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GENERAL STATEMENT

Our institute was established for comprehensive research on genetics and we celebrated our 40th anniversary in June, 1989. The outstanding accomplishments in the advancement of genetics by members of our institute has made it the center of genetic study in our country, and a major institution with world-wide recognition. In addition, six years ago the institute was reorganized into a National Inter-university Research Institute. With the foundation of the Graduate University for Advanced Studies consisting of seven inter-university research institutes, our institute admitted the first graduate students this April, in the Department of Genetics of the Graduate School of Life Science.

Recent rapid progress in genetics with newly developed approaches has greatly shifted the course of study in biology. The establishment of the DNA Research Center some years ago was a timely and proper step to meet the changing demands. It is obvious, however, that this and other actions taken by the institute, are far from sufficient. We shall have many research related problems remaining to be handled. With proper advice and guidance of people in and outside the institute, I would like to lead the institute into more prosperous years ahead.

Regarding personnel changes in the past year, Dr. E. Matsunaga, the fifth director, retired on October 1st. From 1983 to 1989 Dr. Matsunaga had led the members of our institute in six years of drastic reform in the institute. Succeeding him, I accepted the appointment as director. While Prof. S. Nawa (Laboratory of Developmental Genetics) and Associate Prof. T. Endo (Laboratory of Agricultural Genetics) retired, and Associate Prof. D. Ayusawa was transferred to the University of Tokyo, the following six researchers joined us: Dr. K. Horiuchi as professor (Laboratory of Microbial Genetics); Dr. Y. Kohara (DNA Research Center) and Dr. F. Yamao (Laboratory of Mutagenesis) as associate professor, Drs. H. Goto (Laboratory of Cytogenetics), F. Tajima (Laboratory of Population Genetics), and M. Yamagishi (Laboratory of Molelcular Genetics) as research members.

It is a pleasure to note that Prof. A. Ishihama was honored by the Encouragement Award of the Association for the Promotion of Genetic

Studies for his molecular studies on gene expression, and Dr. S. Horai the Encouragement Award of the Japan Society of Human Genetics for his human genetic and molecular evolutionary studies on mitochondrial DNA.

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YAMAGUCHI, Hikoyuki; Manager, Emeritus professor of University of Tokyo

PROJECTS OF RESEARCH FOR 1989

1. DEPARTMENT OF MOLECULAR GENETICS

Laboratory of Molecular Genetics

Studies on regulatory mechanisms of gene transcription in prokaryotes and eukaryotes (ISHIHAMA, FUJITA and NAGATA)
Studies on molecular mechanisms of transcription and replication of animal viruses (ISHIHAMA and NAGATA)

Laboratory of Mutagenesis

Molecular and genetic studies of cell-cycle associated genes (SENO and KANEDA)
Genetic consequences of nucleotide pool imbalance in mammalian cells (SENO, YAMAO and TEZUKA)
Radiation sensitivity in mammals (TEZUK)

Laboratory of Nucleic Acid Chemisry

Molecular mechanisms of RNA 5′-cap formation (MIZUMOTO)
Studies on molecular mechanisms of transcription and replication of Sendai virus (MIZUMOTO)
Phenotypic diversity of mammalian cell cycle mutants defective in ubiquitin-activating enzyme (AYUSAWA)

2. DEPARTMENT OF CELL GENETICS

Laboratory of Cytogenetics

Studies on species differentiation of house mouse from genetic view points (MORIWAKI)
Immunogenetical studies on the mouse MHC (SHIROISHI, GOTOH and MORIWAKI)

Theoretical bases for chromosomal evolution in mammals and ants (IMAI)
Genetic mechanism of meiotic recombination in mice (SHIROISHI, IMAI and MORIWAKI)

Laboratory of Microbial Genetics

DNA replication in *E. coli* (YASUDA, HIGASHITANI and HORIUCHI)
Cellular division in *E. coli* (NISHIMURA and HARA)
Penicillin-binding proteins in *E. coli* (HARA and NISHIMURA)

Laboratory of Cytoplasmic Genetics

Studies on cytoplasmic genes during subspecies differentiation of house mouse *Mus musculus* (YONEKAWA)

3. DEPARTMENT OF ONTOGENETICS

Laboratory of Developmental Genetics

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

Laboratory of Phenogenetics

Studies on development any growth of insect (MINATO)
Genetic studies on insect cells in tissue culture (KURODA and MINATO)
Developmental genetic studies on animal cells in tissue culture (KURODA)
Genetics of somatic mammalian cells in culture (KURODA)
Genetic studies on the life history characters in *Bombyx* (MURAKAMI)
Genetic studies on the nerve system characters in *Bombyx* (MURAKAMI)
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

Electron microscopic studies on differentiation of animal cells (SHIMADA and KURODA)
Theoretical models for pattern formation in hydra (SAWADA, SHIMIZU and

SUGIYAMA)

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 molecular evolution on viruses (GOJOBORI and MORIYAMA)
Computer analysis of DNA sequences (GOJOBORI)
Radiation genetics in mice (TUTIKAWA)
Studies on molecular evolution of Drosophila genes (MORIYAMA)

Laboratory of Theoretical Genetics

Theoretical studies of population genetics and molecular evolution (KIMURA)
Population genetical studies on gene-culture coevolution (AOKI)

5. DEPARTMENT OF INTEGRATED GENETICS

Laboratory of Human Genetics

Genetic and physical mapping of human genome (IMAMURA, FUJIYAMA and NAKASHIMA)
Molecular genetics of human metabolic disorders (IMAMURA and NAKASHIMA)
Molecular biology of oncogenes (FUJIYAMA)
Studies on DNA polymorphisms on human populations (HORAI)

Laboratory of Agricultural Genetics

Evolutionary genetics in wild and cultivated rice species (MORISHIMA, SANO and SATO)
Bio-archaeological analysis of plant remains (SATO and NAKAMURA)

Laboratory of Applied Genetics

Molecular genetics of human immune mechanisms (WATANABE)
Theoretical studies on plant breeding (YONEZAWA)

6. RESEARCH FACILITIES

Genetic Stock Research Center

Genetic studies of speciation in rice (SANO and HIRANO)
Studies on plant gene expression (HIRANO and SANO)
Theoretical studies on breeding techniques (IYAMA)
Documentation of genetic stocks in Japan (IYAMA)
Studies on the management system of genetic stocks information (IYAMA)
Theoretical studies on molecular phylogeny (TATENO)
Evolutionary genetics of *Drosophila* (WATANABE)
Molecular genetics of insect development (UEDA)
Genetic and Molecular studies of cell division mechanism in *E. coli.* (NISHIMURA)
Genetic mechanisms for regulating tumor development in the laboratory and wild mice (MIYASHITA and MORIWAKI)

DNA Research Center

Interaction between proteins and nucleic acids (SHIMAMOTO)
Studies on codon usage (IKEMURA)
Studies on chromosome band structures at the DNA sequence level (IKEMURA and MATSUMOTO)
Sequence and structure analysis of DNA and proteins (MIYAZAWA and HAYASHIDA)
Molecular genetics of insect development (UEDA and HIROSE)

Control of gene expression in eukaryotes (HIROSE and UEDA)

Radioisotope Center

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

Experimental Farm

Molecular genetics of plant development (NAKAMURA)
Genetical analysis of physiological traits in plants (NAKAMURA)

RESEARCH ACTIVITIES IN 1989

I. MOLECULAR GENETICS

Promoter Selectivity of *Escherichia coli* RNA Polymerase, I. Effect of Base Substitutions in Promoter −35 Region on the Promoter Strength

Makoto Kobayashi, Kyosuke Nagata and Akira Ishihama

DNA sequence analyses of a wide variety of prokaryotic promoters have indicated that the promoters for the major form of *E. coli* RNA polymerase ($E\sigma^{70}$) are composed of two conserved hexanucleotide sequences, TTGACA and TATAAT, which are located at 35 and 10 base-pairs upstream of the transcription initiation site, respectively. Among natural *E. coli* promoters, however, there are considerable sequence differences, which might be related to differences in promoter strength (for a review see Ishihama, A. (1988) *Trends Genet.* **4**, 282–288). At present, however, little is known about the role of individual bases within these two regions in promoter function.

For better understanding of the relationship between promoter strength and sequence, we constructed a set of variant promoters derived from the *lac*UV5 promoter, each carrying a single base substitution within the −35 region (nucleotide positions from −36 to −31 relative to the transcription start site). Using this promoter collection and purified *E. coli* RNA polymerase holoenzyme, *in vitro* mixed transcription assays were performed to determine two parameters governing the promoter strength: binding affinity to RNA polymerase (parameter I); and rate of open complex formation (parameter II). The following conclusions were drawn from the experimental results (for details see Kobayashi, M. *et al.*, submitted for publication): (1) Alteration in the promoter strength depends on both the position and base species of substitutions; (2) the consensus sequence (TTGACA) exhibits the highest values for both parameters; (3) base substitutions at nucleotide position −34 cause marked effects on both parameters; (4) cytosine at nucleotide position −32 can not be replaced with other

nucleotide without significant reduction in promoter strength; and (5) base substitution at nucleotide position −31 has little effect on parameter I. All these findings were confirmed by abortive initiation assays. The same type of analyses on the −10 region will provide us with detailed knowledge on the function of each promoter element.

The Promoter Selectivity of *Escherichia coli* RNA Polymerase, II. Random Screening of Promoters and Classification Based on the Promoter Strength

Masayo Kubota, Yukiko Yamazaki and Akira Ishihama

To reveal the moleclar basis of promoter strength differences among natural *E. coli* promoters, we developed the *in vitro* mixed transcription assay system and determined two parameters of the promoter strength for a number of natural promoters which were isolated from known genes (for a review see Ishihama, A. (1988) *Trends Genet.* **4**, 282–288). In parallel with the strength measurement of known promoters, we constructed a collection of all kinds of natural promoters from the total chromosomal DNA of *E. coli*, to classify these promoters based on their strength, and afterward to identify the genes carrying these promoters.

Five hundred and fifty DNA fragments of 100–500 base pairs in length were cloned from total *E. coli* DNA, each capable of inducing the synthesis of β-lactamase when inserted upstream of the *ampC* gene without its own promoter in a promoter-probe plasmid (for details see Kubota, M. *et al.*, submitted for publication). These clones were classified, based on the putative promoter strength as measured by the level of resistance to ampicillin, which ranged from 10 to more than 5,000 μg/ml. Some of the high levels of drug resistance (more than 1,000 μg/ml) were due to not only the insertion of strong promoters but also to an increase in plasmid copy number.

Some of the DNA fragments were mapped on the *E. coli* chromosome by hybridization with the DNA membrane filter of Kohara's miniset clones (the gene mapping membrane was developed in this institute and is being produced by Takara Shuzo). The DNA fragments which produced the highest level of drug resistance were all mapped at 5.7 min on the *E. coli* chromosome and shared the same sequence. In these fragments, an upstream AT-rich sequence was found, in addition to −35 and −10 signals

with a high similarity to the promoter consensus sequence. The gene carrying this strong promoter has not, however, been identified yet.

Promoter Selectivity of *Escherichia coli* RNA Polymerase, III. Structural and Functional Modulations of RNA Polymerase during Growth Phase Transition

Miwako OZAKI, Nobuyuki FUJITA, Akira WADA* and Akira ISHIHAMA

In order to reveal the regulatory mechanisms of growth phase-dependent gene expression in *E. coli*, we examined possible alterations in the promoter selectivity of RNA polymerase during the growth phase transition. Previously we reported that, although core enzyme subunits, α, β and β', of RNA polymerase are synthesized coordinately during exponential growth phase, this coordination is disrupted during the growth transition from exponential growth to stationary phase (Kawakami, K. *et al.* (1979) *Mol. Gen. Genet.* **174**, 107–116). Also, newly synthesized subunits in stationary-phase cells are degraded preferentially (Enami, M. and Ishihama, A. (1982) *Mol. Gen. Genet.* **185**, 373–378).

In addition to the alteration in the synthesis and assembly of RNA polymerase, we found that the preexisting RNA polymerase is converted into at least three different forms during the growth phase transition from exponential growth to stationary phase. All three novel forms of RNA polymerase were eluted from phosphocellulose columns at KCl concentrations lower than that required for the elution of exponential-phase RNA polymerase (for details see Ozaki, M. *et al.*, submitted for publication). The relative levels of these three enzyme forms varied depending on the stage of growth phase transition.

To reveal the altered functions of stationary-phase specific RNA polymerases, the promoter selectivity of each enzyme was tested using an *in vitro* mixed transcription system, in which transcription was performed with mixtures of various *E. coli* promoter DNA fragments. All three RNA polymerases exhibited altered promoter selectivity. In order to identify differences in structure between exponential-phase and stationary-phase RNA polymerases, both enzymes were dissociated into σ^{70} subunit and core enzyme, and a holoenzyme reconstitution was carried out using isolated

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components. The hybrid holoenzyme from the exponential-phase σ^{70} and the stationary-phase core showed essentially the same promoter selection pattern as that of the stationary-phase holoenzyme. The results indicate that the altered function of stationary-phase RNA polymerase is due to an alteration in the core enzyme. These results altogether suggest that the modulation of RNA polymerase might play a major role in the global switch of transcription pattern during the growth transition to stationary phase.

