be required for for translocating peptide fragments of endogenous antigens into the endoplasmic reticulum for association with MHC class I molecules. Because the hotspot is located between the MHC class I gene and genes mentioned above, recombination at the hotspot produce new assortments of the class I molecules and proteins involving antigen digestion, transportation and presentation to the T-cell receptor by the MHC class I molecules.

Gene Dosage Effect of Recombination Hotspots on the Rrate of Recombination Frequency

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

Recocmbinational breakpoints in the MHC class II region are clustered at restricted sites, the so-called recombinational hot spots. At least four different hotspots have been identified in this region. The location of each hotspot differs, depending on the MHC haplotypes in the genetic crosses. For example, recombination occurs preferentially at a hotspot located between the Ab3 and the Ab2 genes when the wm7 and the cas3 MHC haplotypes, both of which are derived from Asian wild mice, are used in the genetic cross, while recombination takes place at another hotspot located between the H-2K and the Ab3 genes when the cas4 haplotype is used in the genetic cross. In these mating experiments, a high rate of recombination frequency at the respective hotspots was observed if one of the parental strains carry the above haplotypes. Thus, we concluded that a high rate of recombination is a genetically dominant trait. In the present study, we attempted to test the gene dosage of the hotspots on the rate of recombination frequency. For this purpose, two genetically different crosses were made. First, the recombination frequency in (wm7×cas3)F1 mice were examined to test the effect of a double dosage of the hotspots at the same position. Second, (wm7×cas4)F 1 were examined for recombination frequency. Our preliminary results indicated that neither of the two crosses yielded augmented recombination frequencies, suggesting that a double dosage of the hotspots does not show an additive effect on the rate of recombination frequency. Further studies are being continued.

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Mouse Visible Mutations of the Intra-MHC Recombinants

Toshihiko SHIROISHI, Tomoko SAGAI, Shigeharu WAKANA* and Kazuo MORIWAKI

Intra-MHC recombinats between wild-derived wm7 and standard laboratory chromosomes have exhibited a high incidence of visible mutation. Since neither of the parental strains, B10. MOL-SGR nor laboratory strains, have yielded any mutation, high mutation rate is characteristic to the intra-MHC recombinants. Preliminary linkage analysis indicated that six independent mutant genes are not linked to the MHC in the chromosome 17. Thus it appeared that the mutations occurred on the genetic background of C57BL/10J strain, because the mouse strain used were B10. H-2 congenic strains. In the present study, we carried out the gene mapping to locate the dominant mutant genes, Ram3, Ram4 and Ram5. The mouse stocks harboring these mutant genes were crossed with DBA/2J strain and resultant F1 mice were successively backcrossed to DBA/2J strain. After the phenotype of the backcross progeny was typed, genetic linkage of respective mutant phenotypes with a series of genetic markers were analyzed based on a length polymorphism of microsatellite sequences which distributes in mouse genome with high frequency. As a result, Ram3 and Ram5 were mapped to chromosomes 11 and 2, respectively. Ram3 is tightly linked to Mpo and Gfap. Gene order and distance between each markers is Mpo-7.4cM-Ram3-4.2cM-Gfap. Ram5 is linked to micarossatellite marker, Mit30. The result of mapping experiment can provide crucial information for guiding molecular cloning of the genes in order to understand the molecular mechanism of a high incidence of mutation in intra-MHC recombinants.

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

Tomoko SAGAI, Norio NAKATSUJI, Toshihiko SHIROISHI and Kazuo MORIWAKI

Embryonic stem (ES) cell lines, established from preimplantation mouse embryos, can colonize both the somatic and germ-cell line ages of chimaeric mice following injection into host blastocysts. These ES cells are accessible for experimental genetic manipulation by homologous recombination in vitro and the modified ES cells can be used to introduce any mutation into the

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germ lines of chimaeric mice.

Such ES cells should provide a useful system for the various studies on B10.H-2 congenic strains. In particular, the study of the molecular mechanism of meiotic recombination in the H-2 region definitely requires this system. Thus far, ES cell lines have been produced from limited mouse strains, mainly 129/Sv, and not from B10.H-2 congenic strains. Therefore we tried to establish new ES cell lines from several different B10.H-2 congenic strains. As the result, we obtained total 46 ES cell lines from 5 B10.H-2 congenic strains (14; C57BL/10, 1; B10.MOL-SGR, 19; B10.A(R209), 7; B10(R233), 5; B10.CAS-TCH). These cells showed fine growth and retained the undifferenciated figure on feeder cells.

For the chimaera formation , the cell lines are desirable to have normal and

Table 1. Karyotype analysis of ES cell line

Exp.	Cell line	Passage	2n%[a]	4n%[b]	Karyotype
1	ES.B10-2	5	92	6	40XY (5)[c]
2	ES.B10-11	7	94	6	40XY (5)
2	ES.B10-12	6	93	7	40XY (6)
2	ES.B10-13	6	90	10	40XY (5)
2	ES.B10-14	7	94	6	40XY (4)
3	ES.SGR-1	8	95	5	40XY (1)
4	ES.R209-1	5	94	6	40XY (3)
5	ES.R209-2	5	93	7	41XY-Ts6[d] (11)
5	ES.R209-4	6	93	7	40XY (4)
5	ES.R209-9	6	91	9	40XY (7)
5	ES.R209-15	7	90	10	40?X? (1)
5	ES.R209-16	4	91	9	40XY (2)
6	ES.R209-17	5	96	4	40XY (3)
6	ES.R209-18	5	88	12	40XY (2)
6	ES.R209-19	6	95	5	40XY (2)
6	ES.R209-20	4	94	6	40XY (3)
7	ES.R233-1	7	98	2	40XY (2)
8	ES.R233-2	5	91	9	40XY (3)
9	ES.R233-6	5	96	4	40XY (5)
10	ES.R233-7	6	94	6	40XY (2)
11	ES.TCH-1	6	89	11	40XY (5), 40?XY (1)
12	ES.TCH-2	8	94	6	40XY (6)
13	ES.TCH-5	5	94	6	40XY (11)

[a),b)] 2n and 4n indicate approximate diploidy and tetraploidy.

[c)] Figure in parentheses represents the number of observations.

[d)] Ts represents trisomy.

male karyotypes. So the karyotypes of 23 cell lines were examined by G-banding method using urea solution. The result showed that 21 of them have normal and male karyotypes and there is no XX cell line. (Table 1)

Generation of a Novel Mutant Mouse by Transgenesis

Hideo GOTOH, Toshihiko SHIROISHI and Kazuo MORIWAKI

The genes for the steroid 21-hydroxylase (21-OHase) and for the fourth complement component (*C4*) are located in the H-2 class III region. Both the genes for 21-OHase and for C4 are duplicated in tandem array. Of the two 21-OHase genes, *Cyp21* and *Cyp-ps*, the *Cyp21* gene is functionally active while the other is inactive. The gene family for *C4* consists of the *C4* and *Slp* genes. Immunogenetic studies have reported that the *C4* gene is haemolytically active, and that the *Slp* gene is inactive.

The C57BL/10SnSlc-*H-2*^{aw18}/*H-2*^{aw18} mouse is lethal in the early postnatal period due to a lack of 21-OHase. We previously introduced DNA fragments of the active form of 21-OHase into the *H-2*^{aw18}/*H-2*^{aw18} homozygous mice, and rescued the lethal mutant mice. The chromosome of the *H-2*^{aw18} haplotype has been reported to have a deletion of about 80 kilobases encompassing the *Cyp21* gene. Further genetic analysis of the rescued mouse has been performed using specific DNA sequences for the *C4* gene and the *Slp* gene. The genome of the transgenic mouse has a DNA fragment specific for the *Slp* gene but lacks one specific for the *C4* gene. Detection of transcripts of the *C4* gene also indicated a deletion of the *C4* gene in the transgenic *H-2*^{aw18}/*H-2*^{aw18} mouse genome.

In humans, several HLA haplotypes containing a null allele for the *C4* gene have been reported. In most cases, homozygocity for the HLA haplotype is related to autoimmune disease, and the correlation between *C4* deficiency and autoimmune disease has been discussed. We produced a novel mutant mouse with a deletion for the *C4* gene through transgenesis. The mouse will provide a novel means to investigate the unknown function of the *C4* protein in relation to autoimmune disease in humans.

Genetic Analysis of Susceptibility to Urethane-induced Pulmonary Adenomas in M.MOL–MSM (MSM) Mice

Nobumoto MIYASHITA and Kazuo MORIWAKI

In mice, it is evident that multiple genes control susceptibility to the development of pulmonary adenomas, because F1 and F2 progeny gave responses intermediate to those of the parental laboratory strains. In crosses between strain A and C57BL mice, resistance to adenoma development in the C57BL strain show marked behavior as a single recessive gene. To analyze the mode of inheritance of adenoma susceptibility, we crossed one of the adenoma-resistant, wild-derived M.MOL–MSM (MSM) strain with the adenoma susceptible A/Wy strain. All of these mice were sacrificed in the fifth month after a single subcutaneous injection of 1.5 mg urethane/g body weight. After the lungs were fixed in ethanol/formaldehyde (9 : 1), the number of adenoma foci were counted.

MSM and A/Wy mice have tumor multiplicities of 0.16 ± 0.06 (range: 0–2) and 27.07 ± 0.96 (10–43), respectively. The F1 generation mice were intermediate to the parental strains in their susceptibility. All of the F1 mice had tumors (range: 1–9) and a tumor multiplicity of 3.91 ± 0.40. A/Wy-backcross mice showed responses intermediate to those of the F1 and A/Wy strain with a range of 4–43. F2 mice showed all gradations in tumor multilicity between the parental strains with a range of 0–27. There is no indication of resistant : susceptible = 3 : 1 distribution as would be expected with a dominant single major factor for resistance in MSM mice. However there is a possibility a major genetic factor expressing no dominance, because 11 out of 56 F2 progeny had no tumors and 14 had more than 9 tumors. Multiplicities of which overlap those of the resistant MSM and susceptible A/Wy mice, respectively.

Comparison of the DNA Sequence in the D-loop Region of Mouse Mitochondrial DNA. II. Two Distinct Clusters in the Fareast Population with *musculus*-type mtDNA

Hiromichi YONEKAWA, Sumiyo WATANABE, Nobumoto MIYASHITA and Kazuo MORIWAKI.

Based on mtDNA haplotype analysis with restriction enzymes, we previously proposed the hypothesis that Japanese wild mouse which had been

recognized as an independent subspecies "*Mus musculus molossinus*", is a hybrid from crosses between ancestral colonies, possibly very small, of *M. m. musculus* and *M. m. castaneus* (Yonekawa *et al.* (1988) *Mol. Biol. Evol.* **5**, 63). To prove this hypothesis, it is necessary to analyze more precisely the population structure of Japanese "*molossinus*" mouse and to examine the evolutionary relationship of mouse populations between Japan and its neighboring countries. Due to very low degree of restriction polymorphism in the Japanese population, it is difficult to analyze them through restriction enzymes. Thus, we introduced a new technique, DNA sequencing analysis combined with PCR technique, to this subject. The D-loop region of mtDNA was chosen for this analysis, because we found that there was a stretch of 200 nucleotides with a very high degree of nucleotide substitutions (approximately 10%) in this region when we compared the entire sequence of mtDNA between *M. m. domesticus* and *M. m. musculus.*

Through this analysis we 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 phylogenic trees using the unweighted pair-groupe 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, Jpn.* **41**, 46–47), although no restriction polymorphisms have been detected in the population (Yonekawa *et al.* (1988) *Mol. Biol. Evol.* **5**, 63).

The next question is how the populations in Japan are evolutionally related to those in its neighboring countries. To address this question, we sequenced 35 mtDNA samples, in total, of *M. m. musculus* collected from China, Korea and Russia including the samples which were collected from Japan and had been sequenced. Using the sequence divergence values among the samples, we constructed a phylogenic tree by UPGMA, and found two distinct clusters, cluster I and cluster II, in the *musculus* populations. Although the degree of sequence divergence values between the two clusters was small, they showed a distinct geographical distribution; e.g. all Japanese mice belong to cluster I, in which all Russian mice are included, whereas all Chinese and Korean mice belong to cluster II. Based on this result, we conclude that Japanese populations with *musculus*-type mtDNA are evolutionally closer to Russian populations than Chinese–Korean ones and the ancestral colony

(ies) of Japanese mice possibly came from Russia.

We also sequenced 18 mtDNA samples of *M. m. castaneus* collected from South East Asia including 5 samples from Japanese mice with *castaneus*-type mtDNA. We have found so far that *M. m. castaneus* did not show any distinct clusters in the phylogenic tree. However, it still remains a question whether or not this is true in populations of *M. m. castaneus*, because *M. m. castaneus* also widely distributes in the Southern part of China with very large populations. To clarify this, the sequence analysis of these Chinese *castaneus* mice is now in progress.

IV. DEVELOPMENTAL AND SOMATIC CELL GENETICS

Cloning of *Hydra* Interstitial Stem Cells Developmentally Restricted to Sperm Production

Chiemi NISHIMIYA-FUJISAWA and Tsutomu SUGIYAMA

Interstitial cells in hydra are undifferentiated stem cells which divide rapidly. At the same time they give rise by differentiation to somatic (nerve, nematocyte and gland) cells in asexual animals, and also to gametic cells in sexually differentiated animals. It is not clear at present whether all members of the interstitial stem cell population can differentiate into both somatic and gametic cells (Bosch and David, 1987), or whether a separate germline subpopulation exists for gametic cell differentiation (Littlefield, 1985).

Strain chim-C1 is a chimeric strain of *Hydra magnipapillata* which contains wild-type epithelial cells and temperature-sensitive interstitial cell lineage (see Marcum, Fujisawa and Sugiyama, 1989). At 18°C, it grows and multiplies by budding normally. If cultured at non-permissive temperature of 25°C, it is expected to lose all interstitial stem cells and their differentiation products (nerve cells and nematocytes), and turn into "epithelial" hydra unable to move or feed.

When Chim-C1 was actually cultured at 25°C for several days, and then returned to 18°C, many polyps turned into epithelial hydra as expected. The same treatment, however, also produced an unexpected type of polyps, termed "pseudo-epithelial hydra". These polyps, like epithelial hydra, lacked nerve cells and nematocytes in the tissue, and could not move or feed. In contrast to epithelial hydra, however, they contained interstitial cells in the tissue. These interstitial cells, however, were apparently unable to differentiate into nerve cells or nematocytes. Similar pseudo-epithelial hydra was also produced from another strain, nem-1, by reducing interstitial cell numbers by hydroxyurea treatment.

Clones of pseudo-epithelial hydra were maintained by force-feeding over 130 days for chim-C1 and over 400 days for nem-1. In both cases, interstitial cells proliferated throughout the entire period without producing any nerve cells or nematocytes. These interstitial cells, however, differentiated into sperms. Thus, the interstitial cells present in pseudo-epithelial hydra were

able to differentiate into gametic but not into somatic cells.

These observations suggest that the interstitial stem cell population in *H. magnipapillata* includes a subpopulation whose differentiation is restricted to gametic cell production. This subpopulation was cloned in this study.

A Nerve Factor(s) Required for Survival and Proliferation of Interstitial Stem Cells in *Hydra*

Toshitaka FUJISAWA

Interstitial cells (I-cells) in hydra are undifferentiated multipotent stem cells which proliferate to maintain their own population as well as differentiate nerve cells and nematocytes during an asexual growth phase. When a small number of I-cells alone are introduced into epithelial tissue which lack all the cell types in the I-cell lineage, the majority of the I-cells cannot survive. In contrast, when I-cells are introduced into epithelial tissue together with nerve cells, they proliferate exponentially. These results indicate that some nerve factor(s) is required for maintaining the I-cell population.