Structural Modulation of Ribosomes during Growth Phase Transition of *Escherichia coli*

Akira WADA*, Yukiko YAMAZAKI, Nobuyuki FUJITA and Akira ISHIHAMA

The decrease in overall translation activity occuring concomitantly with the transition from the exponential growth phase to the stationary phase of *Escherichia coli* was found to be accompanied by the appearance of 100S ribosome (dimer of 70S ribosome monomers). Analysis of ribosomal proteins by the radical-free and highly reducing (RFHR) method of two-dimensional gel electrophoresis indicated that a protein, previously designated protein E, was exclusively associated with 100S ribosome (Wada, A. *et al.* (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 2657–2661). From the results, we proposed that protein E is a "ribosome modulation factor" (RMF), which associates with 70S ribosomes and converts them to a dimeric form. The hypothesis that 100S ribosomes are non-functional stored form of translational apparatus is being tested experimentally. A homology search of the partial amino acid sequence of RMF using the DNA sequence data bases revealed that the *rmf* gene, which encodes RMF, is located next to the *fabA* gene at 21.8 min on the *E. coli* chromosome. This was found to be the case by PCR amplification of this region and direct sequencing of PCR products. Structural and functional modulations of both transcription and translation apparatuses might play a major role in the global control of gene expression during growth phase transitions of *E. coli*.

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The Molecular Anatomy of *Escherichia coli* RNA Polymerase: Functional Mapping of the α Subunit

Kazuhiko IGARASHI, Richard S. HAYWARD*, Nobuyuki FUJITA, Masahiro YAMAGISHI and Akira ISHIHAMA

The DNA-dependent RNA polymerase is composed of at least four different subunits, α, β, β' and σ, each having different functions in gene transcription. The intrinsic functions of individual subunits are, however, expressed only when they are assembled into a complete enzyme. It is therefore important to analyze the molecular interactions among the subunits. The assembly takes place in a sequential manner in the order: $\alpha \rightarrow \alpha_2 \rightarrow \alpha_2\beta \rightarrow \alpha_2\beta\beta' \rightarrow \alpha_2\beta\beta'\sigma$ (for a review see Ishihama, A. (1986) *Adv. Biophys.* **21**, 163–173). Previously, we carried out a molecular anatomy study of the β subunit and identified the region involved in the interaction with other subunits (for a review see Ishihama, A. (1988) *Trends Genet.* **4**, 282–288). Along this line, we have analyzed the region of α involved in subunit assembly.

First, we determined the mutation sites of the RNA polymerase α subunit gene in two temperature-sensitive *E. coli* strains carrying mutations, *rpoA101* or *rpoA112* (Ishihama, A. *et al.* (1980) *J. Mol. Biol.* 137, 137–150), because the assembly of RNA polymerase is defective in the *rpoA112* mutant (Kawakami, K. and Ishihama, A. (1980) *Biochemistry* **19**, 3491–3494). Sequence analysis of PCR-amplified DNA indicated that both of the mutant *rpoA* genes have a single base transition, which leads to a substitution of Cys for Arg at position 191 (*rpoA101*) or 45 (*rpoA112*), respectively. The location of the *rpoA112* mutation raised the possibility that the amino-terminal region of α plays an important role in subunit assembly.

To test this possibility, we constructed a set of deletion mutations of the *rpoA* gene. When carboxyl-terminally truncated α polypeptides were synthesized *in vitro* and examined for their assembly-competence using an *in vitro* reconstitution system, N-terminal fragments of 296, 256, or 235 amino acid residues in length were found to retain the ability to form pseudo-$\alpha_2\beta$ ($\alpha_2\beta$ complex composed of truncated α) and pseudo-$\alpha_2\beta\beta'$ complexes, while those truncated at residues 176 or 150 did not form such complexes.

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Studies of *E. coli* strains producing the same truncated α subunits showed that the 296, 256, and 235 amino acid species can even form pseudo-holoenzyme *in vivo*. These results indicate that the aminoterminal region of the α subunit plays an important role in RNA polymerase assembly. However, *rpoA101* and *rpoA112* mutations could not be complemented by the expression of α-296, α-256, or α-235. Instead, α-256 and α-235 showed inhibitory effects on cell growth, suggesting that, although these truncated α polypeptides lack some essential function associated with RNA polymerase, they interfere with the assembly of intact α subunit.

Promoter Selectivity of *Micrococcus luteus* RNA Polymerase: Identification and Characterization of the Major Sigma Factor

Manabu NAKAYAMA, Nobuyuki FUJITA, Syozo OSAWA* and Akira ISHIHAMA

Promoters of *Micrococcus luteus*, a bacterium whose chromosomal DNA has a high G+C content (74%), differ from the consensus prokaryotic promoter in having GC-rich DNA sequences at less important positions (Nakayama, M. *et al.* (1989) *Mol. Gen. Genet.* **218**, 384–389). In order to compare the promoter selectivity of RNA polymerase between *M. luteus* and *E. coli*, we purified the enzyme from both organisms. The sets of promoters recognized by the two RNA polymerases were found to overlap partly (for details see Nakayama, M. *et al.*, submitted for publication). Some, but not all, *E. coli* promoters were found to be correctly transcribed *in vitro* by the *M. luteus* RNA polymerase as well as the *E. coli* enzyme.

For better understanding of the molecular basis of different promoter selectivity, we attempted to isolate the σ subunit from *M. luteus* and to compare it with the *E. coli* σ subunit. One molecular species of the *M. luteus* σ factor, with an apparent molecular weight of 60 kDa, was isolated from purified RNA polymerase. By the addition of either *M. luteus* or *E. coli* core enzyme, it was reconstituted into an active holoenzyme. The hybrid holoenzyme between the *M. luteus* σ subunit and the *E. coli* core enzyme was able to initiate transcription only from promoters which were recognized by both of the native holoenzymes. These observations indicate that, although the σ subunit plays a major role in promoter recognition,

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the core enzyme also participates in promoter selection. This suggestion is consistent with our previous observation that the promoter selectivity is altered by mutations not only in σ but also in core enzyme subunit genes (Nomura, T. *et al.* (1976) *Mol. Gen. Genet.* **193**, 8–16).

In addition to the major σ factor, we found a minor species of *M. luteus* σ factor, which is different from the major σ factor in both promoter selectivity and molecular size.

Molecular Mechanisms of Transcription and Replication of Phage Q_β: Cloning, Sequencing and Mapping of Host Factor

Masayuki Kajitani and Akira Ishihama

Bacteriophage Q_β contains a positive-strand RNA as the genome, which encodes coat ptotein, maturation protein and RNA-dependent RNA polymerase. The RNA polymerase is composed of four subunits, one phage-coded protein (subunit β) and three host-derived proteins (subunit α= ribosomal protein S1, subunit γ=EF-Tu, subunit δ=EF-Ts). Phage RNA-dependent synthesis of RNA by purified RNA polymerase requires in addition a host protein factor (HF). The nature of this host factor, its role in phage RNA-dependent RNA synthesis, and the physiological role of this protein in uninfected *E. coli* cells all remained unclear.

For better understanding of these problems, we purified HF from uninfected *E. coli* W3350 as the activity stimulating Q_β RNA-directed RNA synthesis with purified Q_β RNA polymerase, determined the amino acid sequence of several parts of purificed HF and, based on the sequence data, prepared probes for hybridization. Using the gene mapping membrane of Kohara's miniset library, we identified the gene encoding HF, cloned the *hfq* gene encoding HF and mapped it on the *E. coli* chromosome (for details see Kajitani, M. *et al.*, submitted for publication). Sequence analysis revealed that HF is a protein of 102 amino acid residues with Mr 11,200. The physiological role of HF is being examined by constructing mutants defective in the *hfq* gene.

Molecular Mechanisms of Transcription and Replication of Influenza Virus RNA, I. Molecular Structure of Virus-Associated RNA Polymerase

Akira ISHIHAMA, Ayae HONDA*, Jun MUKAIGAWA**, Atsushi KATO***, Kyosuke NAGATA, Debi NAYAK**, Susumu UEDA*** and Mark KRYSTAL****

The RNA-dependent RNA polymerase of influenza virus A/PR/8 was isolated from virus particles by stepwise centrifugation in cesium salts. First, RNP (viral RNA-NP-P proteins) complexes were isolated through glycerol gradient centrfugation of detergent-treated viruses and subsequently NP was dissociated from RNP by cesium chloride gradient centrifugation. The P-RNA (P proteins-viral RNA) complexes thus isolated were further dissociated into P proteins and viral RNA by cesium trifluoroacetate (CsTFA) gradient centrifugation. The nature of dissociated P protein complexes was further analyed by glycerol gradient centrifugation and immunoblotting using monospecific antibodies against each P protein (Honda, A. *et al.* (1990) *J. Biochem.* **107**, 624–628).

The three P proteins, PB1, PB2 and PA, sedimented together as fast as the marker protein with a molecular weight of about 250,000 dalton. Upon addition of the template vRNA, the RNA-free P protein complex exhibited the activities of capped RNA cleavage and limited RNA synthesis. For the synthesis of template-sized RNA, however, NP might be needed as previously demonstrated, using native P protein-vRNA complexes and isolated NP (Honda, A. *et al.* (1988) *J. Biochem.* **104**, 1021–1026). When a cell line stably expressing cDNA for three P proteins and NP protein was examined, three P proteins were found to be co-precipitated by treatment with antibodies against the individual P proteins. These results altogether indicate that the influenza virus RNA-dependent RNA polymerase is a heterocomplex composed of one each of the three P proteins and that the RNA-free RNA polymerase can be isolated in an active form from virus particles. Furthermore, P proteins form a complex in the absence of vRNA.

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Molecular Mechanisms of Transcription and Replication of Influenza Virus RNA, II. Function of Influenza Viral Proteins, PB2 and NP, in Viral RNA Transcription

Kunitoshi YAMANAKA, Naotake OGASAWARA*, Masashi UEDA**, Hiroshi YOSHIKAWA*, Akira ISHIHAMA and Kyosuke NAGATA

Ribonucleoprotein (RNP) cores (RNA polymerase-RNA-NP-complexes) are capable of synthesizing template-sized RMA *in vitro*, and of producing infectious viruses when transfected to permissive cells. RNA polymerase, composed of PB1, PB2 and PA, is essential for transcription initiation as well as elongation, while NP is required for efficient elongation of RNA chains. However, the functions and functional domains of each component are not well understood yet.

PB2 has been thought to interact with the cap-1 structure. By comparing dinucleotide- and capped RNA-primed *in vitro* RNA synthesis activities between a wild type and a *ts* mutant carrying mutations in the PB2 gene, it was concluded that PB2 is indeed involved in the recogition of the cap structure. Nucleotide sequence analysis revealed that the amino acid number 417 is essential for the recognition of cap-1 structure and/or the interaction with the catalytic unit of the RNA polymerase (Yamanaka, K. *et al.* (1990) *Arch. Virol.*, in press).

On the other hand, the analysis of RNA binding mode indicated that NP is an oligonucleotide (<15 bases) binding protein without any base-specificity. The RNA-NP complexes reconstituted under the optimized conditions are structurally equivalent to native RNA cores (Yamanaka, K. *et al.* (1990) *J. Biol. Chem.*, 265, 11151–11155).

Thus, protein-protein and protein-RNA interactions play important roles in the regulation of influenza virus RNA transcription. By the same type of genetic and biochemical analyses, we hope to define functions and functional domains of other proteins involved in viral RNA transcription. For detailed analysis of the structure-function relationships of these proteins, we are trying to develop a bacterial expression system for large scale production of each protein.

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Molecular Mechanisms of Transcription and Replication of Influenza Virus RNA, III. Analysis of Transcription Promoters and Replication Origins of Viral Genomes

Kunitoshi YAMANAKA, Naotake OGASAWARA*, Hiroshi YOSHIKAWA*, Akira ISHIHAMA and Kyosuke NAGATA

In order to determine the RNA signal (promoter) for transcription by influenza virus RNA polymerase, we performed *in vitro* transcription experiments using RNA polymerase, NP and various kinds of short model template in collaboration with the Department of Microbiology of Mount Sinal Medical School in New York. The results showed that dinucleotide primer-dependent transcription is directed by the putative promoter located within the 15 nucleotide 3′ terminus of the genome (Parvin, J. *et al.* (1989) *J. Virol.* **63**, 5142–5152). In order to clarify RNA signals for transcription *in vivo*, we developed an infectious RNA-protein complex system.

First, cDNA for the RNA segment 8 of influenza virus A/PR/8/34 was cloned. The region coding for the NS protein was deleted and replaced by the chloramphenicol acetyltransferase (CAT) gene. The resulting DNA sequence was deposited under control of the promoter of T7 RNA polymerase and the antisense RNA (vRNA-sense) to CAT mRNA was produced by transcrbing recombinant DNA with T7 RNA polymerase.

Transfection of cells with this antisense CAT RNA in the presence of helper RNP cores led to no significant production of CAT. In contrast, when the RNA was mixed with purified NP prior to transfection, the efficient expression of CAT activity was observed. Using this infectious RNA-NP complex system, the essential structure of RNA signals required for transcription and replication is being studied.

Molecular Mechanisms of Transcription and Replication of Influenza Virus, IV. Mechanism of Anti-influenza Virus by Mouse Mx Gene

Manabu NAKAYAMA, Kyosuke NAGATA, Akiko YOKOIYAMA, Yoichiro IWAKURA**, Atsushi KATO*** and Akira ISHIHAMA

The inbred mouse strain A2G is known to be resistant to infection by

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influenza virus. This resistance has been shown to be determined by a single dominant allele designated *Mx*, and both cDNA and genomic DNA encoding the Mx protein have been cloned and sequenced. The resistance mechanism by the Mx protein is still unclear. By western blotting and immunoprecipitation analyses using antibodies against synthetic peptides corresponding to either the carboxyl terminus or a region near the amino terminus of the mouse Mx protein, we confirmed that the Mx protein was induced by interferon.

In order to reveal the action mechanism of the Mx protein, we examined the effect of the Mx protein, induced in primary cultures of A2G mouse embryos by interferon, on each step of the influenza viral infectious cycle. It was found that the infection of influenza virus was not affected, whereas viral transcription, viral translation and the production of infectious virus were reduced about 30 fold under the conditions employed. These results suggest that the interferon-induced Mx protein inhibits the synthesis of influenza viral mRNAs or the transport of influenza viral RNA-protein complexes from cytoplasm to the nucleus. However, interferons induce the expression of multiple genes including the Mx gene, which together repress the growth of viruses. Thus, the possibility is not excluded yet that the inhibitory effect is not due to the Mx protein but other interferon-induced proteins. We are trying to establish cell lines which produce the Mx protein in the absence of interferon.

Molecular Mechanisms of Transcription and Replication of Adenovirus: Involvement of Nuclear Factor I in Transcription Regulation

Ken MATSUMOTO*, Fumio HANAOKA**, Akira ISHIHAMA and Kyosuke NAGATA

Recently, evidence has been accumulating indicating DNA replication origins contain transcriptional regulatory elements. Studies using several DNA viruses reveal that trans-acting factors essential for DNA replication are aslo involved in transcription regulation. The replication origin of adenovirus (Ad) DNA consists of three functional domains. The minimum origin is located at the end of the genome, containing an AT-rich region conserved in all human adenoviruses. The nuclear factor I (NFI) binding

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site (FIB site) adjacent to this AT-rich region and the NFIII binding site are essential for Ad DNA replication. We examined the transcription promoter activity within the Ad replication origin in a cell-free system using nuclear extracts of HeLa cells (Matsumoto, K. *et al.* (1989) *Biochem. Biophys. Res. Commun.* **164**, 1212–1219).

Transcription from a cloned inverted terminal repeat of Ad type 5 DNA was found to take place in the opposite direction with respect to that of DNA replication. The major transcript was initiated outside the Ad sequence about 30 nucleotides downstream from the AT-rich region. Competitive transcription experiments using oligonucleotides and isolated NFI revealed that NFI and the FIB site were involved in negative regulation of this transcription. DNase I footprinting experiments indicated that the TATA-box binding protein, TFIID, was capable of binding to the AT-rich region at the minimum origin but not when NFI binds to the adjacent FIB site.

The results of this study indicate that NFI, which is essential for Ad replication and is involved in the positive regulation of transcription of a variety of genes, can also function as a transcriptional repressor possibly dependent on the position of its binding site relative to the TATA-box.

Molecular Mechanisms of Transcription and Replication of Rice Stripe Virus

Akira Ishihama, Pascale Barbier, Mami Takahashi*
and Shigemitsu Toriyama*

Rice stripe virus (RSV) is a member of the newly recognized tenuivirus group of plant viruses. These viruses are able to infect their insect vectors as well as their plant hosts. Biochemical studies have indicated that this group of viruses has a unique genome structure. Purified RSV was found to contain one major (32 kDa coat protein) and one minor (230 kDa putative RNA polymerase protein) as the protein components, and four segments of both single-strand (ss) and double-strand (ds) RNA as the genome. In addition, RNA-dependent RNA polymerase activity was found to be associated with virus particles. As an initial attempt to reveal whether both ss- and ds-RNA are required for virus growth, we stared the purifica-

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tion and characterization of RNA polymerase as well as the determination of the sequence of each RNA segment.

The sequencing analysis of both termini of ss-RNA segments indicated that terminal sequences are well-conserved among the four RNA segments and moreover the 3′- and 5′-terminal sequences are complementary to each other (for details see Fukuhara, M. *et al.*, submitted for publication). These properties suggest the negative-strand nature of ss-RNA segments, indicative of the presence of RNA polymerase in virus particles. To confirm this prediction, several approaches are being employed, including determination of the nucleotide sequences for RNA segments, determination of the amino acid sequence for RNA polymerase, characterization of transcripts formed by RNA polymerase, and identification of RNA polymerase-binding sites on viral RNA segments. Initial attempts have indicated that 230 kDa viral protein, the putative RNA polymerase, can be dissociated from viruses and recovered from vRNA through CsCl centrifugation, the procedure being successfully employed in this laboratory for the purification of virus-associated RNA polymerases. Effort is being made to establish an *in vitro* transcription system using isolated vRNA and RNA polymerase.

Molecular Studies on Evolution, I. Annual and Perennial Differentiation in Wild Rice *Oryza rufipogon*

Pascale Barbier and Akira Ishihama

The Asian wild rice species *Oryza rufipogon* is differentiated into 2 ecotypes: an annual type and a perennial type. In spite of a clear cut differentiation in phenotypic traits, little differentiation has been found in isozymic markers between these two ecotypes (Barbier, P. (1989) *Jpn. J. Genet.* **64**, 259–271; ibid., 273–285).

In order to estimate the divergence time of the two ecotypes, an attempt was made to estimate the phylogeny of eight annual and perennial strains originating from various regions of Asia (India, Indonesia and Thailand), using the nucleotide sequence of three introns located in the phytochrome gene (about 600 base-pairs of introns and as many as exon borders). The related African perennial species *Oryza longistaminata* was also analyzed as a control outgroup. The nucleotide sequence of each of the nine strains was determined without cloning, using PCR amplification of target sequences

and direct submission of purified PCR products to sequencing reactions with the dideoxy method using *Taq* polymerase.

The variation in the phytochrome introns revealed that: i) the difference between *O. longistaminata* and *O. rufipogon* was about 0.042 substitutions per site; but ii) within *O. rufipogon*, strains from geographical origins as remote as India, Indonesia and Thailand, the difference was within the range of 0.0017–0.0050 substitutions per site. These results indicate that extant strains of Asian wild rice diverged as recently as a few hundred thousand years ago, compared to 5–6 million years ago for the divergence of *O. logistaminata* and *O. rufipogon.* Annuals and perennials from the same geographic region (=sympatric strains) seemed to be a little more closely related than allopatric ones. Thus, a monophyletic origin of annuals is excluded. These data also indicate that the annual-perennial differentiation may be even more recent than the geographic differentiation between strains. However, the existence of a shared substitution between a strain from India and a strain from Thailand could also indicate that long-distance migration and introgression between strains cannot be excluded in the evolutionary history of wild rice.

Molecular Studies on Evolution, II. Variation in the Sequence of a Storage Protein Gene Family in Wild Rice

Pascale BARBIER and Akira ISHIHAMA

Variation in the 10 kDa prolamin gene family was probed using PCR amplification and direct sequencing. Eight strains of Asian wild rice (*Oryza rufipogon*) and one strain of African wild rice (*Oryza longistaminata*) were examined (for details see Barbier, P. and Ishihama, A. (1990) *Plant Mol. Biol.*, **15**, 191–195). As PCR allows the amplification in bulk of all copies of a gene family, the extent of heterogeneity among copies may be also investigated. The sequencing of artificial template mixtures showed that the presence of minor variants representing 20% of all copies can be detected by the method employed. The 10 kDa prolamin genes from all the test strains were found to be about 400 bp long, without introns. Within *rufipogon* strains, variants were only detected in two strains: a strain from West India and a strain from Thailand (one and two base substitutions, respectively). How these two strains escaped the homogenization process (concerted evolution) seen in other strains is unknown. Large differences

were found in *O. longistaminata* as compared to *O. rufipogon*, including the presence of two additional codons. No detectable heterogeneity among the 10 kDa prolamin gene copies was found for all the strains analyzed. The number of copies is being determined.

Molecular Studies on Evolution, III. Introgression of Mitochondrial DNA in Land Snail *Luchuphaedusa* (*Ophaedusa*) *ophidoon*

Rei UESHIMA and Akira ISHIHAMA

L. (*O.*) *ophidoon* is a clausilid land snail endemic to Shimokoshiki Island in Kyushu. Studies on morphological and allozymical variation have indicated that at least three genetically distinct taxa, southern large form (LS), southern small form (SM) and northern large form (LN) exist, each carrying characteristic morphological traits and having a different geographical distribution (for details see Ueshima, R. *et al.*, in preparation). The two southern taxa, LS and SM, form a narrow hybrid zone in natural habitats. In order to reveal the evolutionary history of these taxa, with particular focus on gene flow across the hybrid zone, we analyzed variations in mitochondrial DNA (mtDNA) sequences.

We analyzed 87 restriction enzyme sites of mitochondrial (mt) DNA from 236 LS individuals and 100 SM individuals collected from 37 localities. Altogether 17 different haplotypes were identified, among which 16 haplotypes were specific to each taxon (13 to LS and 3 to SM) but one haplotype was shared by both taxa. The phylogeny analysis revealed that these haplotypes could be classified into two distinct mtDNA lineages, LS- and SM-lineages. Only a single haplotype among those present in SM taxon was found to belong to the SM-type lineage. The LS-type mtDNA lineage found in the SM taxon was considered to be introgressed from LS taxon through hybridization. In contrast, the flow of nuclear genes across the hybridization seems to be prevented due to strong post-mating isolation. These observations indicate that mtDNA was transferred from LS to SM long enough ago to accumulate mutations in the transferred mtDNA genome and to spread into most of the SM individuals. To explain the introgression pattern of mtDNA in the land snails on this small island, we proposed a model of two cycles of range contraction and expansion.

Structural and Functional Analysis of the Human Thymidylate Sythase Gene

Sumiko KANEDA, Josephine NALBANTOGLU*, Dai AYUSAWA, Keiichi TAKEISHI**, Kimiko SHIMIZU***, Osamu GOTOH*** and Takeshi SENO

The complete nucleotide sequence of the human thymidylate synthase (TS) gene was determined. Genomic DNA fragments for human TS were cloned using a DNA mediated transformation of mouse mutant cells deficient in TS. The biologically active unit spans about 16 kilo bases (kb) and is composed of seven exons ranging in size from 72 base pairs (bp) to 250bp, and six introns ranging in size from 507bp to 6271bp. The nucleotide sequence of the exons was in agreement with that reported for cDNA (Takeishi *et al.*, 1985, *Nucl. Acids Res.* **13**, 2035). Comparison of the positions of the exons in human and mouse TS genes (Deng *et al.*, 1986, *J. Biol. Chem.* **261**, 16000) showed that their splice junctions are identical; each exon is interrupted at the same amino acid position and each codon is interruted in the same phase in the two TS genes. The gene is G+C-rich and has a high concentration of CpG in the 5′ flanking region, exon 1 and intron 1. The promoter region and the major transcriptional start sites were located within about 400bp and 160–180 bp, respectively, upstream from the ATG initiation codon. The 5′ upstream region lacks the three canonical transcriptional signals, the TATA box, CAAT box, and GC box (GGGCGG), whereas GC boxes are present in the corresponding region of the mouse gene. Instead, there are three GC box esquences in intron 1 and all of them seem to be functional, as determined by gel retardation assay. A new enhancer-like sequence is also present in intron 1 (Kaneda *et al.*, in preparation). There are two polyadenylation signals 288bp apart around the 3′ region, corresponding to the polyadenylation site of the cDNA. In both cases, the polyadenylation signal of AATAAA is approximately 20bp upstream from the sequence CAPyTG which contains the dinucleotide CA, the preferred sites of polyadenylation in eukaryotic genes. The second polyadenylation signal seems to be used in normal fibroblastic cells transformed by SV40, as estimated by 3′ mapping of 7 different TS cDNA clones. In the mouse TS mRNA, the 3′ untranslated region is

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absent, that is, poly (A) is added to the G of the stop codon UAG. Thus, polyadenylation sites are movable and the untranslated region of TS mRNA may have no essential and rigid function.

Functional Analysis of the Human Thymidylate Synthase Gene by Moleucular Dissection

Atsushi TAKAYANAGI, Sumiko KANEDA, Dai AYUSAWA and Takeshi SENO

We reported previously that cell cycle-dependent expression of human thymidylate synthase (TS) is largely regulated posttranscriptionally, that is, in normal human diploid cells the amount of TS mRNA increases about 14 fold from the G0/G1 phase to the S phase, while the initial velocity of transcription in the TS gene is almost the same between the two phases as measured by "nuclear run-off" (Ayusawa *et al.*, 1986, J. Mol. Biol. 190, 559). In order to locate the regulatory sequences responsible for the posttranscriptional regulation within the gene, we constructed a minigene of the human TS and related chimeric genes partially replaced by corresponding SV40 promoter sequences or 3′ regulatory sequences in the Okayama-Berg expression vector and introduced them into a TS-negative mutant of rat 3Y1 cells to establish stable transformant lines. The minigene was composed of about 4kb of a 5′ flanking sequence, a coding sequence with or without interruption by intron 1 and about 1.7kb of a 3′ flanking sequence.

Each transformant line was synchronized in the G0/G1 phase by serum starvation, and then stimulated to traverse into the S phase through the addition of serum. The amount of TS mRNAs was assayed between cells in G0/G1 and S phases by Northern hybridization analysis with a fragment of human TS cDNA clone as a probe. The results showed that the above minigene gave an increment of mRNA in the S phase and showed a repression of it in the G0/G1 phase, whereas the cDNA in the SV40 promoter-driven vector produced an abundant amount of mRNA equally in the two phases. The same minigene, but without an interruption by intron 1, showed the S phase-specific expression pattern but to a lesser extent. These results suggested that the 5′ flanking and/or 3′ flanking genomic sequences in the minigene are responsible for the S phase-specific increase of mRNA. Furthermore it was shown that the presence of the genomic 5′ flanking sequence, but not the 3′ flanking sequence is sufficient for the S phase-specific increment

of mRNA and subsequent repression in the G0/G1 phase by using chimeric genes of genomic and viral sequences. The role of intron 1 in the S phase-specific expression is under investigation. Elucidation of the differential role of the 5′ flanking sequence and intron 1 in the S phase-specific expression of the TS gene is of central interst in understanding the posttranscriptional regulatory mechanism.