In the present study, such a factor was searched for in hydra extracts by examining whether or not they can support the survival of I-cells introduced into epithelial tissue. It was found that methanol-extracts effectively supported the survival of I-cells. The extracts were further fractionated according to molecular weights. The fraction with a molecular weight of around 1,000 daltons was active. This activity was destroyed by proteolytic enzymes. These results prompted us to test known hydra neuropeptides with a similar molecular weight range; head activator (Schaller and Bodenmuller, 1981) and hydra RF-amide (Grimmerikhuijen *et al.*, 1992). Both neuropeptides used here were synthetic substances supplied by Schaller and Grimmerikhuijen, respectively. The head activator but not hydra RF-amide was found to be active. These results indicate that at least the head activator is an active component of the nerve factor(s) which supports I-cell growth.

Hereditary Mosaic (*mo*) as a Functional Disorder in the Differentiation Process of Meiotic Oocytes in *Bombyx*

Akio MURAKAMI

In one batch culture of F_1 hybrid slikworms from a cross between an *mo*

mutant strain female and a wild-type male, 136 sex-mosaic or gynandromorph moths with both male and female sex organs appeared in addition to 37 females and 28 males among a total of 201 adult moths. The occurrence of gynanders is strong evidence of fertilization, an ovum with a sperm in one part of a mosaic individual and a polar-body with another sperm in the other part, must be involved in abnormal development. The latter case is a result of abnormal fertilization between the sperm and polar-body suggests that the polar-body in this *mo* strain has the ability to attract sperm.

In the course of normal meiotic divisions, a mature oocyte results in two types of cells: an ovum and three polar-bodies. The ovum will unite with a sperm in general, with the polar-body being uninvolved. Accordingly, this biological event principally depends on the ability of the ovum to receive a sperm, but the other meiotic products (or polar-bodies) do not retain this ability. Thus, it can be said that the function of the polar-body in the *mo* mutant strain is different from that in the wild-type. The morphology of the polar-body in insects is identical with that of ova, while the polar-body and ova in mammals are generally different in shape. We can assume that the ovum maintains the function of producing a sperm attractant, while other meiotic products acquire an inhibitory function. Thus, the *mo* gene provokes a functional disorder in the polar-body which usually rejects sperm.

Meiosis is the special process of the reductive division of chromosome number and it also provides for an interchange of segments of the chromosome. Normal meiosis in females seems to have in addition a process of functional differentiation of gametocytes into two types of cells in which one cell or ovum accepts a sperm and the other (or one of three polar-bodies) rejects sperm. In other words, the meiotic process is defined as the ovum maintaining the ability to produce a sperm attracting substance and to accept the sperm, while the polar-bodies suspend the functions which results in the rejection of sperm. If this is the case, it is likely that the action of the *mo* gene involves the hindrance of the normal differentation of oocytes into polar-bodies. In short, the *mo* mutant gene is an impediment factor on the suppression sperm attractant production in polar bodies.

In the *mo* mutant, there often appear polyploids, mostly tetraploid females. This biological phenomenon is an additional type of abnormal development probably due to fusions between the doublly fertilized zygotes mentioned. Spontaneous parthenogenesis in *Bombyx* is a common biological event and the resultant parthenotes developed are mostly triploidal females, suggesting

that this abnormal development is a result of nuclear fusions between the secondary oocytes (probably due to an arrest in meiotic division II) and one of the first polar-bodies depending on affinity (or a flexible discrimination) among the meiotic products. Accordingly, the mechanism responsible for the appearance of polyploids and parthenotes is not the same as that in the double fertilization mentioned.

A Novel Recessive Multistar-spot Mutant (*ms*^{A}) in *Bombyx* and Its Genetic Nature

Akio MURAKAMI and Kiyomi AKUZAWA*

A standard *Bombyx* $+^{P}$ has a pair of larval "star-spot" markings on the 8th segment (numbering is assigned by counting all through the thoracic and abdominal segments in this report), besides a pair of "crescent" markings on the 5th segment. In this insect, a new multistar-spot mutant with a high level of embryonic lethality, fixed as a triplicated star-sport type on segments 6, 7, 8, was found twenty years ago in a commercial Japanese race hybrid line, Miyama and Shinshu (Akuzawa, K., 1972). Since then the multistar has been preserved and several multistar types, 7–8, 8–9, 7–8–9, 6–7–8–9–10, appeared in addition to the original 6–7–8 type during the course of numerous breeding generations. In preservation, this mutant has shown a tendency to select larvae having a greater number of segments marked with star-spots. In spite of continued selections, the multistar types have not remained pure, with a fair number of various multistar types in every generation. In addition, some exhibited an unpaired star-spot on one or more segments in certain cases.

To learn the dominant-recessive relationship for this mutant, a test cross between the standard ($+^{P}$) type females and 6–7–8–9–10 type multistar males was made and the resultant offspring were subjected to genetic analyses.

The results of breeding experiments showed that no multistar type larvae appeared in the F_1 generation, but various multistar types corresponding to a little over 20% of the total observed larvae were detected in the F_2 generation. Lethality in embryos obtained from sibmatings of the hexaplicated

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types was about 50% and that of F_1 embryos between the standard type females and hexaplicated type males was about 35%. These findings and other observations clearly indicate that the gene responsible for the new multistar mutant is recessive and that the chromosome on which the gene is located is accompanied by high lethality, indicating that the mutant gene, ms^A (Akuzawa's multistar), is not the same as those of coventional multistar mutants, *ms* (Tanaka, 1963), *msn* (Chikushi, 1953) and E^{ms} (Tsujita, 1953).

The offspring obtained from sibmatings of the hexaplicated type were cultured and subjected an analysis of the spectrum of star-spot marking patterns. The multistar type on the 6–7–8–9 segments was the most common (62.5%) followed by the 6–7–8–9–10 type (26.7%). The frequencies of the 7–8–9 and 7–8–9–10 types were about 8 and 2.5%, respectively. The 8–9 type was found in only two out of the total 562 larvae observed (0.4%). These observations suggest that the expressivity of star-spot markings on the 10th segment is the most unstable followed by those on the 6th segment. It is natural that the star-spot on the 8th segment is the most stable as the normal (standard) trait. Such a feature in the ms^A mutant appears to be almost the same as *ms* rather than *msn* which only affects the 6, 7 and 8th segments. However, the ms^A mutant seems to be associated with a certain chromosomal aberration similar to the mutant E^{ms}.

From the genetic analysis of the ms^A mutant and other related mutants in *Bombyx*, it became clear that the phenotypic expression of the *ms* gene is limited to five segments, with the 8th segment at the center and including the two infront of it (6–7) and the two behind it (9–10). The star-spot expressivity of the ms^A gene on the 8th segment is the most stable and followed by those of the 7 and 9th segments. The 6 and 10th segments, which are more remote from the 8th segment, are unstable. This variance may depend on the action probability of the *ms* gene on each segment and/or the accessibility of each segment to the gene action, inferring that such differences are essentially connected with the time sequence for segment formation or affinity between segments.

A Consideration of the Hereditary Mode of Feeding Behaviour on Artificial Diets in *Bombyx*

Akio MURAKAMI

The domesticated mulberry silkworm (*B. mori* L.) has widely been viewed

as a monophagous insect which feeds only on mulberry leaves. In 1972, Yokoyama detected a polyphagous line named as Sawa J from among Japanese stocks through persistent selection efforts. This polyphagous stock shows a wide spectrum in feeding on various kinds of plant leaves and even some fruits other than mulberry leaves. In addition, the stock feeds well on either a semi-artificial diet containing a high percentage of powdered mulberry leaves or a specifc artificial diet (LT-1) without the mulberry leaves. Cultured larvae with the artificaial diet can be be raised to the adult stage to some extent. It is worth-while to note that the feeding ability of *Bombyx* larvae on artificial diet is a strong racial difference (e. g., Kanda *et al.*, 1988): the Japanese race has a strong ability as a rule, while the Chinese race is poor. The ability of the European race is differs stock to stock. This racial difference is a strong indication of the participation of some genetic factor(s) responsoble for the feeding behaviour.

According to the reports of Fujimori *et al.* (1982) and Kanda *et al.* (1988), the larvae of Sawa J stock fed well on semi-artificial (ca. 100%) and artificial (ca. 89%) diets, whereas, Kansen, one of the Chinese races, showed a low feeding ability (ca. 16%) on the semi-artificial diet and another Chinese stock, Chinese No. 2, did not feed on the atrificial diet (LT-1). These stock differences among the Chinese stocks in feeding response is due to the quality of diets used for the experiments in relation to the presence of powdered mulberrry leaves. It is also likely that the racial difference between the Japanese and Chinese races in the feeding ability depends on a differential response to the smell as to the taste of the diet. Fujimori *et al.* (1982) indicated that the high feeding ability of Sawa J on a semi-artificial diet was dominant over the low ability of Kansen, while the feeding ability on the artificial diet (LT-1) appeared a recessive in relation to the poor ability of Chinese No. 2 (Kanda *et al.*, 1988). This contradiction may have been due to the constituency of the diets used for the tests.

A number of factors, olfaction, gustation, and other senses are involved in feeding behavior. It is natural to assume that the physical properties of the two types of diets used for the experiments did not markedly differ except for a few factors, especially in the presence or absence of powdered mulberry leaves. Thus it is not unreasonable to suppose that olfaction and gustation are the main factors influencing feeding behaviour.

Newly hatched F_1 larvae obtained from a mating between Sawa J and Kansen stocks fed well on the semi-artificial diet and about 68% of the BF_1

larvae [(S×K)×K] fed on the diet (Fujimori *et al.*, 1982), while the F_1 and BF_1 larvae [(S×C)×C] from a cross of Sawa J and Chinese No. 2 did not feed on the artificial diet (LT-1) (Kanda *et al.*, 1988). From these results, it can be assumed that the Sawa J stock larvae acceptance of the artificial diet is due to dull senses of olfaction and gustation and that the genetic factor responsible for the dull sense of smell is recessive (O^-) and for taste it is dominant (G^+), whereas the Chinese stocks are particularly fastidious about the artificial diet on account of keen senses of smell and taste and the former trait is under the control of a dominant factor (O^+), but the latter trait is recessive (g^+).

Differential Susceptibility of Domesticated *Bombyx* silkworms to the Tachina Fly, *Exorista sorbillans* Wied

J. SHIMADA*, T. OHSHIKI* and A. MURAKAMI

The domesticated mulberry silkworm, *Bombyx mori* L., has been developed from its ancestral form through selection and other ways on the basis of human economic needs since before the time of recorded history, while the non-domesticated mulberry silkworm, *B. mandarina* M., seems to have been free of any domestication. F_1 hybrid obtained from a cross between *B. mori* and *B. mandarina* are fertile so that these can be regarded as subspecies. This infers that their common ancestral form had a number of genetic traits which might more closely resemble those of *B. mandarina* than these of *B. mori.* In the wild, *B. mandarina* frequently suffers from infestations of the tachina fly, *E. sorbillans.* The fly is occasionally parasitic on *B. mori* too. Tachina flies infect *Bambyx* silkworms in the daytime, but not in darkness (Prasad, N. R., 1990), suggesting that the fly's visual recognition plays an important role in host selection, among other factors. Thus, the fly may selectively and/or inherently infect *B. mandarina* or some mutant stocks of *B. mori* through its ability to recognize preferential larval-body marking patterns (texture) as well as colour. If so, by putting its instinct to use, the tachina can detect the larval-body marking pattern and colour of *B. mori* which may closely resemble the ancestnal form of silkworms.

Recently, two papers reported independently that the fly has a preference for the larval-body marking patterns in *B. mori* (Ohsiki *et al.*, 1989; Prasad,

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1990), but there seemed to be no consistent tendency for a preference among the mutant stocks used in their experiments.

The present experiment was carried out to clarify the relationship between susceptibility to the tachina fly and *Bombyx* larval-body marking patterns. Eight different mutant stocks in larval-body marking patterns and colour were used. The p^3 (or p^+) stock was used as the standard type. It has a pair of black crescent markings and a pair of black star spots on a white background p (was plain with no crescent or star spot on a white background). p^M (moricaud), p^S (striped), p^B (black), U (ursa-like body marking pattern), p^{Sa} (this trait, sable, to some extent resembles the larval-body marking of *B. mandarina*) and *Ze* (or zebra) were also used in the present study. The host preference of the fly on *B. mori* larvae at the 5th instar was measured on five larvae for each mutant stock on mulberry treetops in the Tsukui Experimental Farm of Tokyo Univ. of Agric. & Technol. A similar procedure was repeated at least three times for each stock.

The result of the experiments showed that the number of eggs laid on the larval-body surface of the mutant stock by the tachnia fly was greater on the p^3 stock larvae than those of the p^3 stock or the mutant stocks p^M, p^S, p^B and U. The body colour in those mutant stocks (p^M, p^S, p^B and U) is prominently black in comparison with the p stock. The *Ze* mutant was susceptible to the fly to the same degree as the p^3 stock. *E. sorbillans* showed a tendency to more frequently to lay eggs on the mutant stock with the sable marking (p^{Sa}) than on the the normal p^3. This finding suggested that the tachina fly has an instinctive preference for silkworn larvae with a larval-body marking pattern similar to *B. mandarina.* Although the marking pattern of the p^{Sa} resembles that of *B. mandarina*, the colour is to some extent different.

In addition to the experiment above, we also examined whether the fly is susceptible to the tone of the larval marking colour in *B. mori.* There is a positive correlation between colour on larval-body marking in *Bombyx* and the concentration of juvenile hormones during the moulting period (Kiguchi, 1972). On the allatectomized p^{Sa} mutant larvae immediately before the 4th moulting, the body marking pattern darkens in the next instar as compared with the sham operated ones, while the larvae aplied with Methoprene (JHA, 20 μg per capita) just before the 4th molt changed into brownish. The allatechtomized p^{Sa} mutant larvae hardly suffered from infection of tachina fly in contrast to the sham operated ones. The JHA applied larvae also did not show any significant change in infection as compared with the non-

treated group. These findings indicated that the tachina fly has a preference for silkworms having a similar colour of the larval-body marking pattern as *B. mandarina*, but does not have a preference on larvae having an extremely deviated tone of colour from the ordinary trait of the p^M mutant, which is like *B. mandarina*.

Analysis 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 mesoderm cell layer, central nervous system and primordial germ cells. For experimental analysis of this period, we utilized various methods including whole embryo culture, manipulation of cultured embryos, and isolation and culture of embryonic tissues and cells (Nakatsuji, N. and Hashimoto, K. (1991) In "*Gastrulation: Movement, Patterns and Molecules*," 43–56). In collaboration with another laboratory, we studied migration patterns of neuroblasts isolated from the central nervous system (Nagata, I. and Nakatsuji, N. (1991) *Development* **112**, 581–590). We are setting up a new laboratory of mammalian development, in which we are planning to analyze morphogenetic events during the postimplantation period of normal and mutant strain mice, by using whole embryo culture and micromanipulation techniques. Particular attention will be paid to the formation of mesoderm cells at the primitive streak and their migration, the appearance and migration of the primordial germ cells, and differentiation of the central nervous system.

Manipulation of Embryogenesis Using Mammalian Embryonic Cells

Norio NAKATSUJI and Yasuaki SHIRAYOSHI

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

We used embryonic stem cells established from mouse blastocysts for analysis of embryogenesis. We isolated a cell line which expresses β-galactosidase, and made chimaeras using these labeled cells. For example, we

analyzed chimaeric brains to study the migration of neuroblasts during the formation of the cerebral cortex (Nakatsuji, N. *et al.* (1991) *Devel. Growth Differ.* **33**, 571–578).

We also studied primordial germ cells which may be used for manipulation of the mouse germ line. As the 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).

In the new laboratory, we continue to use embryonic stem cells and germ cells for analysis of embryogenesis through manipulation of these cells and their reincorporation into embryos or mice. New attention will be paid to primordial germ cells during early stages in postimplantation embryos and cells in the neural plate and neural tube.