Enhancement by *l*-Leucovorin of Thymidylate Stress-Induced DNA Double Strand Breaks in Mouse Cells

Shugo AKAZAWA*, Dai AYUSAWA, Yusuke WATAYA**, Hideo TEZUKA and Takeshi SENO

We previously demonstrated that thymidylate stress (thymine nucleotide starvation) in cultured mammalian cells results in cell death with concomitant DNA double strand breaks associated with ongoing DNA replication, resulting in the accumulation of DNA fragments of a particular size (50–150kb) as measured by pulsed-field gel electrophoresis (Ayusawa, D. *et al.*, 1988, *Mutat. Res.* **200**, 221). These results are consistent with the known effectiveness of fluorinated derivatives of pyrimidine in cancer chemotherapy. Since inhibition of TS is a cause of thymidylate stress, one may expect a positive correlation between the extent of inhibition of TS and that of DNA double strand breaks. In this report we treated mouse mammary FM3A cells with the TS specific inhibitor 5-FdUrd or 5-fluoro-2′-deoxy-3′-O-oleoyluridine-5′-(2-trimethylammonioethyl) phosphate (T506) in combination with *l*-leucovorin which forms a covalent bonded ternary complex with the TS enzyme, and derived 5-FdUMP. The results showed that (1) 5-FdUrd and T506, but not 5-FUra produced DNA double strand breaks as detected by pulsed-field gel electrophoresis; 5-FUra produced DNA strand breaks only at high concentrations, (2) the extent of the DNA double strand breaks increased with time in the drug-treatment, (3) *l*-leucovorin enhanced both cytotoxicity and DNA double strand breaks brought about by 5-FdUrd or T506 treatment, and (4) a clinical test showed that treatment of a patient with 5-FdUrd or T506 produced in the blood for a reasonable period of time, a range of 5-FdUrd concentration comparable to that which brought about DNA double strand breaks in the above *in*

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vitro experiment. In the case of 5-FUra, its concentration in the blood decreased easily to below the level necessary for DNA strand breaks to be induced. Theses results suggest that the presently used combination therapy of 5-FUra and leucovorin should be replaced by that of T506 and leucovorin.

Genetic Analysis of Mammalian Cell Cycle Mutants in a Unique Complementation Group

Dai AYUSAWA, Sumiko KANEDA and Takeshi SENO

So far, a wide variety of conditional cell cycle mutants and non-cell cyle mutants have been isolated from various mammalian cells. Curiously enough, a large number of such mutants fell into a single complementation group based on cell hybrid formation. Some of these mutants are shown to have the temperature-sensitive ubiquitin activating enzyme El.

We purified a human gene which rescued temperature-sensitive traits in several mouse cell mutants through two-rounds of DNA-mediated gene transfer with human genomic DNA. From the DNA library of a secondary transformant, genomic DNA segments derived from the human gene were cloned in a λ phage vector with an *Alu* sequence as a probe. Then, using a DNA segment which detects 3.6 kb mRNA expressed in transformant and human cells, we cloned full-length cDNAs for the mRNA from a human cDNA library. These cDNAs were ligated to a mammalian expression vector and found to have the ability to rescue genetic defects in the temperature-sensitive mouse cell mutants.

Now we can employ a genetic analysis to reveal the mechanism underlying phenotypic diversity in the mutants. The functions of the El enzyme central to various cellular processes will be addressed taking advantage of the use of such mutants and the cloned genes.

Coupling between Replication and Translation by *E. coli* Single-Stranded DNA Binding Protein

Nobuo SHIMAMOTO

E. coli single-stranded DNA binding protein (SSB) specifically binds to a group of mRNA, including its own mRNA, in a cooperative manner. Three RNA segments responsible for the specific and cooperative binding

with homologous 12 base sequences, named SSB box, are located on *ssb* mRNA outside of the coding regions. Genes *alkA* and *secY* mRNA have such sequences. The following evidence implicates a regulatory role of the binding of SSB to mRNA. The binding takes place at physiological concentrations, and a significant amount of SSB was found complexed with RNA in cell lysate. The binding inactivates template activity of such a mRNA in a cell-free translation system *in vitro*. The translation of *recA* and *nusA*, with no such sequences, was not affected by SSB. Thus the binding protein may be a pleiotropic translation factor presumble SSB translational regulons. From these lines of evidence the following picture emerges. When cells grow in a favorable environment, replication forks increases, catching more binding protein. Thus more mRNA is exposed and used in translation. The state of replication can be thus transmitted to a translation system.

To check this model, the intracellular concentration of SSB was changed by overproducing SSB or RNA containing SSB box. The overproduction of either SSB protein or SSB box RNA was lethal. The overproduction of both, however, was not harmful. These results suggest that the amount of uncomplexed SSB is strictly regulated and that the binding of overproduced SSB to DNA and mRNA is toxic. Consistently, several proteins were underexpressed upon the overproduction of SSB. These candidates of SSB translational regulon are now being sequenced.

Kinetic Study on Transcription by Immobilized Operons: Requirement for β, γ-Phosphodiester Bond of ATP in Initiation

Nobuo SHIMAMOTO and Miki FUJIOKA

The requirements for the β, γ-phosphodiester bond of ATP in transcription initiation by *E. coli* RNA polymerase has long been questioned. We devised a novel method for studing these requirements and this method enabled us to substitute substrates during elongation without changing the ionic atmosphere.

A DNA fragment containing an operator and a promoter was fixed to acrylamide beads at its ends. The immobilized DNA was a template as good as free DNA for *E. coli* RNA polymerase. If necessary, transcription was interrupted by rapidly diluting substrates to 0.1 μM and resumed by

adding new substrates. Using ATP analogues as substrates, we clarified that the β, γ-phosphodiester bond of ATP is required for effective transcription in the early stage of elongation. When transcription was started from λ P_R promoter by adding four natural substrates, and eloncated 20 sec to form an elongation complex, this complex resumed elongation upon addition of a substrate set containing a β, γ-unhydrolyzable analogue of ATP. The same substrate set, however, could not support the initiation. The results suggest the presence of a new initiation complex which requires ATP until elongation is established.

Kinetic Study on Transcription by Immobilized Operons: Sliding of RNA Polymerase on DNA

Nobuo SHIMAMOTO

The first step of transcription is the binding of RNA polymerase to a promoter. The enzyme could directly bind to a promoter on a long DNA, or alternatively, first bind to DNA in a nonspecfic 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 confildent evidence. The immobilized operon provides a critical method for determining which case is true. A fragment of DNA with one end immobilized on a slide glass was extended by electric field of 30–100 V/cm and the other end was photocrosslinked on the same slide glass. Fluorescently labeled RNA polymerase was added to the linearly fixed DNA thus prepared and the movement of a single molecule of RNA polymerase was followed through microscopy. The one-dimensional movement was detected and its contribution to the promoter binding is now being evaluated.

Giant G+C% Mosaic Structures of Human Genome Found at a Level of DNA Sequences

Toshimichi IKEMURA, Ken-nosuke WADA, Shin-ichi AOTA,
and Ken-ichi MASTUMOTO

To determine the overall variation of G+C% distribution over long ranges of the human genome, DNA sequences of human genes, which were closely linked genetically or physically, were surveyed in the GenBank

Data Base. A total of 72 sequences longer than 2 kb, which were mutually linked within 500 kb, were identified. The sequences belonged to 17 linkage groups and were ordered in each group according to their genetic positions. Analyses of G+C% distribution along the ordered sequences showed that sequences within each group almost always had similar G+C% levels, but those belonging to different groups often had different levels. Similar analyses of more distantly linked sequences (e. g., > ten megabases) showed mosaic structures of G+C% distribution. These findings are consistent with predictions made from the 'isochore' structures found by Bernardi and his colleagues using CsCl equilibrium centrifugation, in that structures having a homogeneous base composition stretched over at least several hundred kilobases. Two possible boundaries of the giant G+C% mosaic structures were identified between classes II and III in the HLA locus and between X-linked G6PD and F8C. We are now attempting to clone the entire junction of the classes II and III in the HLA in order to clarify the characterisis of the boundaries. For details, see Ikemura *et al.*, *Genomics,* **8** (in press, 1990).

Codon Usage Tabulated from GenBank Genetic Sequence Data

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Fumie Ishibashi, Takashi Gojobori
and Toshimichi Ikemura

In 1986 and 1988, we reported codon usages in a total of 1638 and 3681 protein genes, respectively analyzing all the data available in those days. Now codon usages in 11415 genes can be analyzed using nucleotide sequence data obtained from the GenBank Genetic Sequence Data Bank (Release 62.0, Dec., 1989). Because of the growing size of the database, this year a part of the data is listed in *Nucl. Acids Res.* (1990). [We will send upon request, a magnetic tape, or a hard copy listing codon usages in 11415 genes, to any Institutes of any countries.] To reveal the characteristics of codon use in a wide range of organisms, as well as viruses and organella, the frequency of codon use in each organism for which more than 20 genes were available was calculated by summing up numbers of codon use. Confirming the "genome hypothesis" of Grantham *et al.*, among taxonomically

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related organisms (e. g. among mammals) codon-choice patterns resemble each other but they differ between distant organisms. Synonymous codon-choice patterns in different genes of a single unicellular organism are known to be usually similar to each other regardless of gene functions (dialectal codon-choice pattern found for individual unicellular organisms as pointed out previously). However, codon-choice patterns in one higher vertebrate often differ significantly between different genes: we previously pointed out the diverse codon-choice patterns found among genes of a single higher vertebrate in connection with the evident diversity in the G+C% at the codon third position among the genes. It should be stressed that the characteristic pattern for the mammals mentioned above is obtained only after summing up the genes with varying functions. When codon usages of approximately 10 or more genes with varying functions were summed up for each mammal, they usually resulted in a very similar pattern, regardless of differences in the genes used for the summation. The fact that the pattern common among mammals does not depend on the genes used for the summation, shows that this relates to the general characteristics of their genomes; 1) deficiency of CpG and TpA dinucleotides; 2) paucity of genes in the A+T-rich genome portion, i. e. paucity of genes in A+T-rich isochores and in G/Q bands (thus A- and T-ending codons are less frequent); 3) gross similarity of tRNA populations between different higher vertebrates (our preliminary data); 4) gross similarity of amino acid composition between different proteins, as well as between different mammals. For details, see Wada, K., Aota, S., Tsuchiya, R., Ishibashi, F., Gojobori. T., and Ikemura, T. (1990) *Nucl. Acids Res.*, **18**, Supplement 2367–2411.

G+C% Mosaic Structures of the Higher Vertebrate Genome and Distribution of Dinucleotide Frequencies

Ken-nosuke WADA*, Isamu WATANABE**, Rye TSUCHIYA
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It has become clear that the genome of higher vertebrates is a mosaic of very long DNA sequences, each of which is fairly homogeneous in its

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G+C content with several different levels. We attempted to characterize the distribution of dinucleotide frequencies in different domains of the G+C% mosaic structures of the human genome by comparing it with the distribution along invertebrate (*Drosophila* and nematode) genome sequences. As a sensitive tool for visualizing both long-range and the short-range features of G+C% variation along genome sequences, we proposed the analysis of the frequency distribution of dinucleotides that are composed only of G and/or C, or of A and/or T, as well as the distribution of their differences. For details, see Wada, K., Watanabe, I., Tsuchiya, R., and Ikemura, T. in *New Aspects of Genetics in Molecular Evolution* (editted by N. Takahara) 1990, Japan Sci. Soc. Press.

Effect of DNA Supercoiling on *in vitro* Transcription from the Adenovirus Early Region 4

Hiroshi HANDA*, Hajime WATANABE*, Yoshiaki SUZUKI**
and Susumu HIROSE

Recent studies have indicated that a specific DNA topology is required for the expression of certain eukaryotic genes (see Weintraub, H., 1985, *Cell* **42**: 705–711 for review). To investigate the possibility that superhelical tension of DNA plays a direct role in the regulation of transcription, a cell-free transcription system that maintains superhelical DNA templates has been developed using a *Bombyx mori* silk gland extract (Hirose, S. *et al.*, 1985, *J. Biol. Chem.* **260**: 10557–10562). Using this *in vitro* system, we examined the effect of DNA topology on the regulation of transcription from the early region 4 (E4) of adenovirus type 5. The supercoiled DNAs yield several-fold higher levels of E4 transcripts and more faithfully reflect the regulation of *in vivo* transcription than linear DNAs do. For details, see *FEBS Letters*, **249**: 17–20, 1989.

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Supercoiling of DNA by DNA Topoisomerase II and a Supercoiling Factor Purified from the Posterior Silk Gland of *Bombyx mori*

Tsutomu OHTA and Susumu HIROSE

A protein factor with an estimated molecular mass of 50 kDa was purified to homogeneity from the silk gland of *Bombyx mori.* In the presence of molar excess of this factor and eukaryotic DNA topoisomerase II, relaxed circular DNA was converted to the negatively supercoiled form. Eukaryotic DNA topoisomerase I could not substitute for eukaryotic DNA topoisomerase II in the supercoiling reaction. The reaction was dependent on ATP and was inhibited by VP-16, a specific inhibitor of eukaryotic DNA topoisomerase II. When DNA topoisomerase I was subsequently added to the supercoiling reaction mixture, the supercoiled DNA became relaxed. These results suggest that when both the 50 kDa protein and eukaryotic DNA topoisomerase II are present in excess, unconstrained negative supercoils are introduced into DNA. For details, see *Proc. Natl. Acad. Sci. USA*, **87**: 5307–5311, 1990.

A Sequence-Specific DNA-Binding Protein that Activates *fushi tarazu* Segmentation Gene Expression

Hitoshi UEDA*, Sandra SONODA**, J. Lesley BROWN***, Matthew P. SCOTT** and Carl WU***

The Drosophila segmentation gene *fushi tarazu* (*ftz*) is expressed at the cellular blastoderm stage in a pattern of seven transverse stripes; the stripes lie out of register with the segmental primordia, spanning alternate segmental boundaries. The zebra element, a 740-bp DNA sequence upstream of the *ftz* translational start, directs striped expression of *lacZ* when introduced into the fly genome. We purified to homogeneity a sequence-specific DNA-binding factor, FTZ-F1, which binds to two sites located within the zebra element and to two sites within the *ftz* protein-coding sequence. FTZ-F1

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DNA-binding activity is first detected in extracts of 1.5- to 4-hr embryos, coincident with the time of *ftz* expression in stripes; the activity then diminishes before reappearing during the late embryo, larval, and adult stages. When one of the FTZ-F1-binding sequences in the zebra element is mutated by 2- or 4-base substitutions, the binding to FTZ-F1 is disrupted in vitro, and the intensity of *lacZ* expression is reduced in transformed embryos, especially in stripes 1, 2, 3, and 6. These results suggest that FTZ-F1 is a transcriptional activator necessary for the proper expression of the *ftz* gene.

Cloning and Structural Analysis of the *waxy* (*wx*) Locus Gene of Rice (*Oryza sativa*)

Hiro-Yuki HIRANO and Yoshio SANO

The *wx* locus controls amylose synthesis in rice seeds. Endosperms of deficient mutants of the locus contain starch only composed of amylopectin. In wild-type rice, the amylose content (relative to total starch) varies among varieties or species in the *Oryza* genus and is genetically controlled in each strain. For example, in the Japonica-type rice of *Oryza sativa*, the amylose content is 14–18%, while in Indica-type rice it is 20–27%. In earlier studies, we found the *wx* locus product (Wx protein) through electrophoretic analysis of protein extracts from starch granules. There is also a difference in the amount of Wx protein between the two varietal groups; Indica rice has Wx protein about 10-times as much as Japonica rice. In addition, we indicated that there is a positive correlation between the amount of Wx protein and amylose content, suggesting that the extent of synthesis and accumulation of amylose is controlled by the amount of Wx protein. The molecular mechanism of the quantitative gene regulation of the *wx* gene is of great interest for research. Since amylose content is a major determinant of rice quality for eating or cooking, studies on the *wx* gene expression are not only attractive in terms of understanding gene regulation but also important for applied uses, such as molecular breeding.

To approach the above problems, we tried to clone the *wx* locus gene of rice using the *wx* locus gene of maize as a probe. We obtained DNA clones from both Japonica- and Indica-type rice. Since Northern analysis indicated that RNA, homologous to the cloned rice DNA segment, is

expressed in wild-type rice strains and not in mutants, this strongly suggests that cloned DNA is derived from the *wx* locus of rice. Southern blotting analysis showed that each varieties of rice contains a single copy of the gene. Previous genetic studies support this result. The fact the gene to be examined is single copy in the genome is a great advantage for genetic and molecular biological studies. A cloned 4.5 kb DNA segment, which may correspond to the *wx* locus of Japonica, has been completely sequenced. The *wx* locus gene of rice is composed of many exons and introns as that of maize is. A long open reading frame indicates that the *wx* gene products are about 59 kb polypeptides made up of 532 amino acids. In comparison to the amino acid sequence of the N-terminal region of the mature Wx protein, the precursor protein was shown to have an additional polypeptide of 77 amino acids, which may be a transit peptide necessary for importing the protein into amyloplasts. The size of the predicted polypeptide from the nucleotide sequence is consistent with that of the Wx protein as determined by polyacrylamide gel electrophoresis. The deduced amino acid sequence of the rice *wx* locus gene has 88% homology with the maize gene. Since the maize *wx* locus is a gene for an enzyme, UDP-glucose starch glycosyl transferase, the *wx* locus of rice may also encode the same enzyme, which catalyzes the last step of amylose synthesis.

We purified the rice Wx protein and prepared the anti-sera for it. In immunoblotting analysis of total protein extracts, the quantitative difference in Wx protein expression between Japonica and Indica was also shown as in earlier experiments. These result eliminated the possibility that the difference in amount of Wx protein in previous electrophoretic analysis was due to the reduced affinity of the Wx protein to starch granules and loss during fractionation in Japonica rice. Northern blotting analysis clearly indicated that the diffierences in quantitative expression of the *wx* locus in the two varietal groups is regulated at the transcriptional level.

Studies on the Mechanism of mRNA Capping and the Process of Transcription Initiation by RNA Polymerase II

Kiyohisa MIZUMOTO

Most of the cellular as well as viral mRNA in eukaryotes contain a 5′-terminal cap structure, m^7GpppN. This cap structure is required for the efficient initiation of translation and for RNA splicing. It has also been

suggested that the synthesis of the methylated cap structure may play an important role in transcription itself. The cap structure is synthesized at the initial stage of mRNA synthesis and conserved at the 5′-termini of RNAs while they are processed in the nucleus and transported to the cytoplasm. Consequently, elucidation of the mechanism of the cap structure is important for understanding the molecular mechanism of eukaryotic gene expression.

We characterized the reactions catalyzed by the mRNA capping enzyme (mRNA guanyltransferase), the key enzyme in the cap formation, from various eukaryotic cells. The capping enzyme from animal cells consists of a single polypeptide chain with an approximate Mr of 70,000 containing two catalytic domains for each of the initial two consecutive reactions involved in the cap formation, *i. e.*, those catalyzed by mRNA guanylyltransferase and RNA 5′-triphosphatase. In contrast, the highly purified yeast capping enzyme is composed of two separate chains of 52 kDa (α) and 80 kDa (β), responsible for the activities of mRNA guanylyltransferase and RNA 5′-triphosphatase, respectively (Mizumoto, K. and Kaziro, Y. (1987) *Prog. Nucl. Acid Res. Mol. Biol.*, **34**, 1).

The gene encoding yeast α subunit has been isolated by immunological screening of a yeast genomic expression library in λgtll with antibodies against the yeast capping enzyme. The idetity of the gene was confirmed by expressing it in *E. coli* to produce catalytically active mRNA guanylyltransferase. The gene is present in one copy per haploid genome, and encodes a protein of 459 amino acid residues. Gene disruption experiments showed that the α gene is essential for growth (Mizumoto, K. *et al.* (1990) in Nucleic Acid Methylation (Eds: G. Clawson, D. Willis, A. Weissbach and P. Jones), Alan R. Liss, Inc., NY, pp. 45–56).

We characterized an RNA polymerase II transcription initiation complex formed with HeLa cell extracts and a DNA fragment containing the adenovirus major late promoter in the presence of ATP. An initiation complex with an approximate size of 50S could be isolated by glycerol gradient centrifugation free from the bulk of RNA polymerase II and the capping enzyme. Specific transcription was detected with this complex when supplemented with the remaining nucleoside triphosphates. Analyses of the 5′-terminal structure of the transcript revealed the presence of a cap structure m^7GpppA in more than 50% of the RNA chains. These results indicate that at least two cap-related enzymes, mRNA guanylyltransferase

and mRNA (guanine-7-) methyltransferase, are specifically associated with the complexes. The requirement of ATP hydrolysis for the initiation complex formation is also being studied.

Transcription of Sendai Virus (HVJ) Genome

Kiyohisa MIZUMOTO

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

We developed an efficient and faithful *in vitro* transcription system using purified virus particles, and found that the HVJ mRNA synthesis is almost entirely dependent on the presence of a host factor (s). The host factor essential for the *in vitro* transcription was partially purified from mammalian cells and was shown to be separated into at least two complementary fractions, one of which, surprisingly, could be replaced by highly purified tubulin from bovine brain. Furthermore, it was suggested that tubulin is involved in the formation of transcription initiation complex by viral RNA-dependent RNA polymerase.

The analysis of the 5'-terminal sequences of *in vitro*-synthesized poly $(A)^+$RNA demonstrated that almost all RNA molecules terminate in m^7GpppAmpGpGp-. mRNA was synthesized *in vitro* in the presence of [α- or β-^{32}P] ATP or GTP as the labeled substrates. The α and β phosphates of GTP and the α phosphate of ATP, but not the β phosphate of ATP, were incorporated into the cap structure. These ^{32}P-labeling data were consistent with a unique mechanism of cap formation by condensation of GDP residue from GTP with pA- at the 5' temini of HVJ mRNAs.

II. MICROBIAL GENETICS

Replication of Filamentous Phage f1: Enhancer-independent Mutation of an Initiator Protein (gpII)

Atsushi HIGASHITANI, Nahoko HIGASHITANI and Kensuke HORIUCHI

The plus-strand replication origin of the filamentous bacteriophage f1 has a bipartite structure consisting of a required core origin (domain A) and an adjacent A+T-rich enhancer sequence (domain B). The core origin sequence (about 45 bp) is absolutely required for replication and contains three repeats (β, γ and δ), which serve as the binding site for the phage-encoded initiator protein (gpII: Horiuchi, K. 1986, *J. Mol. Biol.* **188**: 215–223). gpII binds the core origin in two steps, forming a binding intermediate (complex I) and a functional complex for nicking (complex II). Greenstein, D. and Horiuchi, K. (1990. *J. Mol. Biol.* **211**: 91–101) have indicated that complex I contains two gpII molecules per origin, and complex II contains four molecules per origin. The replication enhancer stimulates replication approximately 100-fold (Dotto, G. P. *et al.*, 1984, *J. Mol. Biol.* **172**: 507–512; Johnston, S. and Ray, D. S., 1984, *J. Mol. Biol.* **177**: 686–700) and contains three binding sites (1, 2′ and 2″) for the *E. coli* integration host factor (IHF: Greenstein, D. *et al.*, 1988, PNAS. **85**: 6262–6266). Two kinds of enhancer independent mutations in gpII were isolated, which allow wild-type levels of replication in the absence of either the enhancer sequence or IHF (Dotto, G. P. and Zinder, N. D., 1984, Nature **311**: 279–280; Kim, M. H. and Ray, D. S., 1985, J. Virol. **53**: 871–878; Horiuchi, K., unpublished). One is the mpl mutation in gpII (Met 40→Ile) which increases the co-operativity with which the protein binds to form complex II (Greenstein, D. and Horiuchi, K., 1990, *J. Mol. Biol.* **211**: 91–101). To elucidate the molecular mechanism of enhancer-independent replication by the other gpII mutant, which was termed "Hl1", we determined the mutation site and characterized the Hl1 mutant protein.

The sequence data indicated that the Hl1 mutation was caused by a single amino acid change, Gly 73→Ala in gpII. An overproduction system under the Tac promotor of the Hl1 mutant protein was constructed, and

this mutant protein was purified in mg order per litre culture. We examined the binding properties of the purified Hll mutant protein to the replication origin in vitro. The Hll mutation in gpII increases the co-operativity with which the protein binds to form complex II, but to a less extent than the mpl protein. In addition, the Hll mutant gpII forms both complexes I and II with a DNA fragment containing only two (β-γ) of the three repeats from the core origin sequence as the mpl mutant does, while the wild-type protein forms only complex I with this fragment. These results suggest that both mpl (Met 40→Ile) and Hll (Gly 73→Ala) mutations increase the co-operativity of protein-protein interactions to form a functional initiation complex (complex II), and stimulate DNA replication in the absence of the enhancer domain.

Multicopy Suppression in *E. coli*: Cloning of *sufB*, a Dosage-dependent Suppressor of a Thermosensitive Cell-division Mutation, *ftsB*

Atsushi HIGASHITANI*, Yoshihiro YAMAMOTO** and Yukinobu NISHIMURA

The suppression of a mutation in one gene by amplified copies of a different wild-type gene has been termed 'multicopy suppression' (Berg *et al.*, 1988, Gene **65**: 195). A thermosensitive cell-division mutation, *ftsB84* (Ricard and Hirota, 1973, *J. Bacteriol.* **116**: 314), is allelic to *nrdB* (ribonucleotide reductase B2 subunit gene) which maps at min 48.5 (Higashitani and Nishimura, unpublished work: Kren *et al.* 1987, *J. Bacteriol.* **169**: 14; Taschner *et al.*, 1987, *ibid.* **169**: 19). The growth thermosensitivity of this mutant can be corrected by a multicopy plasmid, pLCl-41, which bears the *purM* region around min 54 (Yamada and Hirota. 1981. this Ann. Rep. **31**: 33). We have named the suppressor *sufB* (suppressor of of *ftsB*) for the dosage-dependent suppressor of *ftsB84*.

To learn more about the structure and function of the multicopy suppressor, we subcloned the *sufB* gene from pLCl-41. The *sufB* gene was within ca. 3 kb fragment obtained by *Pst*I digestion (Fig. 1). Nucleotide sequencing of this DNA fragment is now in progress.

Fontecave *et al.* (1989, *PNAS* **86**: 2147) have found the suppression of *nrdA*, *nrdB* and also *ftsB84* mutations under anaerobic conditions, and

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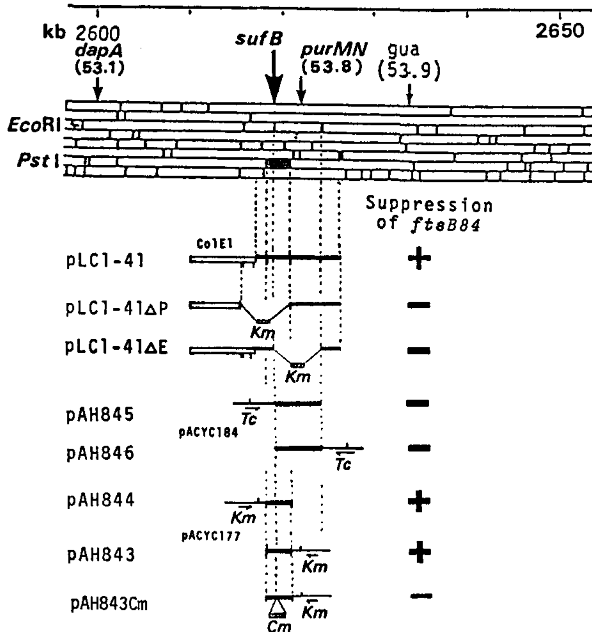

Fig. 1.