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 new laboratory, we have started experiments to approach such problems from their molecular aspects. One example would be to identify genes involved in determination and differentiation of the central nervous system during the earliest stages in postimplantation stage mouse embryos. As a first step, we have been constructing a cDNA library from 7.5–8.5 day mouse embryos, in which the early events of the central nervous system development are initiated, including neural plate induction and neural tube formation.

A cDNA Project on the Nematode *Caenorhabditis elegans*

Yuji KOHARA, Hironobu MITSUKI and Akiko NISHIGAKI

C. elegans is a small, free-living soil nematode, whose biology has been studied extensively. The accumulated information includes an extensive genetic map which now contains about 1,000 genes, the entire somatic cell

lineage from fertilized egg to adult consisting of 959 cells, the complete neural network at the ultrastructural level, and, more recently, the physical map of the 100 Mb (megabase-pairs) genome. A project to sequence the entire genome is now under way at two laboratories in the U.K. and the U.S..

In order to have easy access to all the genes of this organism and to ultimately understand the network of gene expression in its development, we have started to classify and characterize all cDNA species, whose number is now believed to be around 15,000. To this end, the following systematic strategies were taken.

(1) First of all, abundant cDNA species were identified by picking up the clones which gave strong signals in the hybridization of a worm cDNA library with total cDNA as a probe. The 4 most abundant species, which each occupied 1–2% of the library, turned out to be in the *vit* (vitellogenin) gene family. Dozens of other abundant cDNA species were also identified.

(2) We made a cDNA library (λZAPII vector) from the mRNA of a mixed-stage population which included all developmental stages of this organism. To get as full length cDNA as possible, cDNA longer than 1.5 kb were selected on agarose gel before cloning. On a first attempt, we picked up 8,000 independent clones and stored them separately in the 96-well format. They were gridded onto the lawn of indicator bacteria, blotted onto nylon membranes and probed with the abundant clones to discover and remove the abundant ones. As a result, a subset of some 5,000 less abundant or rare cDNA clones was established and we have started to analyze it using the following procedures.

(a) Restriction mapping: A procedure to efficiently make restriction maps of cDNA inserts was developed, applying PCR with one labeled primer and partial digestion with 4 base cutter enzymes. The maps are used as fingerprints for the classification of clones. This was used because we wished to avoid sorting methods which depend solely on DNA-DNA hybridization, considering the potential importance of the gene family and alternative splicing.

(b) Pattern of expression: We amplified virtually all cDNA species from a series of single embryos of various developmental stages and dot-blotted them onto strips of nylon membrane. The stage of expression of a given cDNA was able to estimate simply by probing the strip with the cDNA clone.

(c) Gene mapping: The position of cDNA clones on the genome were determined by hybridization to the "YAC polytene filter", on which the

ordered 956 YAC clones that covered virtually the whole genome were blotted.

(d) DNA sequencing: An efficient protocol for sequencing a cDNA insert directly from a single plaque was established.

(3) After completion of the analysis of the current set of 8,000 clones, we plan to carry out a subtraction between the current set and the larger number of clones, 100,000 or more, from the same library to find out rarer clones which were not presented in the current set and then to continue the above analyses.

Differential Screening of Founder Cells in *C. elegans* Embryo

Hiroaki TABARA* and Yuji KOHARA

A fertilized egg of *C. elegans* divides unequally to give rise to a larger somatic founder cell named AB and a smaller germ-line daughter P1. The P 1 cell further divides 3 times in a stem-cell fashion to produce three other somatic founder cells, EMS, C and D, and the germ-line precursor cell, P4. The founder cells continue to divide to finally give rise to 959 somatic cells at the adult stage. The somatic cell lineage is invariant and is known in its entirety. Accumulated evidence has shown that the fate of the founder cells can be determined both by internally segregating factors and by cell-cell interactions. The fate-determining factors may be some mRNA, protein or small molecules, but the nature of the factors are largely unknown.

Aiming to identify the fate-determining factors, we have started, as a first step, to search mRNA that segregate unequally among founder cells. For this purpose, we established a procedure to isolate individual founder cells from 2- and 4-cell stage embryos using a micromanipulater. cDNA was made and amplified from individual founder cells. The amplified cDNA, which represents most of the mRNA species in individual founder cells, were labeled and used for screening a cDNA library that had been made from a populatiton of early embryos; most were before gastrulation. In preliminary experiments using cDNA probes from AB and P1 cells, we got many differentially positive signals. Analyses of these clones are in progress.

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

Isao KATSURA and Minoru KAWAKAMI

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 the real target of NaF *in vivo* and to elucidate related 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-1 X*, *flr-2 V*, *flr-3 IV*, *flr-4 X* and *flr-5* (linkage group not yet determined). 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. Double- and triple-mutants in the *flr* genes are resistant to 10 mM NaF and almost normal in growth rate and brood size if they contain both class 1 and class 2 mutations, whereas they have the same phenotype as the single mutants if they contain mutations of only one class. Namely, class 1 mutations are epistatic to class 2 mutations concerning the degree of fluoride-resistance but hypostatic concerning growth rate and brood size. We interpret this relationship by assuming a hypothetical metabolic or transport pathway that confers fluoride sensitivity to *C. elegans*. Class 1 mutations give rise to fluoride-resistance by blocking the pathway. At the same time they cause accumulation of a toxic metabolite or an intermediate state of transport that delays growth and decreases brood size. Class 2 gene products act before the intermediate in the pathway, and hence mutations in those genes restore the growth rate and brood size of class 1 mutants by preventing the accumulation of the hypothetical toxic intermediate. The weak fluoride-resistance of class 2 mutants can be explained by the presence of a parallel bypass to the intermediate.

We cloned a 3.7 kb DNA fragment containing one of the *flr-1* mutation sites by the transposon-tagging method and are determining the nucleotide

* Continuation of the work performed at Department of Biology, College of Arts and Sciences, University of Tokyo in collaboration with Dr. Kazunori Kondo and Mr. Tosikazu Amano.

sequence.

Caenorhabditis elegans Larval Lethal Mutations That Cause Gross Morphological Changes*

Isao KATSURA

We isolated 24 mutants which die as larvae with abnormal shapes, to study the essential developmental genes and their functions at the late embryonic and post-embryonic stages. They were classified according to shape into (1) 2-fold short body mutants (3 alleles), (2) mutants with irregular outer shape (2 alleles), (3) a mutant that dies during the first molt (1 allele, low penetrance), (4) mutants that are bloated around the pharynx (2 alleles), (5) 4-fold mutants (3 alleles), (6) coiled mutants (3 alleles), and (7) mutants in which the outer surface of the intestine gradually detaches from the inner surface of the body wall (10 alleles). These mutations were mapped by cross and complementation with known mutants. They were maintained as balanced stocks using known visible mutations that map near the lethal mutations.

Genetic mapping studies showed that most of the mutations classified under (5) and (6) above are in known muscle-related (*unc-15*, *mup-1*) and neural (*unc-17*, *unc-104*) genes, respectively. Most of the mutants of class (1) to (3) seem to be defective in hypodermis or cuticle, judging from the phenotype. Some of the class (7) mutants map in genes that are thought to act in signal transduction systems, such as *let-23* (tyrosine kinase), *let-341*, and *clr-1*. We are now concentrating our study on mutants of this phenotype.

* Continuation of the work performed at Department of Biology, College of Arts and Sciences, University of Tokyo in collaboration with Dr. Kazunori Kondo and Miss Yuko Kawase.

V. CYTOGENETICS

Mutability of Constitutive Heterochromatin (C-bands) During Eukaryotic Chromosomal Evolution and Their Cytological Meaning

Hirotami T. IMAI

A quantitative analysis of the alterations of constitutive heterochromatin in eukaryotic chromosomal evolution was attempted using the accumulated C-banding data available for mammals, amphibians, fish, ants, grasshoppers, and plants. It was found that these eukaryotes could be classified into two types by their C-banding patterns: Type I including mammals, fish, and ants, and Type II including amphibians, grasshoppers, and plants. C-bands were rather scarce in Type I eukaryote chromosomes and were found around the pericentromeric region when found in Type II eukaryote chromosomes. The Type I and II C-banding patterns can best be interpreted by assuming that in the former group of eukaryotes the saltatory increase in constitutive heterochromatin, occurs preferentially at the pericentromeric regions of telocentric chromosomes induced by centric fission, with C-bands being eliminated almost completely through centric fusion and/or pericentric inversion. On the other hand, C-bands appear in Type II eukaryotes both interstitially and in the telomeric regions of chromosomes, and there may be no effective mechanism for eliminating these bands once they are integrated. For details see *Jpn. J. Genet.* **66**, 635–661, 1991.

Nucleolar Organizer Regions (NORs) in the Australian Ant *Myrmecia croslandi* (=*M. (pilosula) n=1*)

Hirotami T. IMAI, Hirohisa HIRAI*, Yoko SATTA, Toshihiko SHIROISHI, Masa-aki YAMADA and Robert W. TAYLOR**

Localization of NORs on metaphase chromosomes of the Australian ant *Myrmecia croslandi* (*previously discussed* as *M. (Pilosula) n=1*) were examined using the rDNA (*Drosophila*): DNA *in situ* hybridization (FISH method). It was revealed that in individuals with $2K=1M_{(1+2)}+1SM_1+1M_2$

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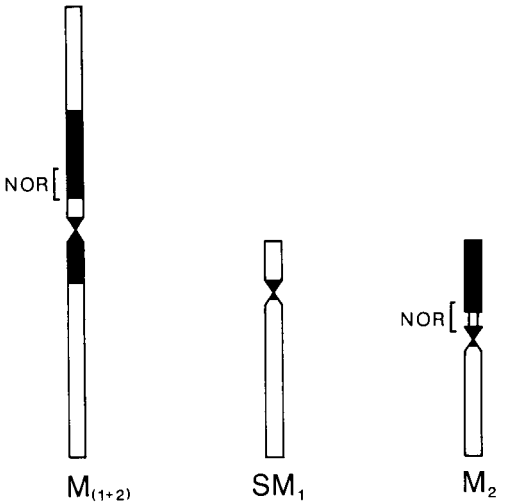

Fig. 1.

(2n=3) NORs are located at the proximal region of the short arm heterochromatin block in $1M_{(1\cdot 2)}$, and at the chromosomal gap in the heterochromatic short arm of M_2 (Fig. 1). These findings were supported cytologically by silver staining, i.e., nucleoli appeared exactly at the expected NOR sites. There was no silver staining at any of the metaphase NORs in this ant, but instead the kinetochores stained faintly.

Cloning of rDNA of the Ant *Myrmecia croslandi*

Masa-Toshi YAMAMOTO*, Keiji OGURA* and Hirotami T. IMAI

We tried to isolate DNA fragments of *Myrmecia croslandi* homologous to rDNA of *Drosophila melanogaster.* Southern hybridization analysis of *M. croslandi* revealed a strongly hybridized 6.0 kb band, possibly a doublet, and a weakly hybridized 7.0 kb fragment when probed with a mixture of internal coding region of 18S and 28S of *D. melanogaster* at a lower stringency. Genomic DNA of *M. croslandi* extracted from a sample HI89-030 with 2n= 2 by Y. Satta (1989) was digested with *EcoRI*, and the DNA fragments were

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ligated into a phage vector λ ZAP II. Eight positive clones were isolated through plaque hybridization using the same probe as that for the Southern blot analysis. Two of them were isolated and purified as recombinant plasmids named pMc·r1 and pMc·r2. Restriction maps of the inserted fragments have been determined. They however both lack a 18S coding region but carry that of the 28S and the spacer region. It may be necessary to clone the entire unit of rDNA repeats in the future but the two clones with the 28S region will be valuable molecular markers for analyses of karyotype evolution in ants.

VI. MUTAGENESIS AND RADIATION GENETICS

Abnormal Differentiation and Associated Radiosensitivity of Erythroid Progenitor Cells in the Wasted Mutant Mouse

Hideo TEZUKA, Dai AYUSAWA and Takeshi SENO

Erythropoietic cell differentiation and its relationship to radiation induced cell death was studied in a mouse mutant, "*wasted*". Previous reports noted that bone marrow cells of the *wasted* mouse became hypersensitive to ionizing radiation in an age dependent fashion, and the radiosensitivity increased four-fold over that in a control mouse with erythroid colony forming units (CFUE) but not with other progenitor cells. Here we report that the increase in radiosensitivity is closely associated with abnormal erythroid differentiation in the *wasted* mouse. Stem cells (day8 spleen colony forming units, an ancestor of CFUE) increased in number, followed by decreasing numbers of the stem cells and CFUE, and lastly CFUE responsiveness to erythropoietin (Epo) decreased. In temporal accordance with the change of Epo responsiveness, the radiosensitivity of the CFUE increased. Experimental results showed that radiosensitivity in the *wasted* and control marrow CFUE is dependent on the Epo concentration in culture, and the radiosensitivity of the *wasted* CFUE is probably due to a decrease in Epo responsiveness in the cells themselves. The *wasted* mutation is closely related to the development of hematopoietic stem cells and erythropoietic progenitor cells. This mutant mouse may be a good model animal for studying the mechanism of hematopoiesis and of radiation induced cell death in mice.

VII. POPULATION GENETICS

Role of Diversifying Selection and Gene Conversion in Evolution of Major Histocompatibility Complex Loci

Tomoko OHTA

Genes at the major histocompatibility complex (MHC) in mammals are known to have exceptionally high polymorphism and linkage disequilibrium. In addition, these genes form highly complicated gene families that have evolved through gene conversion and unequal crossing-over. It has been shown recently that amino acid substitution at the antigen recognition site (ARS) is more rapid than synonymous substitution, suggesting some kind of positive natural selection working at the ARS. It is highly desirable to know the interactive effect of gene conversion and natural selection on the evolution and variation of MHC gene families. A population genetic model is constructed that incorporates both selection and gene conversion. Diversifying selection is assumed in which sequence diversity is enhanced not only between alleles at the same locus but also between duplicated genes. Expressed and nonexpressed loci are assumed as in the class I gene family of MHC, with gene conversion occurring among all loci. Extensive simulation studies reveal that very weak selection at individual amino acid sites in combination with gene conversion can explain the unusual pattern of evolution and polymorphisms. Here both gene conversion and natural selection contribute to enhancing polymorphism. For details, see *Proc. Natl. Acad. Sci. USA* **88**, 6716–6720.

Multigene Families and the Evolution of Complexity

Tomoko OHTA

Higher organisms are complex, and their developmental processes are controlled by the sequential expression of genes that often form multigene families. Facts are surveyed on how functional diversity of genes is related to duplication of genes or segments of genes, by emphasizing that diversity is often enhanced by alternate splicing and proteolytic cleavage involving

duplicated genes or gene segments. Analyses of a population genetics model for the origin of gene families suggest that positive Darwinian selection is needed for acquiring gene families with desirable functions. Based on these considerations, examples that show acceleration of amino acid substitution relative to synonymous change during evolutionary processes are surveyed. Some of such examples strongly suggest that positive selection has worked. In other cases it is difficult to judge whether or not acceleration is caused by positive Darwinian selection. As a general pattern, acceleration of amino acid substitution is often found to be related to gene duplication. It is thought that complexity and diversity of gene function have been advantageous in the long evolutionary course of higher organisms. For details, see *J. Mol. Evol.* **33**, 34–41.

Overdispersed Molecular Clock at the Major Histocompatibility Complex Loci

Naoyuki TAKAHATA

The extent of amino acid differences of major histocompatibility complex molecules within species is unusually high, consistent with the finding that some pairs of alleles have persisted for more than ten million years and the view that the polymorphism has been maintained by natural selecution. The disparity between synonymous and nonsynonymous substitutions in the antigen recognition site, however, suggests that some nonsynonymous sites have undergone a number of substitutions whereas others have little or none. To describe statistically such an overdispersed underlying process, commonly used Poisson processes are inadequate. An alternative process leads to the surprising conclusion that each nonsynonymous site has accumulated as many as 2.6 substitutions, on the average, in the two lineages leading to humans and mice. The standard deviation is also very large (6.6) and the dispersion index (the ratio of the variance to the mean) is at least 17. The substitution process thus inferred qualitatively agrees with the disposition (a boomerang pattern) of substitutions between HLA-A2 and Aw68 alleles, and quantitatively agrees well with that expected where the evolution of major histocompatibility complex molecules has long been driven mostly by balancing selection. (See *Proc. R. Soc. Lond. B* **243**, 13–18, 1991 for details.)

A Trend in Population Genetics Theory

Naoyuki TAKAHATA

Recent studies in theoretical population genetics focus on retrospective properties of stochastic processes occurring in finite populations in conjunction with DNA sequence data from which the ancestry of homologous genes is inferred. Under neutrality (selective equivalence), two stochastic theories (the coalescent and the lines-of-descent) have been developed and extensively applied to various population genetics problems. There can, however, be a third category of such stochastic processes which may be called allelic genealogy that describes the ancestral relationships among different allelic lines. Here, I summarize several results of this stochastic process subjected to fairly strong selection, although neutral allelic genealogy is also considered. (See *New Aspects of the Genetics of Molecular Evolution*, eds. M. Kimura and N. Takahata, pp. 27–47. Japan Sci. Soc. Press, Tokyo/Springer-Verlag, Berlin, 1991 for details.)

Statistical Models of the Overdispersed Molecular Clock

Naoyuki TAKAHATA

The most commonly used statistical model to describe the rate constancy of molecular evolution (molecular clock) is a simple Poisson process in which the variance of the number of amino acid or nucleotide substitutions in a particular gene should be equal to the mean and henceforth the dispersion index, the ratio of the variance to the mean, should be equal to one. Recent sequence data, however, have shown that the substitutional process in molecular evolution is often considerably overdispersed and have called into question the generality of using a simple Poisson process. Several efforts have been made to develop more realistic models of molecular evolution. In this paper, I will show that the spatial (site-specific) variation in the rate of molecular evolution is an improbable cause of the overdispersion and then review various statistical models which take the temporal variation into account. Although these models do not immediately specify what the mechanisms of molecular evolution might be, they do make qualitatively different predictitions and give some insight into the inference. One way to distinguish them is suggested. In addition, effects of selected substitutions

that presumably occur after a major change in a molecule are quasi-quantitatively examined. It is most likely that the overdispersion of molecular clock is due either to a major molecular reconfiguration (fluctuating neutral space) led by a series of subliminal neutral changes or to selected substitutions fine-tuning a molecule after a major molecular change. Although the latter possibility, of course, violates the simplest neutrality assumption, it would not impair the neutral theory as a whole. (See *Theor. Pop. Biol.* **39**, 329–344, 1991 for details.)

Sampling Errors in Phylogeny

Naoyuki TAKAHATA and Fumio TAJIMA

The sampling variance of nucleotide diversity or branch length in a phylogenetic tree constructed by any distance method provides a criterion to judge whether a deduction or an inference made from data is statistically significant. However, computation of the sampling variance is usually tedious, particularly when the number of operational taxonomic units (OTUs) or DNA sequences is large, and must rely on computers. Recently, Nei and Jin (1989) have developed a computer algorithm, but it can be applied only to a simple substitution model. In this paper, we derive simple formulas for the minimum and maximum values of the sampling variance, which are independent of underlying substitution models. Application of these formulas demonstrates satisfactorily accurate estimates of the sampling variances and therefore their practical use. (See *Mol. Biol. Evol.* **8**(4), 494–502, 1991 for details.)

Genealogy of Neutral Genes and Spreading of Selected Mutations in a Geographically Structured Population

Naoyuki TAKAHATA

In a geographically structured population, the interplay among gene migration, genetic drift and natural selection raises intriguing evolutionary problems, but the rigorous mathematical treatment is often very difficult. Therefore several approximate formulas were developed concerning the coalescence process of neutral genes and the fixation process of selected mutations in an island model, and their accuracy was examined by computer simulation.

When migration is limited, the coalescence (or divergence) time for sampled neutral genes can be described by the convolution of exponential functions, as in a panmictic population, but it is determined mainly by migration rate and the number of demes from which the sample is taken. This time can be much longer than that in a panmictic population with the same number of breeding individuals. For a selected mutation, the spreading over the entire population was formulated as a birth and death process, in which the fixation probability within a deme plays a key role. With limited amounts of migration, even advantageous mutations take a large number of generations to spread. Furthermore, it is likely that these mutations which are temporarily fixed in some demes may be swamped out again by non-mutant immigrants from other demes unless selection is strong enough. These results are potentially useful for testing quantitatively various hypotheses that have been proposed for the origin of modern human populations. (See *Genetics* **129**, 585–595, 1991 for details.)

Trans-species Polymorphism of HLA Molecules, Founder Principle, and Human Evolution

Naoyuki TAKAHATA

There are three main hypotheses for the origin of *Homo sapiens*, called the candelabra, multiregional and Noah's Ark. Their essential difference is in the role and extent of gene migration which might have occurred during the Middle and Upper Pleistocene. The candelabra assumes no migration and thus parallel evolution of *H. sapiens* in several regional localities while the Noah's Ark assumes the complete replacement of *Homo erectus* populations in the Old World, around 200,000 years ago, by anatomically modern humans from Africa. The multiregional hypothesis allows continuous but presumably infrequent gene migration throughout the Pleistocene. It had been difficult to distinguish these hypotheses based solely upon morphological characters, because of limited amounts of fossil records and little knowledge about the evolutionary mechanisms. This situation in the study of human evolution has recently been changed dramatically by the introduction of DNA technology. It seems now possible to examine more deeply the history of organisms and the population dynamics including speciation processes. It is argued that although the data of mitochondrial (mt) DNA sequences

sampled from human populations contradict the candelabra hypothesis, they can be consistent with the other alternatives and do not provide further information about what might have happened during the Middle and Upper Pleistocene. In contrast, *HLA* polymorphisms exhibit trans-species modes of evolution and therefore have a great potential in examining the human evolution in much longer time scales. The *trans*-species polymorphisms clearly contradict the extreme form of the Noah's Ark as well as the founder principle as being important in speciation. Combined with DNA sequence data of *HLA*, population genetics theory predicts that the long-term effective size of the human population is of the order of 10^5. This figure is about ten times larger than that inferred from the allele frequency data of other proteins and enzymes, suggesting either that the population had been structured, not to a small extent, until the Late Pleistocene, or that the number of breeding individuals *per se* had been large. (See NATO ASI Series, Vol. H59 *Molecular Evolution of the Major Histocompatibility Complex*, Edited by J. Klein and D. Klein, Springer-Verlag Berlin Heidelberg, 1991 for details.)

Calibrating Evolutionary Rates at Major Histocompatibility Complex Loci

Yoko SATTA, Naoyuki TAKAHATA, Christian SCHÖNBACH, Jutta GUTKNECHT, and Jan KLEIN

Unlike alleles at many other loci, major histocompatibility complex (*Mhc*) locus alleles often differ by nucleotide substitutions at more than one site, often as many as 88 sites. The substitutions accumulate gradually during evolution by the same process that leads to the divergence of genes in two biological species. The difference between the inter-and intraspecific variation is that in the former, substitutions become fixed in the population (reach a frequency of 1.0), whereas in the latter, they reach polymorphic frequencies (≥ 0.01, < 1.0). Since accumulation of interspecific differences is believed by many geneticists to proceed with a clock-like regularity within certain taxonomic groups, there is no a priori reason why the same should not be true for the accumulation of polymorphic differences. Here we demonstrate the validity of this assumption by comparing alleles at the *Mhc-DRB1* and *Mhc-DQB1* loci of different primate species. We then estimate the evolutionary rates at the *DRB1* and *DQB1* loci; the overall rates of these loci are 0.97

± 0.17 and 1.2 ± 0.39 (site/billion years), respectively. However, the rate of the sites (both synonymous and nonsynonymous) encoding the peptide (antigen)-binding region (PBR) is 4 to 7 times higher than in the rest of the gene. As previously suggested, the enhanced nonsynonymous rate at the PBR is most likely due to balancing selection, but the PBR as a whole may be a hot spot of nucleotide substitutions. (See NATO ASI Series, Vol. H59 *Molecular Evolution of the Major Histocompatibility Complex*, Edited by J. Klein and D. Klein, Springer-Verlag Berlin Heidelberg, 1991 for details.)

Persistence of Repeated Sequences That Evolve by Replication Slippage

Hidenori TACHIDA and Masaru IIZUKA*

The evolution of short repeated sequences by replication slippage under the assumption of selective neutrality is modeled using a linear birth and death process. The equilibrium distribution, the distribution of the life expectancy of a repeated sequence when the process starts from two repeats, the age distribution of repeats, the probability of obtaining two genes with i and j copies which diverged t generations ago and the conditional variance of copy number given the repeat number is more than one are computed. The distributions of life expectancy and age are shown to have long tails. Also the statistic which estimates the conditional variance is shown to have a large coefficient of variation. Using these theoretical results, we develop an approximate test of our model and analyze persistent repeated sequences found in the primate β-globin gene region and *Oenothera* chloroplast DNA which are polymorphic within species. We found one sequence in *Onenothera* chloroplast DNA which does not fit to our neutral model. For details, see *Genetics* **131**: 471–478.

Genetic Variability and Geographical Structure in Partially Selfing Populations

Kishiko MARUYAMA** and Hidenori TACHIDA

In order to clarify the effect of selfing on genetic variation in geographical-

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ly structured populations, Wright's island model is generalized to include the effects of extinction, mutation, selfing, and migration of pollen and seed. The equilibrium probabilities of identity by descent for two genes are obtained. It is found that selfing causes a decrease of the variation within colonies and an increase of the variation between colonies. Our model partly explains the "heterozygosity paradox" but other factors such as selection are necessary to fully account for the paradox. For details, see *Jpn. J. Genet.* **67**: 39–51.

Determination of Window Size for Analyzing DNA Sequences

Fumio TAJIMA

DNA sequences are generally not random sequences; the G+C content may not be the same for all regions of a sequence, and there may be conservative and variable regions in it. Such nonrandomness can be tested by using several statistical methods. On the other hand, to show such nonrandomness visually, data are often plotted as moving averages for a certain length of window slid along sequence. For example, if the window size is 5, the first point is the average over nucleotide sites 1–5, the second is that of 2–6, the third is that of 3–7, and so on. In this case, however, the window size is arbitrary and no method is known for determining it. Here a simple algorithm is presented for determining the window size and for finding a nonrandom region of sequence. This method can be applied to various studies in a wide field.

Consider a DNA sequence with N nucleotides. If the window size is L and if the window is moved one by one, then we have $N-L+1$ average points. If these points are independent, we can test whether or not each point deviates from the average over these points in a usual way such as binomial test. When $L=1$, all points are independent, so that we can use an ordinary test. In this case, if C is the confidence level ($=1-$significance level) for the entire sequence, then $C^{1/N}$ is the confidence level for each point. To simplify the algorithm, I suggest $C^{1/N}$ to be used as the confidence level even for $L>1$, although this causes the test to be conservative when the window size is large. The two-tailed test for nonrandomness can be conducted by using the binomial, normal, or Poisson distribution, depending on the data and the accuracy we seek.

To determine the window size, we first compute for each L how many

average points significantly deviate from the average for the entire sequence by using the above algorithm. Then we choose the window size that has the largest number of significantly deviated points. In the case where there are two or more window sizes that have the same largest number, we choose the smallest window size among them. This procedure causes the smaller window size to be chosen, noting also that the expected number of significantly deviated points for the entire sequence is approximately given by $(N-L)(1-C^{1,N})$ if the sequence is made by a random distribution of nucleotides. Since a large window size may overlook small regions which deviate from randomness, this tendency may be preferable. When the sequence under consideration is long, it may be better to divide the sequence into a certain number of units with equal size to simplify the computations. In this case N and L become the total number of units in the sequence and the window size in terms of the number of units, respectively.

The present method for determining the window size and for finding nonrandom regions in the sequence is quite simple, and the computations required are straightforward, so that we can easily apply this method to various cases not only in biology but also in many fields of science. The computer program is available on request. The statistical properties of this method, however, remain to be solved. One way to study them is to use the bootstrap resampling method. In any case there might be room for improvement and more extensive studies on this subject must be done. For details, see *J. Mol. Evol.* **33**, 470–473.

Recent Development of the Neutral Theory

Motoo KIMURA

In sharp contrast to the Darwinian theory of evolution by natural selection, the neutral theory claims that the overwhelming majority of evolutionary changes at the molecular level are caused by random fixation (due to random sampling drift in finite populations) of selectively neutral (i.e., selectively equivalent) mutants under continued inputs of mutations. The theory also asserts that most of the genetic variability within species at the molecular level (such as protein and DNA polymorphism) are selectively neutral or very nearly neutral and that they are maintained in the species by the balance between mutational input and random extinction. The neutral

theory is based on simple assumptions, enabling us to develop mathematical theories based on population genetics to treat molecular evolution and variation in quantitative terms. The theory can readily be tested against actual observations. The neutral theory was proposed nearly a quarter century ago. Since then, strong opposition against the theory has been expressed by the neo-Darwinian establishment. However, supporting evidence for the theory has gradually accumulated as time went on. Particularly, with outpouring of DNA sequence data starting some fifteen years ago, many pieces of favorable evidence for the theory have been obtained. They include such observations as high evolutionary rates of pseudoglobin genes of the mouse, αA-crystallin genes of the blind mole rat and genes of influenza A virus. In addition, it was shown that the evolution of the exceptional, non-universal coding system in *Mycoplasma capricolum*, as discovered by S. Osawa and his group, can readily be explained by the neutral theory. Recently, the famous physicist, F. Dyson proposed a new theory on the origin of life, in which stochastic processes involving neutral evolution plays a crucial role. Also, I recently proposed what I called the "four-stage scenario" theory of macroevolution in which neutral evolution plays an essential role. Based on these theoretical considerations as well as favorable observations for the neutral theory, I claim that neutral evolutionary changes have predominated over Darwinian evolutionary changes, at least in number, throughout the whole history of life on Earth. For details, see Kimura, M. (1991) *Jpn. J. Genet.* **66**, 367–386.

VIII. EVOLUTIONARY GENETICS

Analysis of HLA Data at the 11th International Histocompatibility Workshop

Takashi GOJOBORI, Tadashi IMANISHI, Yasuo INA, Naruya SAITOU, Yoshio TATENO and Hidetoshi INOKO

We tried to compile all the data that were sent to the Central Data Analysis Committee by the participants of the 11th International Histocompatibility Workshop. Although we could successfully list most of the raw data of HLA typing at the serological and DNA levels in the data book, we could not include all score data of serological typing and other information. This is mainly because we had to deal with an extremely large amount of data, too large to be in one volume of reasonable size, and because of a shortage of time for processing the data. Thus, the data book was regarded as a tentative version of the data for the Workshop. It was also possible that the data from some laboratories were missing from the book. Despite the incompleteness mentioned, we still believe that the information in the book was very helpful for discussion by all the participants at the Workshop.

To provide the participants with more comprehensive data, we compiled them in the form of CD-ROM. The CD-ROM was distributed to the all participants together with this data book. It was noted, however, that the data compiled in the CD-ROM were also tentative, possibly including some errors. For details, see the Data Book of the 11th International Histocompatibility Workshop, Volumes 1 and 2.