Table 1. Multicopy suppression in *E. coli*

Mutation	Suppressor	Reference
ilvE	*avtA*, *aspC*	*Gene* 65: 195 '88
lon	*alp*, *ompT*	*J. Bact.* 171: 3348 '89
topA	*toc*	*Mol. Microbiol.* 3: 531 '89
cheA	*cheY*	*PNAS* 81: 5056 '84
dnaA	*groE*, *sudA*	*MGG* 202: 435 & 446 '86
dnaB	*dnaC*	*J. Bact.* 146: 418 '81
ftsB (*nrdB*)	*sufB*	*Ann. Rep. NIG* 31: 33 '81
ftsH	*ftsI*	*J. Bact.* 169: 5776 '87
ftsM (*serU*)	*ftsZ* (*sulB*)	*J. Bact* 171: 2090 '89
ftsI	*sufI*	*J. Bact.* 170: 3967 '88
secA, *secY*	*groE*	*Nature* 342: 451 '89
htrA (*degP*)	*sohA*	*J. Bact.* 172: 1587 '90

identified a novel ribonucleotide reductase (Nrd) that specifically functions under anaerobic conditions. We speculate that the *sufB* gene product be

this particular Nrd. Tests for this possibility are under way.

Diverse examples of multicopy suppression have been reported in *E. coli* (Table 1) as well as other procaryotes such as *S. typhimurium* (Wasserman *et al.* 1983. *J. Bacteriol.* **153**: 1439; van Dyk *et al.*, 1989, *Nature* 342: 451) and *Xanthomonas* (Thorne *et al.*, 1987, *J. Bacteriol.* **169**: 3593) or eucaryotes such as yeast (Hinnebusch and Fink, 1983, *PNAS* **80**: 5374) and nematode *C. elegans* (Maruyama *et al.*, 1989, *MGG* **219**: 113).

It has often been suggested that gene amplification plays an important role in evolution since gene duplication may represent the initial event in the evolution of new function and in the formation of multigene families. Following this initial step, one member could change by mutation or gene conversion or by exon shuffling (Gilbert 1978, *Nature* **271**: 501) to produce new functional genes. In view of this, we believe that it is important to elucidate the molecular mechanism of the multicopy suppression in *E. coli* as a model system.

A Novel DNA Topoisomerase Essential for the *E. coli* Chromosome Partition

Jun-ichi KATO*, Yukinobu NISHIMURA, Sota HIRAGA**, and Hideho SUZUKI***

Twin chromosomes produced upon completion of one round of replication are likely to be linked with each other in a catenane form, as previously demonstrated for small replicons (Sakakibara *et al.* 1976. JMB 108: 569). The primary event in chromosome partitioning should involve the topological resolution of the catenated chromosomes through the decatenase activity of a DNA topoisomerase. DNA gyrase participates in the resolution of the catenated *E. coli* chromosomes (Steck and Drlica, 1984, *Cell* **36**: 1081). Three kinds of DNA topoisomerases have so far been found in *E. coli*: Topoisomerase I (Topo I), II (gyrase), and III (Topo III) (Wang, 1985, *Ann. Rev. Biochem.* **54**: 665; DiGate and Marians, 1988, *JBC* 263: 13366; and see Fig. 1). Two *par* mutant phenotypes described as ParA and ParD have been ascribed to *gyrB* and *gyrA*, respectively (Kato *et al.*, 1989, *MGG* **217**: 178; Hussain *et al.*, 1987, *Mol. Microbiol.* **1**: 259). The

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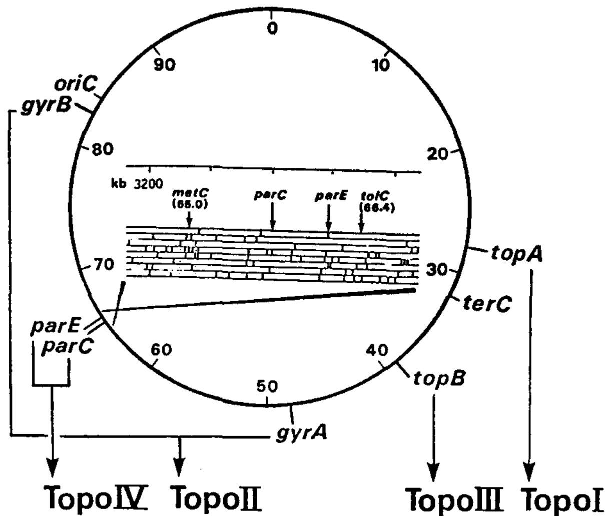

Fig. 1.

parC mutation is not related to *gyr* (Kato *et al.*, 1988, *J. Bacteriol.* **170**: 3967).

The nucleotide sequence of the *parC* gene has now been determined. The deduced amino acid sequence was homologous to that of the A subunit of gyrase (Fig. 2). The homology was particularly remarkable around the tyrosine residue in the active site for gyrase activity. The *parC* protein was shown to bind DNA.

Another new gene was identified in the upstream region of the *parC* gene. The nucleotide sequence indicated that the product of that gene was homologous to the gyrase B subunit. Four mutants were found in the gene by surveying our *E. coli* mutant bank. They showed the typical Par phenotype, and we named this gene *parE*.

A Topo I defect was compensated for by introducting simultaneously two multicopy plasmids, one with the *parC* gene and the other with the *parE* gene. Thus, *toc*, a suppressor of *topA* (Dorman *et al.*, 1989, *Mol. Microbiol.* **3**: 531), is most probably *parC* plus *parE*. We now believe that *parC* and *parE* genes code for a novel topoisomerase, Topo IV, which is essential for chromosome partition (Fig. 1). For details, see Kato *et al.*, Cell in press.

```
E.coli ParC    1'                            MYVIMDRALPFIGDGLKPVQRRIVYAMSELGLNA
                                             * **..**** , ******.***.***..**...
B.subt GyrA    1" MSEQNTPQVREINISQEMRTSFLDYAMSVIVSRALPDVRDGLKPVHRRILYAMNDLGMTS

       ParC   35' SAKFKKSARTVGDVLGKYHPHGDSACYEAMVLMAQPFSYRYPLVDGQGNWGAPDDPKSFA
                  .   .*****.**.*.********** **.** *** *.*** ****.**.*. *. .. *
       GyrA   61" DKPYKKSARIVGEVIGKYHPHGDSAVYESMVRMAQDFNYRYMLVDGHGNFGSVDGDSA-A

       ParC   95' AMRYTESRLSKYSELLLSELGQGTADWVPNFDGTLQEPKMLPARLPNILLNGTTGIAVGM
                  ******.*.** *   .*......* *.   *.**.  .**  ..*.*.**.*.**..******
       GyrA  120" AMRYTEARMSKISMEILRDITKDTIDYQDNYDGSEREPVVMPSRFPNLLVNGAAGIAVGM

       ParC  155' ATDIPPHNLREVAQAAIALIDQPKTTLDQLLDIVQGPDYPTEAEIITSRAEIRKIYENGR
                  **.****.* *. ....*. ..*..*. .*.....***.**...*. .*..*** **.**
       GyrA  180" ATNIPPHQLGEIIDGVLAVSENPDITIPELMEVIPGPDFPTAGQIL-GRSGIRKAYESGR

       ParC  215' GSVRMRAVWKKED-----GAVVISALPHQVSGARVLEQIAAQMRNKKLPMVDDLRDESDH
                  **. .**  . *.        . .....**.**. *...*.**. .*.**.   ..*******.
       GyrA  239" GSITIRAKAEIEQTSSGKERIIVTELPYQVNKAKLIEKIADLVRDKKIEGITDLRDESDR

       ParC  270' ENPTRLVIVPRSNRVDMDQVMNHLFATTDLEKSYRINLNMIGLDGRPAVKNLLEILSEWL
                  ..   *.**  *.. .. . ..*.*.   *.*..*. *** .   .**.* * .* . *...*
       GyrA  299" TG-MRIVIEIRRD-ANANVILNNLYKQTALQTSFGINL-LALVDGQPKVLTLKQCLEHYL

       ParC  330' VFRRDTVRRRLNYRLEKVLKRLHILEGLLVAFLNIDEVIEIIRNEDEP---KPALMSRFG
                     .. ..*** .* * *.  * ****** **. ..*.**..***....     ...*...*.
       GyrA  356" DHQKVVIRRRTAYELRKAEARAHILEGLRVALDHLDAVISLIRNSQTAEIARTGLIEQFS

       ParC  387' LTETQAEAILELKLRHLAKLEEMKIRGEQSELEKERDQLQGILASERKMNNLLKKELQAD
                  ***.**.***...*..*. **  ** .*  .* *  ..*..***.* *. .....** .
       GyrA  416" LTEKQAQAILDMRLQRLTGLEREKIEEEYQSLVKLIAELKDILANEYKVLEIIREELTEI

       ParC  447' AQAYGDDRRSPLQEREEAKAMSEHDMLPSEPVTIVLSQ--------MGWVRSAKGHDIDA
                   . ..*.**. . . .. ......*.. .* ....*..          .  **.*  . ..
       GyrA  476" KERFNDERRTEIVT-SGLETIEDEDLIERENIVVTLTHNGYVKRLPASTYRSQKRGGKGV

       ParC  499' PGLNYKAGDSFKAAVKGKSNQPVVFVDSTGRSYAIDPITLPS-AR-GQGEPLTGKLTLPP
                  .*.. ...*  .  ...........* ...*. *  .  ..*. .* ..* *... *..
       GyrA  535" QGMGTNEDDFVEHLISTSTHDTILFFSNKGKVYRAKGYEIPEYGRTAKGIPIINLLEVEK

       ParC  557' GATVDHML--ME-SDDQKLLMASDAGYGFVCTFNDLVARNRAGKALITLPENAHVMPPVV
                  *. .. ..  * ...  *..... * .   ....... ...*   ..*.*....*   .
       GyrA  595" GEWINAIIPVTEFNAELYLFFTTKHGVSKRTSLSQFANIRNNGLIALSLREDDELMGVRL

       ParC  614' IEDASDMLLAITQAGRMLMFPVSDLPQLSK-GKGNKIINIPSAEAARGEDGLAQLYVLPP
                  ........... *..* ...** .*...... . * * *......... * . *..
       GyrA  655" TDGTKQIIIG-TKNGLLIRFPETDVREMGRTAAGVKGITLTDDDVVVGMEILEEESHVLI

       ParC  673' QSTLTIHVGKRKIKLRPEELQKVTGERGRRGTLMRGLQRIDRVEIDSPRRASSGDSEEX

       GyrA  714" VTEKGYGKRTPAEEYRTQSRGGKGLKTAKITENNGQLVAVKATKGEEDLMIITASGVLIR

       GyrA  774" MDINDISITGRVTQGVRLIRMAEEEHVATVALVEKNEEDENEEEQEEV
```

Fig. 2. Amino acid sequence homology of *E. coli* ParC with *B. Subtilis* GyrA (Moriya *et al.*, 1985, *Nucleic Acids Res.* **13**: 2251). Identities are represented by asterisks and conservative substitutions by dots. Active site Tyr is boxed.

Identification and Cellular Localization of the Product of *prc*, a Gene Involved in the C-Terminal Processing of Penicillin-Binding Protein 3 of *Escherichia coli*

Hiroshi HARA and Hideho SUZUKI*

The maturation of penicillin-binding protein 3 (PBP 3), an enzyme essential for cell division in *Escherichia coli*, involves the proteolytic cleavage of the C-terminal part of the precursor (Hara *et al.*, 1989, *J. Bacteriol.* **171**: 5882–5889). We have found a *prc* mutant defective in this unique processing reaction (Hara *et al.*, 1988, *Natl. Inst. Genet. Ann. Rep.* **38**: 48–49), and have cloned the *prc* gene (Hara and Suzuki, 1989, *Natl. Inst. Genet. Ann. Rep.* **39**: 52–54). The recombinant plasmids pHR53 and pHR61 carrying the *prc* gene are shown in Fig. 1.

To identify the *prc* gene product, proteins synthesized in maxicells and in an *in vitro* coupled trancription-translation system under the direction of pHR53 and pHR61 were analyzed by sodium dodecylsulfate (SDS) gel electrophoresis. A faint band of a protein migrating at the position of about 80 kilodalton (kDa) was detected for pHR61, but not for pHR53. It was previously inferred that *prc* was transcribed in the direction from left to right in Fig. 1, and thus, both in pHR53 and in pHR61, *prc* would be placed next to the promoter of the drug resistance genes of the vectors in the opposite orientation. We recloned the 2.6 kb *Eco*RI-*Pst*I fragment

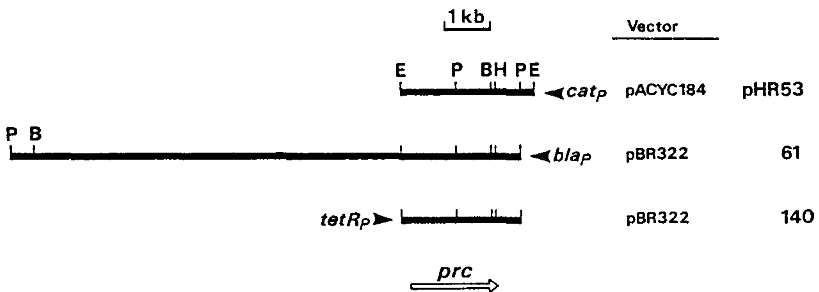

Fig. 1. The plasmids used for the identification of the *prc* gene product. Only the cloned chromosomal regions are shown. Wedges denote the direction of the promoters on vectors, and an arrow denotes the inferred location and orientation of the *prc* gene. B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; and P, *Pst*I.