Evolutionary Origin of Numerous Kringles in Human and Simian Apolipoprotein (a)

Kazuho IKEO, Kei TAKAHASHI, and Takashi GOJOBORI

Human apolipoprotein (a) has a great size heterogeneity and consists of 38 kringle domains in the amino terminal and a serine protease domain in the carboxyl terminal. All but one kringle of apolipoprotein (a) are homologous to the fourth kringle of plasminogen. However, the 38th kringle resembles the fifth kringle of a plasminogen and it seems to have been deleted in simian

species. The phylogenetic trees suggested that an ancestral apolipoprotein (a) may have started with a duplicate of a plasminogen type protein. It also implies that deletion of the three kringles in the amino terminus followed, and that one of the remaining two kringles was duplicated in both human and simian species and the other was processed by a deletion in simian species, after species separation. Thus, the number of kringles in other mammals not yet studied may vary considerably from species to species. For details, see *FEBS Lett.* **287**, 146–148.

Mutation Patterns of Human Immunodeficientcy Virus Genes

Etsuko N. MORIYAMA, Yasuo INA, Kazuho IKEO, Nobuaki SHIMIZU and Takashi GOJOBORI

Human immunodeficiency viruses (HIVs) show extensive genetic variation. This feature is the fundamental cause of pathogenicity of HIVs and thwarts efforts to develop effective vaccines. To understand the mutation mechanism of these viruses, we analyzed nucleotide sequences of *env* and *gag* genes of the viruses by use of molecular evolutionary methods and estimated the direction and frequency of nucleotide substitutions. Results obtained showed that the frequency of changes between A and G was extremely high and the mutation pattern of HIVs was distinct from those of nuclear genes of their host cells. This distinction may be caused by the characteristics of the reverse transcription of HIVs. The mutation pattern obtained would be helpful in constructing effective antiviral drugs. For details, see *J. Mol. Evol.* **32**, 360–363.

Two-Color Cytofluorometry and Cellular Properties of the Urokinase Receptor Associated with a Human Metastatic Carcinomatous Cell Line

Kei TAKAHASHI, Takashi GOJOBORI and Masaki TANIFUJI

Purified human urokinase was labeled with either fluorescein isothiocyanate or iodine-125 and used as a probe for binding to the human metastatic carcinomatous cell line, Detroit 562. Cytofluorometry showed that the ligand bound preferentially to cells that had been exposed to acidic pH. The binding was competitive and decreased after mild tryptic digestion. The bound ligand

could be removed by restoration of the cells to a low pH. Therefore, the cells had specific binding sites. The bound urokinase was involved in the breakdown of fibrin. Two-color cytofluorometric maps were constructed by counterstaining with propidium iodide. Results suggested that there were different cell populations that had different numbers of receptors and amounts of DNA. We cloned cells and found that single clones had homogeneous levels of receptors with different dissociation constants (from 10^{-13} 10^{-11} mol/mg protein) for different clones. Cells of one clone, C5, which had high levels of receptor production, moved characteristically on a glass substratum coated with gold particles and reacted with wheat germ agglutinin, but not with concanavalin A. The receptors were found together with adhesion proteins at the sites where the cells adhered to the substrate. These results and the data obtained by zymography of the cellular proteins suggested that the urokinase-type plasminogen activators were bound to the receptors. The membrane-associated activator may stimulate local proteolysis, facilitating the migration of the tumor cells across the substrate. For details, see *Exp. Cell Res.* **192**, 405–413.

Evolutionary Pathways of N2 Neuraminidases of Swine and Human Influenza A Viruses: Origin of the Neuraminidase Genes of Two Reassortants (H1N2) Isolated from Pigs.

Kuniaki NEROME, Yumi KANEGAE, Yasuyuki YOSHIOKA, Shigeyuki ITAMURA, Masatoshi ISHIDA, Takashi GOJOBORI and Akira OYA

The complete nucleotide sequences of the neuraminidase genes of two reassortants (H1N2) and two H3N2 influenza A viruses isolated from pigs, were determined. The phylogenetic relationships between these genes and the previously reported N2 NA genes were investigated. On the basis of pairwise comparisons of nucleotide sequences, the NA genes of two reassortants, A/sw/Kanagawa/2/78 and A/sw/Ehime/1/80, were most closely related to those of human influenza A virus strains isolated in 1972 and the earliest available swine H3N2 influenza A viruses, respectively. Phylogenetic trees showed that the NA genes can be separated into three groups, (i) swine strains, (ii) the earliest human strain, and (iii) recent human strains. The evolutionary tree for the 11 amino acid sequences suggested that the NAs of A/sw/HK/4/76 and A/sw/Kanagawa/2/78 belong to the lineage of recent

human viruses. In contrast, the NA genes of the A/sw/HK/3/76 and H1N2 reassortant A/sw/Ehime/1/80 viruses were found to be of a swine lineage. The swine virus NA genes were further characterized by the cocirculation of two distinct lineages. Although the rates of synonymous (silent) substitutions for the swine and human viruses were nearly equal (0.00946 to 0.00884 per site per year), the rate of nonsynonymous (amino acid changing) substitutions for swine virus NA genes was about 60% of that for the human virus. For details, see *J. Gen. Virol.* **72**, 693–698.

Molecular Evolution of Hemagglutinin Genes of H1N1 Swine and Human Influenza A Viruses

Shigeo SUGITA, Yasuaki YOSHIOKA, Shigeyuki ITAMURA, Yumi KANEGAE, Keiko OGUCHI, Takashi GOJOBORI, Kuniaki NEROME and Akira OYA

The hemagglutinin (HA) genes of influenza type A (H1N1) viruses isolated from swine were cloned into plasmid vectors and their nucleotide sequences were determined. A phylogenetic tree for the HA genes of swine and human influenza viruses was constructed by the neighbor-joining method. It showed that the divergence between swine and human HA genes might have occurred around 1905. The estimated rates of synonymous (silent) substitutions for swine and human influenza viruses were almost the same. For both viruses, the rate of synonymous substitution was much higher than that of nonsynonymous (amino acid changing) substitution. It is the case even for only the antigenic sites of the HA. This feature is consistent with the neutral theory of molecular evolution. The rate of nonsynonymous substitution for human influenza viruses was three times the rate for swine influenza viruses. In particular, nonsynonymous substitutions at antigenic sites occurred less frequently in swine than in humans. The difference in the rate of nonsynonymous substitution between swine and human influenza viruses can be explained by the different degrees of functional constraint operating on the amino acid sequence of the HA in both hosts. For details, see *J. Mol. Evol.* **32**, 16–23.

Molecular Cloning and Characterization of a Novel Glycoprotein, gp34, that is Specifically Induced by the Human T-Cell leukemia Virus Type I Transactivator $p40^{tax}$

Shigeo MIURA, Kiyoshi OHTANI, Noboru NUMATA, Masaru NIKI, Kazuyuji OHBO, Yasuo INA, Takashi GOJOBORI, Yuetsu TANAKA, Hideki TOZAWA, Masataka NAKAMURA and Kazuo SUGAMURA

We cloned and sequenced a cDNA encoding gp34, a novel glycoprotein expressed in cells bearing human T-cell leukemia virus type I (HTLV-I). HTLV-I has a *trans*-acting transcriptional activator, $p40^{tax}$, that is thought to be implicated in leukemogenesis through the activation of cellular enhancers. With a subline (JPX-9) of the human T-cell line Jurkat, in which $p40^{tax}$ is inducible, gp34 was shown to be of cellular origin and to be transcriptionally activated by $p40^{tax}$. It was also demonstrated that two species of mRNA are generated from one copy of the gp34 gene and that these mRNAs encode the identical gp34 product and differ in the 3′ untranslated region. Analysis of the deduced amino acid sequence of gp34 showed that it lacks typical signal peptides; however, it has a hydrophobic stretch for membrane anchoring and four possible N-linked glycosylation sites at the carboxy-terminal portion, indicating that it belongs to the family of membrane proteins whose carboxy-terminal portion protrudes out of the cell. The gp34 gene displayed relatively delayed induction compared with other genes activated by $p40^{tax}$. Added to the observation of the dependence of gp34 expression on HTLV-I $p40^{tax}$, unlike other $p40^{tax}$-dependent genes, such as those for the interleukin-2 receptor α chain and c-*fos* which are expressed or induced under physiological conditions, these results imply that the mechanism involved in the induction of gp34 expression by $p40^{tax}$ is distinct from and more intricate than those for previously characterized genes. For details, see *Mol. Cell. Boil.* **11**, 1313–1325.

Reconstruction of Molecular Phylogeny of Extant Hominoids from DNA Sequence Data

Naruya SAITOU

Evolutionary distance matrices of the extant hominoids are computed from DNA sequence data, and hominoid DNA phylogenies are reconstructed by applying the neighbor-joining method to these distance matrices. The chim-

panzee is clustered with the human in most of the phylogenetic trees thus obtained. The proportion of the distance between human and chimpanzee to that between human/chimpanzee and orangutan is estimated. Both mitochondrial DNA and nuclear DNA show a similar value (0.44), which is close to values derived from DNA-DNA hybridization data. For details, see *Amer. J. Phys. Anthropol.* **84**, 75–85, 1991.

Statistical Methods for Phylogenetic Tree Reconstruction

Naruya SAITOU

Reconstruction of the phylogeny of organisms is one of the most important problems in evolutionary study. A phylogeny is usually illustrated by a tree-like figure. Thus we call it "phylogenetic tree" or simply "tree." Previously phylogenetic trees were reconstructed mostly by using morphological data. With the advent of the study of molecular evolution, however, it is now customary to construct phylogenetic trees from molecular data, especially from nucleotide sequences. We are therefore concerned primarily with nucleotide sequence data in this review. Theoretical aspects of phylogenetic trees are first discussed, and distance matrix methods and character-state methods are explained in the followings. Lastly, the efficiency of different methods is discussed. For details, see *Statistical Methods for Biological and Medical Sciences* (C. R. Rao and R. Chakraborty eds.), pp. 317–346, Elsevier Science Publishers B.V., Amsterdam, 1991.

Evolutionary Rate of Immunoglobulin Alpha Noncoding Region Is Greater in Hominoids than in Old World Monkeys

Shohji KAWAMURA*, Hideyuki TANABE*, Yuji WATANABE*, Kunihiko KUROSAKI*, Naruya SAITOU and Shintaroh UEDA*

Recent studies on the molecular evolution of primates show that the evolutionary rate among hominoids is considerably slower than that among nonhominoid primates. However, this observation at the nucleotide sequence level is restricted to the β-globin family region. In this study, we sequenced orthologous immunoglobulin alpha ($C\alpha$) genes of chimpanzee, gorilla, orangutan, and crab-eating macaque (an Old World monkey) and

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compared them with that of the human by using noncoding regions for analysis. Since significant differences in rates among hominoids were not found by using the relative rate test, we evaluated the ratio (R) of the evolutionary distance between Old World monkey and human to the distance between orangutan and human. The R value (1.12) for the Cα gene was much smaller than the expected value (1.38–2.33), showing that the nucleotide substitution rate (=mutation rate per year under selective neutrality) of the Cα gene is greater in the human lineage than in the Old World monkey lineage. We also did a similar analysis for the γ1-, γ2-, $\psi\eta$-, and δ-globin genes and found a considerable heterogeneity (1.12–2.37) among the R values, including that for the Cα gene. This indicates that the hominoid slowdown of the evolutionary rate is not a universal phenomenon in primate evolution. For details, see *Mol. Biol. Evol.*, **8**, 743–752, 1991.

Integration of a SINE-like Retroposon into a Specific Locus in Rice Evolution

Hiro-Yuki HIRANO, Kayoko MOCHIZUKI*, Masaaki UMEDA*,
Hisako OHTSUBO*, Eiichi OHTSUBO*,**
and Yoshio SANO

p-SINE*1*, which is found in the *wx* locus of rice (*Oryza sativa*), belongs to a class of retroposon transcribed by RNA polymerase III such as the *Alu* or B1 family in mammals (Umeda, M., Ohtsubo, H., Ohtsubo, E. (1991) *Jpn. J. Genet.* **66**, 569–586). Retroposons transcribed from the master member are believed to be integrated into another position via a cDNA intermediate. This event of retroposition and amplification is quite rare and has been occurred in the evolutionary time scale. The time of retroposition in the evolutionary process can be inferred by analyzing the distribution of a retroposon at a specific locus among related species at present, because retroposons appear not to be deleted. Rice is a very suitable organism for this analysis, because many strains of various species have been collected from all over the world and stocked at the Genetic Stock Research Center in our institute, and their relationships have been well described.

Two members of p-SINE*1* were located at the untranslated 5′-region (p-SINE*1*-r1) and at the 9th intron (p-SINE*1*-r2) of the wx^+ gene of rice.

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We examined the distribution of p-SINE*1* in seven related species carrying the AA genome in the *Oryza* genus by polymerase chain reaction (PCR), and found that a member of p-SINE*1* (p-SINE*1*-r2) was present in only two closely related species, *O. sativa* and *O. rufipogon*, but not in the other species. This result indicates that p-SINE*1*-r2 was integrated into the *wx* locus after *O. sativa* and *O. rufipogon* diverged from the other species with the AA genome. In contrast to p-SINE*1*-r2, another member (p-SINE*1*-r1) at the untranslated 5′-region was present in all species, indicating that p-SINE*1*-r1 has been integrated into that position before the divergence of all rice species with the AA genome. These results, therefore, demonstrate that each p-SINE*1* member retroposed to a specific position at a different period in rice evolution.

Cytogenetic Localization of *Zygotic hybrid rescue* Gene (*Zhr*) of *Drosophila melanogaster* which Rescues Interspecific Hybrids from Embryonic Lethality

Kyoichi SAWAMURA and Masa-Toshi YAMAMOTO

Hybrid females from a cross between *D. melanogaster* males and females of its sibling species, *D. simulans*, *D. mauritiana* or *D. sechellia* die as embryos. Their lethality is supposed to be caused by incompatibility between the X chromosome of *D. melanogaster* and the maternal cytoplasm. *Zygotic hybrid rescue* (*Zhr*) prevents this embryonic lethality and has been genetically mapped at a proximal region of the X chromosome of *D. melanogaster*, and cytologically it has been suggested that it is localized in the centromeric heterochromatin. Thus, we carried out a high resolution cytological mapping using deficiencies and duplications in the X heterochromatin. A deletion of the Zhr^+ gene from a hybrid genome exhibits the *Zhr* phenotype. On the contrary, the addition of the gene to the hybrid genome causes embryonic lethality regardless of sex. In order to minimize variation in tests on the viability of hybrid females, interspecific crosses were performed using *D. sechellia* females and *D. melanogaster* males since escapers rarely emerged from them. The *Zhr* locus has been narrowed down to the region covered by *Dp(1;f)1162* but uncovered by *Dp(1;f)1205*, a chromosome carrying a duplication of heterochromatin slightly distal to the $In(1)sc^8$ heterochromatic breakpoint.

IX. HUMAN GENETICS

Genetic Polymorphisms in the Polyadenylate Tract of Alu Repetitive Elements in the Human Genome

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

As a step toward constructing the complete genetic linkage map of chromosome 18, we made a gene library from a mouse hybrid cell line with an extra human chromosome 18, using the pWE15 cosmid cloning vector. We have attempted to place these clones at distinct bands of chromosome 18 using in situ hybridization techniques and high sensitivity microscopic fluorescent imaging apparatus. Genetic polymorphisms in regions that are identified with these clones were analysed with some of the clone located either on the short or on the long arm of chromosome 18. To identify DNA polymorphism that are abundant in the human genome and are detectable by polymerase chain reaction amplification of genomic DNA, we tested the hypothesis that the polydeoxyadenylate tract of the Alu family of repetitive elements is polymorphic among human chromosomes and found that ten examined thus far were polymorphic. This novel class of polymorphisms is indeed representing one of the most useful and informative group of DNA markers in the human genome. Mapping of the human genome is greatly facilitated by the enormous normal variability of the DNA sequences between two randomly chosen homologous chromosomes. This DNA polymorphism provides the basis for the large number of markers currently being used to construct linkage maps and search for the location of unknown genes that causes hereditary disorders.

In this study we explored the possibilities that the tracts of polyadenylate residues within the 3′ ends of Alu repetitive elements in the human genome contain a variable number of adenine residues and a short reiterated units on the different chromosomes. The Alu sequences examined were isolated from cosmid clones mapped on Chromosome 18p13 region, and those in the HPRT gene region on the Xq28. The locus-specific oligonucleotide primers were synthesized according to the information obtained by sequencing the subclones or by searching for that in the DDBJ and EMBL DNA database. In

order to detect non-radioactively the different alleles, we utilized the method of asymetric polymerase chain reaction followed by SSCP detection on polyacrylamide gel electrophoresis. For the amplification of specific single stranded DNA fragments it was impoartant to use the repetitive and non-repetitive oligonucleotides at a concentration of 1 : 100. Because Alu repeats comprise about 1% of the human DNA, a large number of Alu variable poly (A) polymorphism may exist in the human genome, which should serve as a major marker system in almost any DNA fragment that contains Alu repetitive elements.

Toward a Transcriptional Map of the Human Chromosome 21

Hitoshi NAKASHIMA, Masako SAKAI, Rie INABA and Takashi IMAMURA

The present study is an approach for identifying genes within the human genome. While considerable progress has been made in genetic and physical mapping of the human genome, approaches are now needed to identify the protein-coding sequences contained therein. The ultimate goal would be an integrated genetic, physical and transcriptional map of the genome, combining information in the position of a gene, its sequence and its pattern of expression. Three basic techniques have been used to identify protein-coding sequences from genomic DNA: particular restriction enzymes such as NotI can be used to search for regions rich in CpG residues (HTF islands), which are frequently associated with the 5′ ends of constitutively expreessed genes; genomic DNA of different species such as mouse can be probed with a genomic clone (zoo blots) to detect highly conserved sequences, which are likely to be protein-coding; and northern blots and cDNA libraries can be directly probed with a genomic clone. We chose for this study the cosmid clones of genome DNA that have been mapped by the method of fluorescent *in situ* hybridization on the long arm of chromosome 21 (21q22). Among 8 of these clones examined, 5 were apparently expressed in the mouse brain, liver, and kidney cells. Interestingly, these clones positive for zoo blot testings had at least one NotI site within the insert. Although the results await further confirmation, these clones may possibly encode genes on the human chromosome 21.

While these strategies are feasible for small number of genomic clones, new approaches are necessary for rapid detection of transcribed sequences in

longer streches of DNA, whole chromosomes or entire genome. Other approaches to generate region-specific cDNA libraries used hybridization of cDNA inserts to genomic DNA from specific chromosomes, and subsequent cloning of annealed cDNAs. In one application of this scheme, the human brain cDNA library was hybridized to biotinylated DNA fragments from the human-mouse hybrid cell line (NA08854) containing chromosome 21 as the only human chromosome. Hybrid molecules were then immobilized on the magnet-coated beads, non-specific hybrids were eliminated, and selected cDNA inserts were amplified by PCR before cloning. Using this strategy, substantial enrichment of a low abundance cDNA encoded by a gene on a human chromosome 21 was performed. Because of the problem of carry-over of non-specific cDNAs, the cDNA library has to be subtracted for cDNA clones encoded by the human genomic material. We will continue this line of research, aiming that the resulting cDNA library will consist of expressed sequences from one chromosome. Each clone may then be mapped on the restricted part of the chromosome by *in situ* hybridization.

Beta-Thalassemia Major Resulting from Compound Heterozygosity for the β-globin Gene Mutation: Further Evidence for Multiple Origin and Migration of the Thalassemia Gene

Hitoshi NAKASHIMA, Yutaka CHIFU and Takashi IMAMURA

Thalassemias are a heterogeneous group of inherited disorders of hemoglobin synthesis, all being characterized by the absence or reduced output of one or more of the globin chains of hemoglobin. This leads to an imbalanced globin chain synthesis. Thealassemias are the most common single gene disorder in the world population. Genetic studies at the DNA level disclosed more than 40 different molecular varieties of thalassemia. Thus, it is common for individuals to receive genes for more than one type of thalassemia. We have encountered a patient with severe hypochromia and hemolysis resulting from heterozygosity for two types of β-thalassemia. One mutation was a C-to-T transition at IVS-2 nucleotide position 654 on the backgroud of Mediterranean RFLPs haplotype IX. Another mutation was a G-to-A transition at IVS-2 nucleotide position 1, associated with a novel haplotype XI. The occurrence of these mutations on various chromosomal backgrounds provides strong evidence for an interplay of gene migration, inter-

allelic gene conversion, and multiple origins of the same mutation.

The IVS-2 654 mutation is commonly found in the Chinese population, associated with either haplotype I or II, which predicts the β-globin gene framework (Fr1). We have previously reported the same mutation in a Japanese family, which was associated with framework (Fr1) on the background of haplotype I. Although the presence of IVS-2 654 mutant in two different haplotypes I and IX is consistent with recurrence of the mutation, interchromosomal recombination between and β-globin genes is more likely in view of the nonrandom assortment of the 5′ and 3′ collection of polymorphic restriction sites in the β-globin gene cluster. Interestingly, all three haplotypes I, II and IX found so far associated with the IVS-654 mutation are grouped into the same β-globin gene framework (Fr1). Thus, the presence of the same mutation in a restricted region of the world (China and Japan) might be explained by gene migration in populations, and subsequent interallelic gene conversion or recombination 5′ to the β-globin gene changing haplotype linkage. The IVS-2 1 mutation has been found in several Mediterranean families associated with haplotypes I (Fr1), III (Fr1a) and V (Fr2), but until now there had been no report in Asiatic population. The occurrence of identical mutation on different ethnicity, and the association of the mutation with a variety of frameworks strongly suggest multiple, independent origins of this mutation.

Time of the Deepest Root for Polymorphism in Human Mitochondrial DNA

Masami HASEGAWA and Satoshi HORAI

A molecular clock analysis was carried out on the nucleotide sequences of parts of the major noncoding region of mitochondrial DNA (mtDNA) from the major geographic populations of humans. Dates of branchings in the mtDNA tree among humans were estimated with an improved maximum likelihood method. Two species of chimpanzees were used as an outgroup, and the mtDNA clock was calibrated by assuming that the chimpanzee/human split occurres 4 million years ago, following our earlier works. A model of homogeneous evolution among sites dose not fit well with the data even within hypervariable segments, and hence an additional parameter that represents a proportion of variable sites was introduced. Taking account of

this heterogeneity among sites, the data for the deepest root of the mtDNA tree among humans was estimated to be 280,000±50,000 years old (±1 SE), although there remains uncertainty about the constancy of the evolutionary rate among lineges. The evolutionary rate of the most rapidly evolving sites in mtDNA was estimates to be more than 100 times greater than that of a nuclear pseudogene. For details, see *J. Mol. Evol.* **32**, 37–42, 1991.

A New mtDNA Mutation Associated with Mitochondrial Myopathy, Encephalopathy, Lactic Acidosis and Stroke-like Episodes (MELAS)

Yu-ichi GOTO, Ikuya NONAKA and Satoshi HORAI

MELAS (mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes) is a clinical entity of mitochondrial diseases. Recently, a transition mutation in the mitochondrial $tRNA^{Leu(UUR)}$ gene was found in 26 of 31 MELAS patients and by a comparative analysis on the affected and normal cultured muscle cell lines from a patient. We identified another 9 MELAS patients and found that 32 of the 40 patients (80%) had this mutation, but the remaining 8 patients did not. We, therefore, tried to detect other molecular abnormalities specific to MELAS. Sequence analysis was performed in all mitochondrial tRNA regions as described previously, and we found T-to-C transition mutation at nucleotide position 3271 in three unrelated patients.

To develop a simple test to detect this mutation, we synthesized a modified PCR primer, with a G-to-T mismatch at bp3275 and a T-to-C at bp3276. If there was a transition mutation at bp3271, the PCR products (170 bp) with this mismatch reverse primer and the forward primer, could be cleaved into two fragments (140 and 30 bp) by endonuclease Afl II because a new recogmition site emerged. This test revealed that three patients had mutation DNA in heteroplasmic fashion. However, 32 MELAS patients with the most common mutation at 3243, 6 MERRF (myoclonus epilepsy associated with ragged-red fibers) with the mutation at 8344, 32 and 6 CPEO (chronic progressive external ophathalmoplegia) with and without large scale deletions, respectively, and 50 normal controls from different racial groups did not have this nutataion.

The nucleotide in the anticodon stem are not invariant in contrast to the

most common mutation in the dihydrouridine loop during evolution. However, the preserved nucleotides forming the anticodon stem are complementary to each other, e.g., nucleotides T at 3271 and A at 3261 in normal human individuals. As the tRNA with the most common mutataion at 3243 in MELAS patients is deprived of the hydrogen bond to T-residue in the amino-acid acceptor arm, the new transition should have a similar adverse effect on the structure and the function of tRNA Leu(UUR). Accordingly, this mutation is thought to be an additional candidate for genetic defects in MELAS. For details, see *Biochimica et Biophysica Acta* **1097**, 238–240, 1991.

Heteroplasmy and Polymorphism in the Major Noncoding Region of Mitochondrial DNA in Japanese Monkeys: Association with Tandemly Repeated Sequences

Kenji HAYASAKA, Takafumi ISHIDA and Satoshi HORAI

We have sequenced the major noncoding region of mitochondrial DNA (mtDNA) of four Japanese monkeys and have found length polymorphism in the sequenced region. The length polymorphism resulted from two tandem duplications of 160-bp sequences which contained the conserved sequence blocks 2 and 3 and the light-strand transcription-promoter region. We also found polymorphisms in this mtDNA region among 100 Japanese monkeys from 12 localities, 90 of which were analyzed using DNA amplified through the polymerase chain reaction. In two localities, we found individuals with heteroplasmic mtDNAs which had different numbers of the 160-bp repeats mentioned above. The 100 samples were classified into six types in term of length and presence/absence of the recognition site of two restriction enzymes in the major noncoding region. For details, see *Mol. Biol. Evl.* **8**(4), 399–415, 1991.

A New Type of Mitochondrial DNA Deletion in Patients with Encephalomyopathy

Shigeaki MIYABAYASHI, Hiroshi HANAMIZU, Hitoshi ENDO, Keiya TADA and Satoshi HORAI

We performed Southern blot analysis of muscle mitochondrial DNAs from

47 patients who were clinically diagnosed as having mitochondrial disorders, and found deletions in five patients. Four patients with mitochondrial DNA deletion had muscle weakness, myoclonic seizure, sensorineuronal hearing loss, brain atrophy, delay of neurological development or neurological deterioration, but neither external ophthalmoplegia nor ragged-red fibers in biopsied muscle tissues. The sites of deletions in mtDNAs from KSS were clarified at hotspot regions found by Schon *et al.* or between the nucleotide positions from 5,770 to 16,020. The size of deletion ranged from 1.3 to 7.6 kb. The deletions were flanked mainly by perfect direct repeats, located at the edges of the deletion. The deleted mtDNAs from our patients were larger in size, and surprisingly included the origin of light-strand replication in three patients. The D-loop region was preserved as well as that in the previously described patients with mtDNA deletion. Three patients with the deletion including the origin of light-strand replication were characterized as having an early onset of symptoms before the age of one year and a subsequently severe clinical course. The pathogenesis of the mitochondrial DNA, including the origin of light-strand replication, was unclear. However, these deleted mitochondrial DNA may be made during the process of the replication of normal mitochondrial based on abnormalities within the nuclear DNA or on derangements of the "cross-talk" between the nuclear and the mitochondrial genomes, because they are thought to be unable to replicate in vivo. For details, see *J. Inher. Metab. Dis.* **14**, 805–812, 1991.

Phylogenetic Affiliation of Ancient and Contemporary Humans Inferred from Mitochondrial DNA

Satoshi HORAI, Rumi KONDO, Kumiko MURAYAMA, Seiji HAYASHI, Hiroko KOIKE and Nobuyuki NAKAI

Nucleotide sequence analysis of the major noncoding region of human mitochondrial DNA (mtDNA) from three major races was extended with data from 27 contemporary Mongoloids (20 from Southeast Asia, 7 from America) and 11 ancient Japanese bones (5 from Jomon Age; 3,000–6,000 years B.P., 6 from the early modern Ainu; 200–300 years B.P.). In both cases, the sequence was determined directly from the PCR products. Based on a comparison of the 482-bp sequences from a total of 128 contemporary humans, the nucleotide diversity is estimated to be 1.46%, which is three

times higher than the corresponding value estimated from restriction-enzyme analysis of the whole mtDNA genome. The phylogenetic tree revealed that all lineages are classified into at least five clusters designated as C1 to C5. C 1 consists exclusively of Africans, and the majority of Asians and Europeans formed C2, C3, C5 and C4, respectively. Phylogenetic analysis also indicated that part of the Asians, including the Japanese, subsequently diverged from the majority of Africans, and that overall Asians can therefore be separated into two distinct groups. Native Americans, on the other hand, appeared only in C3 and C5, suggesting that the size of the founder population was not so large during the peopling of America. Nucleotide sequences derived from ancient bones in a highly polymorphic region were also compared with those of contemporary humans. The nucleotide diversity among the 139 sequences in the region was estimated to be 2.26%. A group of ancient Japanese, including both Jomon peoples and the Ainu, showed a close phylogenetic affiliation with one group of contemporary Japanese and Southeast Asians. Moreover, all of the ancient Japanese were clearly placed in a larger cluster, indicating their genealogical difference from another group of contemporary Japanese. This observation supports the immigration theory, which postulates that immigrants from the Asian continent made a considerable contribution to the formation of modern Japanese. Finally, the presence of an Asian-specific deletion was examined through PCR analysis of native American samples. The deletion was found in one mummy sample. This, together with the present sequence analysis and published information, clearly shows that the Circum-Pacifican populations (Asians, Oceanians and native Americans) can be separated into two distinct mitochondrial lineages. For details, see *Phil. Trans. R. Soc. Lond. B* **333**, 409–417, 1991.

Extracellular Matrix Protein Tenascin-Like Gene Found in the Human MHC Class III Region

Ken-ichi MATSUMOTO, Nanayo ISHIHARA, Asako ANDO*, Hidetoshi INOKO* and Toshimichi IKEMURA

We previously reported a cluster of fibronectin type III repeats located ca. 20–50 kb centromeric of CYP21B in the class III region which showed sequence similarity with human tenascin. Because of the high degree of

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similarity and general parallelism of their organizations, the existence of a tenascin-like gene was proposed. Tenascin is a multi-domain and multi-functional extracellular matrix glycoprotein and is made up of heptad, EGF, and fibronectin type III repeats, and a fibrinogen domain, from the N- to C-terminus. If the newly found gene in MHC is actually a tenascin-like gene, heptad and EGF repeats may exist upstream of the clustered type III repeats. Both heptad and EGF repeats, showing evident similarity with those of tenascin, were found in the 2.6 kb sequence ca. 60 kb centromeric of CYP21 B. There were 4 heptad and 18.5 EGF repeats. This work thus presented evidence that a tenascin-like gene exists in the human MHC class III locus. We recently found RNA transcripts from this gene and designated the gene as "tenascin-MHC". The existence of an extracellular matrix protein gene in the MHC locus is of interest because not yet identified disease-susceptible genes for such A diseases as narcolepsy are expected to be present within or near the MHC class II region. For details, see *Genomics* **12**, 485–491 (1992) and also *Immunogenetics* (1992, in press).