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containing *prc* into pBR322 to construct pHR140 (Fig. 1), in which the direction of transcription from the promoter originally for a *tet* repressor gene matched the inferred direction of *prc*. A remarkable overproduction of the 80 kDa protein was observed with pHR140. Insertion of a synthetic translation terminator (Maruyama *et al.*, 1989, *Gene Anal. Techn.* **6**: 57–61) into the *Pst*I site within *prc* eliminated the 80 kDa protein. When the terminator was inserted into the *Bam*HI site, which was also within *prc*, a product of almost normal size was observed. These results indicate that the 80 kDa protein is the *prc* product and that the direction of transcription is from left to right in Fig. 1 as inferred. Plasmid pHR53 complemented the *prc* mutation but did not direct the synthesis of the 80 kDa product to a detectable level, probably owing to a weak promoter of the *prc* gene, or to the transcription from the *cat* promoter counteracting the transcription of *prc*, or to both. The 80 kDa product was detected with pHR61 although the *bla* promoter could counteract the *prc* transcription. Transcription from somewhere in the 9.5 kb *Pst*I-*Eco*RI fragment upstream to *prc* might proceed beyond the *Eco*RI site and contribute to the higher *prc* expression. Weak expression of *prc* in pHR53 was enough to complement the *prc* mutation. Hyperexpression of *prc* by a strong promoter was rather deleterious to cell growth.

The location of the *prc* product in a cell was investigated in maxicells harboring pHR164 which carried the genes *prc*, *bla*, and *tet*. When the maxicells were disintegrated by sonication and fractionated by centrifugation, the *prc* product was observed both in the soluble fraction and in the insoluble membrane fraction. The membrane fraction was further treated with a detergent, Sarkosyl, and the *prc* product was found in the solubilized cytoplasmic membrane fraction. The *bla* and *tet* products were recovered, as expected, in the soluble fraction and in the cytoplasmic membrane fraction, respectively. When the maxicells were converted to spheroplasts by treatment with lysozyme and centrifuged, the *prc* product was fractionated both to the supernatant periplasmic fraction and to the pelleted spheroplasts. These results may indicate that the *prc* product is distributed both in the periplasm and in the cytoplasmic membrane. However, it does not appear that any major processing occurs in the *prc* product, because no difference in electrophoretic mobility was observed between the product synthesized *in vitro* and that in maxicells, when tested in SDS gels of several polyacrylamide concetrations. When the maxicells were fractionated by the

method of Russel and Model, which consists of treatment with 0.1 N NaOH followed by centrifugation and precipitates membrane proteins (Russel and Model, 1982, *Cell* **28**: 177–184), the *prc* product partitioned into the NaOH pellet together with the *tet* product, leaving the *bla* product in the supernatant. It seems probable that the *prc* product is loosely associated with the periplasmic side of the cytopolasmic membrane and easily released into soluble fractions during fractionation procedures. It should be noted that overproduction of the product in maxicells may affect its distribution. Localization of the *prc* product outside the cytoplasmic membrane is consistent with the notion that the C-terminal processing probably occurs after PBP 3 is translocated across the cytoplasmic membrane (Hara and Suzuki, 1988, *Natl. Inst. Genet. Ann. Rep.* **39**: 56–57).

Nucleotide Sequence of the *prc* Gene of *Escherichia coli*

Yoshihiro YAMAMOTO*, Yukinobu NISHIMURA and Hiroshi HARA

The *prc* gene of *Escherichia coli* is involved in the unique processing reaction which cleaves the C-terminal part of the precursor form of penicillin-binding protein 3, an enzyme essential for septum formation (Hara *et al.*, 1989, *J. Bacteriol.* **171**: 5882–5589). The gene was located in a 2.6 kilobase (kb) *Eco*RI-*Pst*I fragment at min 40.3 on the *E. coli* chromosome (Hara and Suzuki, 1989, *Natl. Inst. Genet. Ann. Rep.* **39**: 52–54; Hara *et al.*, 1989, *ibid.* 55), and the product was identified as a protein of about 80 kilodalton (kDa) as described in the accompanying report (p. 49–51).

We determined the nucleotide sequence of a 3178 base-pair (bp) chromosomal fragment, the 2.9 kb *Eco*RI fragment cloned in pHR53, plus 220 bp in the contiguous 9.5 kb *Pst*I-*Eco*RI fragment in pHR61 (see Fig. 1 on p. 49). Only one major open reading frame (ORF) of significant length, starting with an AUG initiation codon either at nucleotide (nt) 287 or at nt 293 and ending with a UAA termination codon at nt 2330, was identified. Its location and direction were consistent with our previous results. We do not know at present which of the two AUG codons is used as the initiator. The ORF could code for a polypeptide of 682 or 680 amino acids with a predicted molecular weight of 76,677 or 76,432, in good agreement with the size of the gene product estimated previously. The

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product, as deduced from the identified ORF, showed a relatively low polarity. Several hydrophobic regions were found near the N terminus and towards the middle of the polypeptide. The N-terminal hydrophobic sequence might serve as a signal directing the product to the outside of the cytoplasmic membrane, but it does not appear to be cleaved, as described in the accompanying report (p. 49–51).

When the *prc* gene was placed under the control of the λ p_L promoter and the temperature-sensitive *c*I857 repressor, production of a large amount of the *prc* product was induced upon temperature upshift to 42°C. Under these conditions the rate of processing of PBP 3 was increased. The *prc* product is possibly a processing enzyme, although we cannot completely eliminate other possibilities, e. g. it may be an activator of the processing enzyme. The deduced amino acid sequence exhibited no significant homology to any proteases, peptidases, or other proteins in the European Molecular Biology Laboratory protein database.

A Mutant Producing Temperature-Sensitive Farnesyl Diphosphate Synthase of *Escherichia coli*

Shingo FUJISAKI*, Tokuzo NISHINO**, Hirohiko KATSUKI***,
Hiroshi HARA and Yukinobu NISHIMURA

A reaction utilizing isopentenyl diphosphate (IPP; C_5) and farnesyl diphosphate (FPP; C_{15}) is a branch point in the synthetic pathway of various isoprenoids. *Escherichia coli* provides an excellent model system for the study of non-sterol isoprenoid synthesis because it is much simpler in the isoprenoid composition than eukaryotic cells. We screened Hirota's temperature-sensitive (ts) mutant stock of *E. coli* for mutants defective in isoprenoid synthesis, and found a mutant, JE11046, having a ts defect in FPP synthase activity. FPP synthase condenses IPP with dimethylallyl diphosphate (C_5) or geranly diphosphate (C_{10}) to yield FPP. The mutation for this defect was named *ispA*. Results of Hfr crosses and F′- and P1-mediated transductions indicated that the map location of *ispA* was about min 10 and that the gene order was *tsx-ispA-lon*. The *ispA* mutation

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was recessive to the wild-type allele.

An isogenic pair of *ispA*$^-$ and *ispA*$^+$ strains was constructed and their properties were examined. Unexpectedly, the *ispA* mutant showed no growth defect compared to the wild type both at 30°C and at 42°C, although the *in vitro* activity of partially purified FPP synthase was significantly lower for the mutant than for the wild type (21% at 30°C and 5% at 42°C). The amounts of major isoprenoids in the mutant cells were almost the same as those in the wild-type cells at 30°C and at 42°C except that the level of ubiquinone was moderately lower (66%) in the mutant cells at 42°C. FPP synthase may not be rate-limiting in general isoprenoid synthesis, or the mutant FPP synthase may be stabilized *in vivo*. The possibility cannot be excluded that there may be another short-chain prenyl diphosphate synthase which can supply a priming substrate for synthesis of long-chain prenyl diphosphate. The is growth of JE11046 appears to be due to some mutation(s) other than *ispA*.

We suggest that *ispA* represents a structural gene for FPP synthase, because FPP synthase partially purified from the *ispA* mutant was ts and wild-type FPP synthase was not inhibited by the addition of the enzyme fraction of the mutant. For details, see Fujisaki *et al*., 1989, *J. Bacteriol.* **171**: 5654–5658.

Mapping of a Whole Set of Cell Division Genes in *Escherichia coli* K-12

Akiko Nishimura, Reiko Irobe, Keiko Suzuki and KiyokoUeyama

One of the possible approaches to elucidate the cell division mechanism in *E. coli* is to identify the whole set of genes involved in cell division, and to analyze their functions and relations among their expression. Such an appeoach would also make possible to find key genes and key reactions in the cell division cycle.

Up to now only a few scores of cell division genes have been identified. However, *E. coli* is expected to have more than one hundred cell division genes as judged from statistical analyses on the occurrence of known mutations (Maruyama, personal communication). We have developed a system of procedures to identify a whole set of cell division genes in a short time by use of three different types of *E. coli* culture bank.

The strategy is as follows: i). Screening mutants from the "Hirota's

TS mutant bank", which form filamentous cells at 40°C without immediate arrest of DNA synthesis and of cell mass increase. ii). Screening plasmids from the "Clarke & Carbon's pLC-plasmid collection", complementing TS defects of these mutants, which represent putative cell division genes. iii). Identification of the map position of the insert in each pLC-plasmid by plaque hybridization to "Kohara's clone bank". The system provides a convenient screening procedure for identifying plasmids harboring a cell division gene, and its mutant strain at the same time, and for carrying out structural analyses of these genes. The 282 of cell division mutants were mapped on the physical map of *E. coli*. For details, see "Control of Cell Growth and Division" 1991, H. Yoshikawa and A. Ishihama (eds.).

The present study was focused on the analysis of cell division genes. However, we notice that our physical mapping of pLC-plasmids would provide by itself a useful tool for genetic analyses and gene cloning in *E. coli*. (in preparation).

The *secA* Gene of *Bacillus subtilis*

Yoshito SADAIE, Hiromu TAKAMATSU*, Kouji NAKAMURA*
and Kunio YAMANE*

We determined the nucleotide sequence of the 4.1 kb *Cfr*13I chromosomal fragment from *ρ*11-*div-341*$^+$ DNA which carries the wild type *div-341*$^+$ gene of *Bacillus subtilis*. Three open reading frames were found in the same orientation. The biggest frame consisted of 841 codons and codes for a protein with a molecular weight of 95,529. The protein shared a 50% identity in amino acid sequence with the *secA* protein of *Escherichia coli* as shown in Fig. 1. The two genes shared a 47.2% identity in their nucleotide sequences.

The wild type *div-341*$^+$ gene of *B. subtilis* is required for sporulation, secretion of extracellular enzymes, autolysis, competence development, and spore outgrowth as well as for cell division. By analogy with the function of the *E. coli secA* gene, the wild type *B. subtilis div-341*$^+$ gene is responsible for the secretion of certain extracellular enzymes and translocation of certain proteins on the cell surface which are involved in cell division, sporulation, autolysis and uptake of exogenous transforming DNA. (*Mol. Gen. Genet.* **190** (1983) 176–178; *J. Bacteriol.* **163** (1985) 648–653; *Jpn.*

* Tsukuba University.