Giant G+C% Mosaic Structures in the HLA Locus and a Border between the Mosaic Domains

Ken-ichi MATSUMOTO, Nanayo ISHIHARA, Asakao ANDO*, Hidetoshi INOKO* and Toshimichi IKEMURA

We previously showed that the genomes of higher vertebrates are composed of G+C% mosaic structures and that the mosaic structure is a factor producing G+C% diversity at the codon third position. The correlation of this mosaic structure with chromosome band structures and with the 'isochore' proposed by Bernardi was also established. An example of borders of the giant G+C% mosaic structures of the human genome was identified fairly precisely between class II and III of the MHC locus: The contiguous classes I and III belong to the evidently GC-rich domain which appears to correspond to T band sequences, and the G+C% levels of class II were significantly lower than those of classes I and III. Thus a border between the G+C% mosaic domains was assigned at the junction area between classes II and III. About 350 kb of this junction area has not yet been sequenced, and we are now attempting to clone the entire junction in order to clarify the

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characteristics of the border region of the G+C% mosaic structures (e.g., how sharp the boundary is and how precisely it can be defined). At present, the border was localized within 100 kb. For details, see *HLA1991* (ed. T. Sasazuki) Oxford Univ. Press, in press.

X. APPLIED GENETICS

Responses of Wild and Cultivated Rice Populations to Four Different Races of Pathogen *Zanthomonas campestris*

Hiroko MORISHIMA and Toshie MIYABAYASHI

Comparative studies of host-pathogen interaction in natural and agricultural ecosystems can provide perspectives on the evolutionary dynamics of plant-pathogen systems. We are studying the genetic variation of wild and cultivated rice populations in response to infection by *Xanthomonas campestris* pv. *oryzae* (*Xco*). *Xco* causes serious disease in rice plants (bacterial leaf blight), and different pathogenic races are known.

We have already reported that wild and landrace populations of rice are more or less polymorphic in response to *Xco*. In 1990–1991, a number of populations collected in Thailand and Bangladesh were tested for resistance to four different *Xco* races which are the major pathogen races prevailing in Japan. These plant materials were collected in delta areas in Thailand and Bangladesh which are deeply flooded in rainy season. Deepwater condition should favor epidemics this disease.

It was found that perennial populations of Thai wild rice adapted to deepwater condition are generally resistant to all four races, while annual-type populations adapted to drier condition in the same area contained individuals susceptible to different races. In the Bangladesh materials, ten different infection types in response to the four races were found. Cultivars grown in rainy season (aman) tended to show higher resistance than those grown in dry season (boro). Weedy wild rice populations associated with aman cultivars, which are most probably hybrid derivatives of natural crosses between wild and cultivated rices, were found to be highly polymorphic in responses to four *Xco* races. The host-pathogen interactions we studied are interactions between Japanese pathogens and exotic plants which have never encountered each other. Yet, the above results indicate that the genetic variation dealt with in this study has been subjected to certain kinds of natural selection. Further study is planned using pathogen races prevailing in the area from which host plants were collected.

To determine the genetic basis for this disease resistance, resistant and

susceptible plants sampled from an annual wild population were crossed, and the F_2 population was investigated. It was confirmed that at least one dominant major gene responsible for resistance segregated. In addition, parent-offspring regression was detected in a cultivar population which showed continuous variation in resistance. This suggests that polygenes controlling quantitative resistance exist.

Constraints in Using Wild Relatives in Breeding: Lack of Basic Knowledge on Crop Genepools

Yoshio SANO

The utilization of alien germplasm became an urgent problem after recent developments in technology. Abnormalities in hybrids have resulted from disharmonious interactions of genes expressed in various developmental stages. Genetic comparisons of internal barriers suggest that different species carry genes leading to genetic discontinuities. A profound barrier can develop from an accumulation of genes with relatively small effects. Gene complexes often show complicated features of incompatibilities depending upon the accumulated genes. Despite the complexity of developmental constraints, adequate selection makes it possible to transfer a simply inherited gene from distantly related taxa.

Plant breeders use local elite lines rather than wild relatives since it is not easy to select favorable gene combinations through hybridization. The nature of the primary genepool in crops predicts the importance of gene flow between subdivided populations. Isolating barriers act as genetic sieves which protect balanced gene sets for the developmental patterns preserved in species. Nevertheless gene flow through the genetic sieve seems to play a significant role in crop evolution. Law genetic materials supplied from alien species could be utilized for the acquisition of new characteristics with other genetic modifications, even though it may appear slightly deleterious. Local cultivars have probably received some alien genes accompanying genetic assimilation under natural conditions. If so it may be easy to utilize such genes in crop improvement. When we consider that genetic erosion is accelerating all over the world, there is no reason why we should wait to utilize alien genes until the primary genepool absorbs them by means of naturally occurring hybridization and selection. This study will appear in

International Crop Science I (1992).

Inheritance of Low Temperature Resistance at the Young Seedling Stage in Rice

Hiroko Morishima

The Japonica and Indica types are the two major varietal groups of Asian cultivated rice which can be distinguished by several diagnostic character and by genes. The former type is known to be less damaged by low temperature treatment followed by exposure to high temperature at the young seedling stage, than the latter type is. Strains of wild rice (*Oryza rufipogon*), except for some Chinese strains, are generally more susceptible to the above-mentioned treatment than cultivars.

An upland cultivar collected in Yunnan, China, was found to be highly polymorphic in various Indica-Japonica diagnostic character including the above mentioned low temperature resistance, within the population. To study the inheritance of these key characters, five lines derived from different individuals of the original population sample were intercrossed, and six F_2 populations were studied regarding low temperature resistance. Five to ten progeny seedlings (1.0–1.5 cm length) derived from each F_2 plant were kept at 0–1°C for 4 days and at 30°C for the following 3 days. The degree of injury was scored individually, dividing into 4 classes (0 : resistant–1–2–3 : susceptible), and the average value was used as the index for resistance of the corresponding F_2 plant. Each F_2 population tended to show a bimodal segregation. Four susceptible (S) × resistant (R) crosses segregated into resistant and susceptible plants giving 10R : 54S or 1R : 3S ratios, an S × S cross giving a 1R : 15S ratio, and a T × T cross giving a 7R : 9S ratio. These results suggest that recessive genes with a complementary nature are responsible for this resistance. If we assume that when at least two out of three loci carry recessive alleles in a homozygous state the carrier exhibits resistance, Then the segregation patterns observed in the six F_2 populations can be consistently interpreted.

To confirm the genetic basis of this resistance, further analysis is under way using F_3 lines. Various types of low temperature resistance so far reported in rice seedlings are all under the control of a single dominant gene. The resistance dealt with in this report seems to be controlled by a different genic

system.

Genetic Control of Fertility Reversions in Cytoplasmic Male Sterility of Rice

Yoshio SANO, Mitsugu EIGUCHI, and Hiro-Yuki HIRANO

The present study was carried out to examine the genetic mechanism responsible for reversions to a fertile phenotype which were detected in cytoplasmic male-sterile plants of rice. The *cms-bo* cytoplasm of Chinsurah boro II gave rise to male-sterility in plants without a gametophytic restorer gene (Rf_1). Taichung 65 (T65A) is known to be a maintainer which carries no restorer. However, Taichung 65 preserved in our laboratory (T65B) showed partial fertility (about 8% seed set) when crossed with male-sterile plants. Unexpectedly, the seed fertility gradually increased with repeated selfings and almost fully fertile plants were obtained in the F_6 generation. Cytoplasmic substitution lines revealed that reversions to a fertile phenotype resulted from mutational events at the nuclear level. Genetic experiments indicated that the partial fertility observed in the F_1 hybrid was controlled by a dominant gene, Ifr_1, which was carried by T65B. The results obtained suggested that Ifr_1 itself is associated with the instability of fertility restoration in the presence of *cms-bo* cytoplasm, since partially fertile plants carrying Ifr_1 always showed a tendency toward gradual increases in fertility in later generations. Backcrossings are under way to introduce Ifr_1 into male-sterile plants. Although the backcross generation is still BC_2 at present, the resultant plants with Ifr_1 have shown a gradual increase in selfings.

Pollen gametophytic selection is apparently more effective than sporophytic selection in leading to fertile revertants. An advantage of gametophytic selection may be minimization of segregational cost. Such an intensified selection could be carried out automatically in the case of self-fertilizing plants. New mutations could be effectively selected for and maintained in the presence of *cms-bo* cytoplasm only through intensified gametophytic selection, otherwise they quickly become extinct. This might give one of the possible explanations for diversified genotypes preserved in plant populations in relation to complex features in fertility relationships. Whatever the mechanism for instability is, the present results confirmed that intensified gametic selection combined with instability leads to a rapid change in the

genetic content of rice. For details, see *Genetical Research* **60** (No. 3), 1992.

Japonica Rice was Born in East China

Y. I. SATO, X. TANG*, L. YANG** and L. TANG***

The middle and lower basins of Yangtze river are argued to be the place of origin for cultivated rice. We examined ancient rice spikelets excavated at Homedu site in Zhejiang province (as old as 7,000 yrs.) to examine whether wild rice existed at this site. It was found under a scanning electric microscope (SEM) that five out of eighty-five spikelets examined, had characteristics of wild rice, although previous observations had not indicated this. Some other seeds showed combined characteristics of wild and cultivated rices, suggesting the frequent occurrence of natural hybridization between wild and cultivated rice or the existence of an intermediate type.

Archaeological records of the excavations in China show that initial rice cultivation began in the middle and lower basins of Yangtze river seven to nine thousand years ago. Records from tropical Asia suggests that it was practiced also in multiple areas of the tropics. Rice cultivation appears to have begun at different sites in Asia independently.

Data taken from the genetic viewpoint also support a hypothesis of a diphyletic origin of Asian rice. Restriction fragment length polymorphisms (RFLPs) of chloroplast DNAs suggest that *indica* and *japonica* evolved from different strains of on ancestral species, *O. rufipogon*, and that strains of *O. rufipogon* having *japonica*-like chloroplast DNAs are largely inhabitants of China (Dally and Second 1990). Combining our results and previous work, we propose a new hypothesis that *japonica* was born in the middle and lower basins of Yangtze river seven to nine thousand years ago.

Developmental Regulation of Phenotypic Plasticity in Deepwater Rice

Mitsugu EIGUCHI, H.-Y. HIRANO and Yoshio SANO

Plants utilize environmental signals to alter the timing of gene expression so as to respond to changing environments. A drastic change in morphology

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and physiology due to changing environments is known as phenotypic plasticity which is particularly important in plants because of their sessile life style. Although phenotypic plasticity has been assumed to play a significant role in evolution, more information is needed about the genetic control of plasticity as well as plastic changes which occur during ontogeny.

Submergence in water induces drastic changes in the morphology of deepwater or floating rice, including a rapid internodal elongation. A floating habit is widely observed in *Oryza rufipogon* which is the wild progenitor of Asian cultivated rice, *O. sativa*. The wild progenitor shows an annual-perennial continuum and the perennial type generally grows in deep water, while the annual type grows in temporal swamps which are parched in the dry season. Experiments were carried out using a perennial wild rice accession with deepwater tolerance and a non-deepwater rice cultivar. Selection for deepwater tolerance was performed under 1 m-deepwater conditions and backcrosses were made up to the BC_5 generation to transfer a gene(s) for deepwater tolerance into the intolerant cultivar. The results indicated that a recessive gene (dw_3) was responsible for deepwater tolerance and plants with dw_3 induced internodal elongation in response to submergence rather than enhancement of elongation. Plants without dw_3 showed no internodal elongation and soon died.

A most striking difference between submerged and air-grown T65dw_3 was the number of elongated internodes. When submerged before 10 weeks of age, 8 to 9 internodes elongated. After 10 weeks of age the number of elongated internodes became almost the same (5 to 6) as that of air-grown plants. Panicle primordia covered with bract hairs were first recognized on shoot apices at 11 weeks of age in both air-grown T65 and T65dw_3, suggesting that floral initiation occurred between 10 and 11 weeks of age. After floral initiation, elongation of developing internodes was similarly enhanced by submergence, irrespective of the presence or absence of dw_3. This indicated that the dw_3 gene changes the timing of internodal elongation depending on environmental conditions in the vegetative phase. A mutation changing the relative timing of developmental events is known as a heterochronic gene. Plants are metameric or modular in their growth and the plant body is indeterminant in its growth, giving an opportunity for a high level of plastic response to environments. The elongation patterns of T65dw_3 in response to flooding markedly changed around the time of floral initiation. This suggests that plastic response by dw_3 in rice is restricted to the vegetative phase or to

the stage prior to the determination of panicle formation in the shoot apical meristem. This study will appear in *J. Heredity*, 1993.

An Altitudinal Cline of Genetic Variation Found in Bhutan Rice Cultivars

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

Rice plants are cultivated in diverse environments ranging from low lying delta to mountainous highland. One-hundred and fifty eight rice strains collected in three major valleys in Bhutan (1200–2600m alt.) were investigated regarding various key characters and genes which have been used for distinguishing the two subspecies Asian rice (*indica* and *japonica*). Samples contained cultivated as well as weedy (naturally propagated in cultivated fields) types of rice.

The following were pointed out.

1. Samples from high altitudes (2200 m<), which were collected in Paro and Thimpu valleys, tended to have *japonica* characters, such as $KClO_3$ resistant, and negative phenol reaction (*ph*), while samples from lower altitudes (1600 m>), collected in Punakha valley, tended to have *indica* characters.
2. Isozyme analysis clearly separated all the samples into *japonica* and *indica*. It was confirmed from this analysis that samples from high altitudes were mostly *japonica* and those from low altitudes were mostly *indica*.
3. Apiculus hair length, which is another *indica-japonica* key character, did not show altitudinal variation.
4. In $KClO_3$ resistance, the frequency of the *ph* gene and length of the basic vegetative phase, variations in cultivar populations and in coexisting weedy populations were positively correlated among collection sites, but no such trend was found with apiculus hair length and grain shape.

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On the Distribution Patterns of Selfing- and Outcrossing-Inclined Genotypes in Plant Populations

Katsuei YONEZAWA

Development of spatial patterns in plant populations of selfing-inclined (highly fertile with selfing) and outcrossing-inclined (showing no or low fertility with selfing) genotypes was theoretically investigated based on a one dimensional population model where three genotypes, i.e., selfing-inclined (aa), outcrossing-inclined (AA), and intermediate (Aa) genotypes were distributed with decreasing population density from central toward peripheral regions. Mating among plants was assumed to be random with a limited dispersion distance for pollen and seeds. The equilibrium distribution of the genotypes was calculated based on an initial state of the three genotypes being randomly distributed in the population. Four major types of distribution patterns were derived according to combinations of the parameters constituting the population model; in two of the four, selfing- or outcrossing-inclined genotypes occupied the entire population (gene a or A became fixed), and in the other two, selfing- and outcrossing-inclined genotypes coexisted in a mixture, or separately with selfing-inclined genotypes predominating in peripheral regions. It was found that the distribution pattern was determined primarily by the dispersion distance of pollen rather than that of seeds. Population size also regulated the spatial pattern. Populations with a large size tended to be more selfing-inclined.

Analysis of Chloroplast DNA from Ancient Rice Seeds

Ikuo NAKAMURA, Wen-Bing CHEN and Yo-Ichiro SATO

We have reported that DNA could be extracted from ancient rice seeds preserved under anaerobic conditions and the extracted DNA could be useful as a template for PCR amplification (*Ann. Rep.* **41**). We could specify the subspecies of the ancient rice into *indica* or *japonica* by the amplification of a particular chloroplast DNA fragment from the ancient rice seed.

Restriction fragment length polymorphisms of chloroplast DNA between *indica* and *japonica* were reported by Ishii *et al.* (1988) and Dally and Second (1990). We focused on the Pst-12 fragment of *japonica* chloroplast DNA, because the RFLP data suggested the presence of a small deletion inside this

fragment of *indica* chloroplast DNA. Two oligonucleotides (5′-AGTCC-ACTCAGCCATCTCTC-3′ and 5′-GGCCATCATTTTCTTCTTTAG-3′) were synthesized and used as primers for PCR reactions. A 1.0 kbp fragment was amplified from *japonica* cultivars and a 0.9 kbp fragment was found in *indica* cultivars. The detail restriction analysis of amplified fragment showed that a small deletion of about 100 bp was present in *indica* chloroplast DNA, corresponding to the ORF 100 region of *japonica* chloroplast DNA. We considered this small deletion a convenient DNA marker for distinguishing the subspecies of ancient rice or rice cultivars, because rice chloroplast DNA is transmitted by maternal inheritance and no recombination events between maternal and paternal chloroplasts is known.

DNA was extracted from a total of 11 ancient rice seeds, 90–1300 years old, which were excavated and found from four different archaeological sites in Japan and about 1 ng of DNA was used as a template for PCR reactions. Amplification of chloroplast DNA fragments was carried out in a thermal programer for 42 cycles under these conditions: 1 min. denaturation at 92°C; 1 min. annealing at 37°C; and 2 min. polymerization at 72°C. Aliquots of four μl were analyzed by agarose gel electrophoresis. The results showed that a 1.0 kb fragment was detected in all ancient rice seeds, revealing that all 11 ancient rice seeds had *japonica* type chloroplast.

Many ancient rice seeds and plant remains have been excavated from archaeological sites in east and south Asia. In the Tai-hu area of China, there are many excavations where rice seeds 5,000–7,000 years old have been found. If the genotypes of such ancient rice seeds could be identified by the DNA analysis described as above, it would greatly contribute to the investigation of domestication of wild rice and varietal diversification of rice cultivars.

An Accelerated Internode Overgrowth Mutant in Rice

Ikuo NAKAMURA

A rice mutant of potential biochemical interest, showing greatly accelerated internode overgrowth characteristics, was isolated in the cultivar Koshi-hikari, following treatment with γ-irradiation.

This mutant named Awa-odori was isolated as follows; forty-thousand M_3 seeds which were descendants derived from seeds irradiated with 25 kR or 30

kR γ-ray were kindly provided by Professor H. Nakai of Shizuoka University. These seeds were directly and densely sown into a paddy field and grown to maturity. A total of 21,190 panicles, with a single mature panicle derived from each stock, were collected and then ten seeds were randomly picked from every panicle. The resulting *ca* 200,000 M_4 seeds were germinated in plastic cases and screened for stature of seedling growth. Only one plant showed extreme extension growth of the internode and leaf at the seedling stage. This mutant plant did not form an panicle or panicle-like structure, but it remained alive for a period as long as normal plants. Since the mutant could not produce seeds, two heterozygous plants were identified in the same panicle from which the overgrowth mutant was derived. A progeny test of heterozygosity yielded a segregation ratio of 3 normal: 1 mutant, indicating a monofactorial recessive inheritance of the mutant phenotype.

The mutant phenotype is characterized by very rapid extension growth of internodes and by extremely long and narrow leaf blades both in the seedling and adult plant stages. The mature plant is tall, slender, extremely lax, and unable to support itself. At the four leaf stage the mutant plants are up to 2.5 times as tall as these with a normal phenotype. The phenotype of Awa-odori mutants resembles that of the *slender* mutant in barley (Foster 1977). Awa-odori mutant plants like barley *slender* do not respond to exogenous gibberellin, whereas non-mutant sibs respond in the usual manner with rapid stem and leaf sheath extension growth occurring. Mesocotyle and first and second internodes elongate in the *slender* mutant, but these organs do not show such elongation in the Awa-odori mutant. In the case of Awa-odori, the higher internodes over second node show rapid extreme extension growth.

XI. DATABASE

DNA Database Release 10 from DNA Data Bank of Japan

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

The DNA database Release 10 includes 59,317 entries which correspond to 77,805,556 bases. Unlike past releases, this release contains the newest data prepared by DDBJ, GenBank and the EMBL Data Library as of January, 1992. Thanks to international collaboration between the three data banks, the International Nucleotide Sequence Database was organized, resulting in a unified database published in this release.

All the entries designated by the accession numbers with a "D" have been collected and processed by DDBJ, and the rest have been prepared by GenBank and the EMBL Data Library. It should particularly noted that due to unresolved technical problems, at present some entries in the three databases overlap. We will resolve the problem by working together with the other data banks.

Because the release contains unified data, all the entries have been annotated with common feature keys. The feature keys used in the previous release were updated beforehand. 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 the International Nucleotide Sequence Database is not yet prepared for this.

Building and Searching Biological Information based on a Relational Database System

Hajime KITAKAMI and Yukiko YAMAZAKI

The DNA database has been managed in a flat-file system at the DDBJ

since 1985. The flat-file system is inadequate for building and searching the DNA database which is receiving an explosive increase entries. We carried out a transformation from the flat-file system to the relational database system with GenBank staff. The schema of the relational database was designed as follows:

(1) Decomposing the DNA data into both structuralized and non-structuralized data.
(2) Partitioning large tables into small tables without redundancies.
(3) Making a flexible relationship among tables to represent complex data.

This schema provided the capability for building and searching the DNA database with less memory on the relational database system. However, the schema was implemented as a complex network structure with about 60 tables. It is difficult to use the SQL search language of the relational database system with this schema.

We defined and simplified the schema for easy use of the commands using the view function of the relational database system on the existing schema. The simplified schema implemented in the view function was defined as LOCUS, DEFINITION, ACCESSION, KEYWORDS, SOURCE, REFERENCE, FEATURE, ORIGIN, and SEQUENCE tables which are virtual tables without storing real data. It represents aspects of the traditional DDBJ/EMBL/GenBank data format which are familiar to biologists using the flat-file system.

Users can easily join these virtual tables using attribute storing accession numbers. Since we developed the simplified schema, users are able to use the SQL search command easily and get quick response in DNA data searches.

Codon Usage Tabulated from GenBank Genetic Sequence Data

Ken-nosuke WADA*, Yoshiko WADA**, Fumie ISHIBASHI, Takashi GOJOBORI and Toshimichi IKEMURA

Codon usages in 22361 genes were analyzed using nucleotide sequence data obtained from the GenBank Genetic Sequence Data Bank (Release 69.0, Sept., 1991). The database is called the CUTG Database, and is distributed on EMBL CD-ROM (December 1991; CODON by Wada *et al.*) as a member of the NAR Sequence Supplement Databases. The CUTG codon database is also available with on-line access to DDBJ. For details, see *Nucl. Acids Res.* **20**, Supplement, 2111–2118 (1992).

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ABSTRACTS OF DIARY FOR 1991

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338th–Feb. 1	Models for genomic rearrangements (David Sankoff)
339th–Feb. 27	Promoter analysis of influenza RNA transcription and replication (Baik L. Seong)
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342nd–Mar. 22	Initiation of transdifferentiation in jelly fish (Volker Schmid)
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orders (Partha P. Majumder)

355th–Dec. 13 Yeast RNA polymerase (Masayasu Nomura)

Mishima Geneticists' Club

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377th–Jan. 28 *Mariner*: a transposon found in Drosophila species (Akihiko Koga)

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379th–Feb. 5 Transcription regulation of Moloney Leukemia Virus during differentiation of mouse embryonal carcinoma cells (Toshio Tsukiyama)

380th–Feb. 15 Analysis of embryogenesis using cell culture and gene transfer of early embryonic cells (Norio Nakatsuji)

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Oct. 4, 1990–Aug. 3, 1991	Jotun J. Hein, University of Montreal, Canada
Jan. 9, 1991–	Christopher J. Basten, North Carolina State University, U.S.A.
Jan. 28	Rudolf Balling, Max-Planck Inst. of Biophys. Chem., Germany
Feb. 1	David Sankoff, University of Montreal, Canada
Feb. 18–Mar. 10	Baik L. Seong, Oxford University, U.K.
Mar. 1–May 31	Ashok Kumor, University of Edinburgh, Scotland, U.K.
Mar. 10–31	Woojin Jeong, Korean Advanced Institute for Science and Technology, Korea
Mar. 18–20	Philip W. Hedrick, Pennsylvania State University, U.S.A.
Mar. 19	Douglas E. Berg, Washington University, U.S.A.
Mar. 22	Volker Schmid, University of Basel, Switzerland
Mar. 28–	Engelbert Hobmayer, University of Munchen, Germany
Mar. 29	Tominori Kimura, MRC Laboratory of Molecular Biology, U.K.
Apr. 2	Xu Xin-Lai, Biologivsl Development Center, China
Apr. 2	Xing Rui-Chang, Inspective Institute of Medicines & Chemical Reagents & Biological Products, China
Apr. 2	Xu Zhen-Lan, Jing Feng Institute of Medicine & Laboratory Animal, China
Apr. 2	Feng Zhao-Liang, International Cooperative Department of National Science & Technology Commission, China
Apr. 5	David Benton, NC.H.G.R., National Institutes of Health, U.S.A.
Apr. 17	Ning Lei, Lanzhou Institute of Biological Product, Ministry of Public Health, China
Apr. 17	Song Kai Zhong, Gan Su Sheng Science and Technology Committee, China

Apr. 17	Sun Yi Fang, Lanzhou Institute of Medicine, China
Apr. 17	Wang Zhong Yu, Gan Su Sheng Institute for New Medicine and Pharmacy, China
Apr. 17	Zhao He, Lanzhou Institute of Biological Product, Ministry of Public Health, China
Apr. 19	Larry Weber, National Science Foundation, U.S.A.
Apr. 27–May 25	William B. Provine, Cornell University, U.S.A.
May 17	Mary F. Lyon, MRC Radiobiology Unit, U.K.
Jun. 15–Sept. 14	Karim Sharif, The City University of New York, U.S.A.
Aug. 23–26	G. Second, International Rice Research Institute, Philippines
Aug. 26–28	Wang Bin, Institute of Genetics, Academia Sinica, China
Sept. 1–	Aseem Ansari, Northwestern University, U.S.A.
Sept. 6–7	Gunther S. Stent, University of California, U.S.A.
Sept. 27	Flemming G. Hansen, The Technical University of Denmark, Denmark
Sept. 27	Tove Atlung, The Technical University of Denmark, Denmark
Oct. 11	Masayori Inouye, University of Medicine and Dentistry of New Jersey, U.S.A.
Oct. 13–Nov. 16	James F. Crow, University of Wisconsin, U.S.A.
Oct. 31	Michael Cinkosky, Los Alamos National Laboratories, U.S.A.
Nov. 14	Wesley Brown, University of Michigan, U.S.A.
Nov. 14	Thomas Whittam, Pennsylvania State University, U.S.A.
Nov. 14	Bruce Walsh, University of Arizona, U.S.A.
Nov. 14	Austin Hughes, Pennsylvania State University, U.S.A.
Nov. 14	Charles Aquadro, Cornell University, U.S.A.
Nov. 14	Chung-I. Wu, University of Rochester, U.S.A.
Nov. 14	Colm OhUigin, Max-Planck Institut für Biologie Abteilung Immungenetik, Germany
Nov. 14	Richard Hudson, University of California, U.S.A.
Nov. 14	Andrew Clark, Pennsylvania State University, U.S.A.
Nov. 14	Brain Golding, York University, Canada
Nov. 17–19	Walter Bodmer, Imperial Cancer Research Laborato-

	ries, U.K.
Nov. 17–19	Colm OhUigin, Max-Planck Institut für Biologie Abteilung Immungenetik, Germany
Nov. 19–Dec. 6	Masatoshi Nei, Pennsylvania State University, U.S.A.
Nov. 21–22	James C. Wang, Harvard University, U.S.A.
Dec. 5	Partha P. Majumder, Indian Statistical Institute, India
Dec. 12–13	Masayasu Nomura, University of California at Irvine, U.S.A.
Dec. 26	Kiyoshi Mizuuchi, NIDDK, National Institutes of Health, U.S.A.

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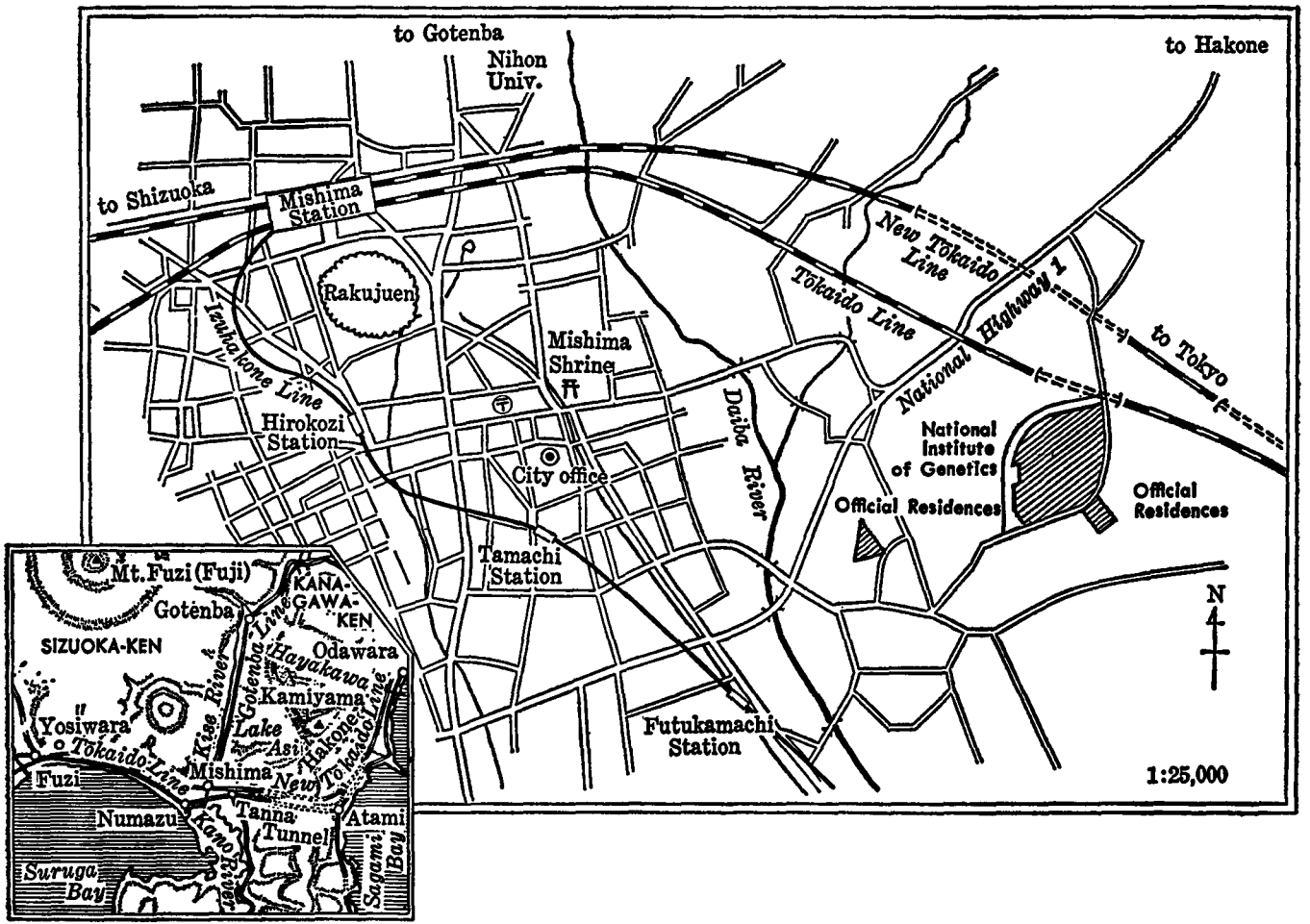
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