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ML-giLnKmF-dptkRTLnRyeKiaNdIdAirgdyEnLSDdaLKhKTiEF
keRLEKGattddLlvEAFAVVREASrRVtGMfpFkVQLmGGvaLhdgnIA
EMkTGEGKTLTsTLPvYLNALTGKGVHVVTVNeYLAsRDAEqmgkiFEFL
GLTVGlNLnsMskdeKREAYAADITYsTNNElGFDYLRDNMvlykEqmVQ
RpLHfAviDEVDSILIDEARTPLIISGqAakStklYvqaNafvr------
-----TlkaEkdytyDiKtkaVqLTEeGmtkaEkaf---GI----dnLfd
vkhvaLnHHinqALkAHvamqkDVDYvVeDGqVvIVDsfTGRlMkGRRyS
eGLHQAiEAKEGleIQNEsmTLAtITFQNYFRmYEKLAGMTGTAkTEeeE
FrnIYnmqvVtiPTNRPvvRdDrPDLiYrTmegKfkAvaEDvaqRymtGQ
PVLVGTvavEtSELiSklLknkGIpHqVLNAKnHerEAqIieeAGqkgAV
TIATNMAGRGTDIkLGeG------------------------------Vk
ElGGLavvGTERHESRRIDNQLRGRSGRQGDpGitqFYLSMEDeLMRrFg
aeRtmaMldrfGMddstpIqskmVsrAvessQkrVEgnNFDsRKQLLqYD
DVlrqQRevIYkQRfEviDsenlrEivenmiksslera IaAYtPreeLpE
eWkldGLvdlinttyldegaLeksdifgKEP----demlelImdriItkY
neKEEqfGkEqMReFEKvivLravDSkWmdHidAMDqLRQGIHLRaYAQt
nPlrEYqmEgFaMFehMiESiedEVakfvmKaei-----ennLEreevvq
gqttAh----qpQegDdnkkAkkApvrkvvdiGRNaPChCGSGKKYKnCc
GRte
```

Fig. 1. The amino acid sequence of the *Bacillus subtilis div-341*$^+$ gene. Capital letters indicate common amino acids with *E. coli secA* protein, which consists of 901 amino acids, 60 amino acids longer than the protein from the wild type *B. subtilis div-341*$^+$ gene.

J. Genet. **64** (1989) 111–119; *Gene* in press.)

III. IMMUNOGENETICS

Fine Mapping of Recombinational Hotspots in the Mouse MHC by Direct Sequencing of PCR Amplified DNA

Toshihiko SHIROISHI, Tomoko SAGAI, Hideo GOTOH
and Kazuo MORIWAKI

Meiotic recombinations within the major histocompatibility complex (MHC) of the mouse occur in clusters at restricted sites, the so-called, recombinational hotspots. So far four hotspots have been identified in the proximal region encompassing 400 kb of DNA in the mouse MHC. Japanese wild mouse derived haplotype, wm7, exibits enhanced recombination frequency in this region in genetic crosses with standard laboratory strains. Recombinational breakpoints were confined to one of the hotspots which is 1 kb of DNA between the $A_{\beta 3}$ and $A_{\beta 2}$ genes. Molecular characterization of this hotspot and previously identified hotspot located in the E_{β} gene revealed that two independent hotspots share a very similar molecular organization, consisting of two molecular elements, mouse middle repetitive MT-family and tetramer repeated sequences (Shiroishi *et al.*, *Immunogenetics* **31**, 79–88, 1990).

In order to elucidate the spatial relationship between the location of the recombinational breakpoints and these two elements, we attempted to make a more fine mapping of recombinational breakpoints. For this purpose, DNA segments including the hotspots were amplified by the polymerase chain reaction (PCR) method from twelve independent recombinants. After further amplification using one of two primers to yield the single stranded DNAs, approximately 1 kb of amplified DNAs including the breakpoints were directly sequenced. Comparison of these sequences and those of parental strains made it possible to assign the fine location of the breakpoints. All recombinants from the cross of wm7 and b MHC haplotypes have breakpoints in approximately 300 bp of DNA located upstream from the tetramer repeated sequence, while most recombinants from the cross of wm7 and k haplotypes placed their breakpoints in 500 bp of DNA located downstream from the tetramer repeated sequence. Thus, all recombinational breakpoints are clustered around

the tetramer repeated sequence. The significance of this association of breakpoints with the tetramer sequence remains to be determined.

Characterization of Asian Specific H-2K^u Class I Antigen

Tomoko SAGAI, Toshihiko SHIROISHI and Kazuo MORIWAKI

Among the polymorphic H-2K class I antignes, H-2K^f and K^d distribute at high frequencies (K^f; 29%, K^d; 31%) in Asia and have been observed at cerain frequencies in Europe (K^f; 6.5%, K^d; 15%) as well. A detailed characterization of the H-2K^f antigens identified in both populations, revealed that they include many minor variants related to each other. Nevertheless, an indistinguishable K^f related molecule was detected in European (*M. m. domesticus*) and Asian (*M. m. musculus, M. m. castaneus* and *M. m. molossinus*) mouse populations, although they are genetically separated to large extent. The geographical survey of the minor variants suggested that the various H-2K^f-related molecules have been generated before and after *M. musculus* subspeciation and coexisted in wild mouse populations during their long evolutionary process. These results support a positive selection hypothesis for increasing the number of MHC alleles.

Another H-2 class I antigen, H-2K^u showed a high frequency (22%) in Asia, but it has been hardly detected in Europe (0.3%). The geographical distribution of H-2K^u almost overlaps the habitat of *M. m. castaneus* and *M. m. molossinus* which is considered to be a hybrid of *musculus* and *castaneus* subspecies. A characterization of H-2K^u antigens using monoclonal antibodies and a DNA probe showed that those observed in Japan, Taiwan and China are very similar to each other and are clearly distinguishable from other H-2 haplotypes. In contrast to H-2K^f molecules, the H-2K^u molecules seemed to share a framework of molecular structure with those of other antigen molecules, such as K^q and K^s. These results suggest that the ancestor of the H-2K^u antigen molecules was generated in Asian population after the subspeciation of *M. musculus*.

IV. DEVELOPMENTAL AND SOMATIC CELL GENETICS

Studies on Freeze Preservation of Embryos of *Drosophila melanogaster*

Yukiaki KURODA and Yuko TAKADA

The freeze-preservation of *Drosophila* embryos is a useful procedure for maintaining many wild-type and mutant stocks of *Drosophila* which saves enormous cost, labor and time spent for maintaining them at 20–25°C in ordinary procedures. There are, however, some barriers to be cleared up to accompolish freeze-preservation. One such barrier may be the introduction of freeze-protective agents, glycerol and dimethyl sulfoxide (DMSO), into embryos. The vitelline membrane of embryos prevents the agent from entering embryos. Many attempts have been made to abolish the non-permissible property of the vitelline membrane. One procedure is to use a laser microbeam to perforate the vitelline menbrane. The procedure proved partially successful in obtaining living larvae and adults after freeze-preservation at a very low frequency.

The other procedure is chemical treatment and this might be accompolished with some enzyme treatment. The enzymes used were proteases such as dispase (Boehringer Manheim), pancreatin (Difco), pectinase (Sigma), neuraminidase (Sigma) β-galactosidase (Sigma) and cellulase (Sigma). Eggs were dechorionated by treatment with 3% sodium hypochloride solution for 6 minutes and then treated with these enzymes at various concentrations for 1–3 hours. After treatment with enzymes, eggs were examined for the entrance of neutral red and their hatchability after incubation at 25°C.

β-galactosidase and pancreatin had almost no effect on the permeability of the vitelline membrane. Neuraminidase, pectinase and cellulase enhanced the permeability of the vitelline membrane. The treatment of eggs with 1% cellulase for 3 hours was especially effective in enhancing permeability and had no significant effect on the hatchability of eggs. More effective and useful treatments with these enzymes are under investigation.

Detection of SR Spiroplasms in Embryonic Tissues of *Drosophila melanogaster* by an Electron Microscope

Yukiaki KURODA, Kugao OISHI* and Yutaka SHIMADA

In the previous study, it was found that sex-ratio (SR) spiroplasms might produce some brown necrotic changes in cultured epithelial cells of embryos of *Drosophila melanogaster*, when they infected embryonic cells *in vitro*. Under an electron microscope, embryonic cells infected with SR spiroplasms contained many spiroplasm-like structures in intercellular spaces and the cytoplasm of necrotic cells.

In the present experiment, whether SR spiroplasms might selectively infect any specific type of embryonic cells in normal development was examined. Eggs were collected 3.5, 7.5, 12 and 13 hours after oviposition from adult flies of the Oregon-R strain infected with SR spiroplasms. They were pre-fixed in glutaraldehyde and paraformaldehyde and fixed in 1% osmic acid. Epon embeded eggs were sectioned and observed under an electron microscope.

The results indicated that some necrotic cells were found in the brain area and in some neuroblast cells under the hypoderm cells of embryos. In embryos at 20 hours after oviposion, some structures which might be SR spiroplasms were detected in intercellular spaces. These findings suggest that SR spiroplasms may have a specific affinity for the brain and nervous tissues and produce necrotic changes in them.

Developmental Genetics of *E*-Pseudoallelic Domain in *Bombyx*
1. Genetic characteristics

Akio MURAKAMI

It was nearly half a century ago when the suggestion was made that *E*-pseudoallelic gene loci (6–0.0) in *Bombyx* could be used as important experimental material for studies concerning the basic body structure of embryo. Receiving impetus from recent studies on the homeotic gene in *D. melanogaster*, the analysis of *Bombyx E*-pseudoalleles has been started again in developmental genetics, using molecular biological techniques. A total of about 30 mutant members which comprise the *E*-allele have been found, including spontaneously induced members and artificially induced ones

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created through the use of ionizing radiation etc. The main genetic characteristics of these members are their dominant characteristic and the fact most of them are embryonic lethals. They are also characterized by a versatile expression of various visible and invisible characteristics from the embryonic stage to the larval stage; including fewer abdominal legs, larval pattern marking, morphological abnormality of gonad and malformation of larval epidermis. In addition, the allelic group can be induced through ionizing radiation which has a chromosome-breaking ability as described above, and recombination can be observed clearly in females of the allelic group, though the recombination frequency is low compared to males (crossing over occurs generally only in males in the case of *Bombyx*).

On the basis of these characteristics, it seems logical to assume that the hereditary nature of the *E*-allelic mutation originated from chromosome aberration rather than gene mutation. Also, the size of the *E*-domain is fairly large and random recombination of chromosomal DNA (unequal crossing over) occurs at chromosome brekage (or easily breakable) areas independent of sex, thus causing the creation of various types of *E*-member group constituents.

Developmental Genetics of *E*-pseudoallelic Domain in *Bombyx*
2. Developmental Characteristics

Akio MURAKAMI

The expression of a supranumeral leg is a degeneration of phylogenesis: There are many reports on the relationships among embryonic lethality, abnormal numbers of larval abdominal legs and abnomality of larval body marking pattern in *E*-pseudoallelic domain mutants, but the mechanism remains unclear. Such a subject is interesting also in terms of its relationship to the determination of the structural plan of *Bombyx*. Results of rearrangement and analysis of observation data so far accumulated on the relationship between visible character and embryonic lethal have indicated the possibility that an excess (or reduced) number of abdominal legs has a strong relationship to embryonic lethality on the basis of the existing patterns of crescent marking and star spot marking.

In normally developed early stage embryos, appendices for the development of future larval organs, such as abdominal and thoracic legs, exist in all of the 18 segments except for the extreme tip segment which becomes a

caudal leg in the future. These appendices, excluding those which become head organs, thoracic legs and abdominal legs of *Bombyx* larvae in the future, disappear just after the so-called "blastokinesis" when organogenesis is almost completed. Among *E*-allelic mutants, however, a mutant having the ability to express supranumeral abdominal leg exists as it is (sometimes in an incomplete form) and in particular has an excess of larval legs. A normal developmental programme results in a silkworm larval body having three pairs of abdominal legs. It can be concluded that a mutant of the *E*-pseudoallelic group shows a morphology similar to that of *Myriapoda* which is a progenic type of insect, because of the abnormal developmental programme caused by structural abnormality (probaly a deficiency) in its *E*-domain chromosome as descrived elsewhere (Murakami, 1989). It seems that the high frequency of embryonic lethality is caused not only by direct chromosomal abnomality and indirect abnormality of the structural plan, but also by functional abnormality. In conclusion, it can be said that the supranumeral leg in the *E*-allelic mutant is a type of embryological degeneration.

Structural plan of Bombyx leg deduced from the distribution of supranumeral legs: As described in the preceding section, supranumeral abdominal legs of a larva appear in the anterior abdominal part of abdominal segments ($A_{1,2,\cdots 7,8}$) but not in the posterior part of abdominal segments ($A_{9,10,11}$). Naturally, such legs do not appear in the thoracic part ($T_{1,2,3}$) or the head which is comprises of four segments. The frequency of supranumeral abdominal legs at two abdominal segments (A_7 and A_8) is quite low (below 1%).

In embryogenesis of *Bombyx*, similar to that of other insects, the head part is formed from the first to fourth embryonic segments and the following three segments form the thoracic part (5th, 6th and 7th segments). The remaining 11 segments form the abdominal part. The formation of the abdominal part starts almost at the same time as head formation. The head formed by the fusion of the first to fourth segments (or ganglia). Interestingly, the formation of the A_{10-7} segments (or ganglia) of the abdominal part as well as the caudal leg (A_{18}) are accomplished in a similar way through the repeated fusion of two segments (or ganglia) until the A_7 segment os reached. On the other hand, the A_6-A_3 segments of the abdominal section are formed smoothly through the fusion of segments and ganglia. Therefore, in normal embryogenesis, the abdominal part (abdominal com-

plex: ABC) is divided into anterior ABC (ABC^a) comprising the 8th to 13th segments and posterior ABC (ABC^p) which comprises the 14th to 18th segments. When the condition of supranumeral legs is taken into consideration, ABC^a can be divided further into a part which includes the 10th to 13th embryonic segments (ABC^{a-2}) where the appearance (decrease) of normal abdominal legs is observed and a second part comprising the 8th and 9th segments (ABC^{a-1}) where supranumeral abdominal legs appear at a high frequency in *E*-allelic mutants. In the same manner, it is possible to classify ABC^p into a segment (ABC^{p-1}) where supranumeral legs appear, though at a low frequency and another part comprising the 16th to 18th segments (ABC^{p-2}) where supranumeral legs do not appear.

Genetic Studies on the Diapause Phenomenon in *Bombyx* 1. Seasonal Adaptation of the Tropical Multivoltine Race in the Temperate Zone

Akio MURAKAMI

In *Bombyx mori* L., univoltine races never change their genetically determined voltinism in the climate of Japan, while bivoltine races easily change their inherent voltinism(s) between bi- and univoltine types. It is of interest to note that tropical multivoltine races have in general an unusually high adaptability: when these races are introduced to the temperate zone including Japan, from their native tropical or subtropical zones, they easily acquire a high embryonic diapause state for only one or two generations after introduction from their native places to the temperate zone. This short term of adaptability of seasonal changes seems to be not in accordance with general adaptation theory. However, this unusual phenomenon can be explained by the detection of a gene (or system) which is responsible for the abnormal phenomenon. Tropical multivoltine races including strain *Cambodge*, however, change remarkably their generation times in a year from the multivoltine type (7–8 times) to the univoltine one (a single time) in Japan. In fact, the strain *Cambodge* is highly adaptable to changes in climatic conditions in Japan and is sensitive to changes of seasons and/or photoperiods (Murakami, 1988, '89, '90). Having foreknowledge of unfavorable environmental conditions to come in the near future, the moths of multivoltine races raised in Japan tend to lay diapausing (and/or hibernating) eggs for life existence and/or species maintenance.

The embryonic diapause phenomenon of the multivoltine race in the temperate zone seems to be one of certain motive powers for expanding their ecological niche. It was revealed by author that these biological phenomena are under the control of a sex-linked gene *npnd* (non-pigmented and non-diapausing egg) detected in the strain *Cambodge*. Of course, the gene itself never changes but only changse its phenotypic expression depending on climatic conditions.

About twenty years ago, an autosomal recessive multivoltine gene *pnd* (pigmented but nondiapause egg) was isolated from the strain *Cambodge* by Katsumata (1968) and its linkage was analyzed (Yamamoto *et al.*, 1972). The insect having the gene *pnd* produces only nondiapause eggs throughout the year regardless of change in environmental conditions. According to our genetic analyses of interactions between these two multivoltine genes, the gene *npnd* is epistatic to *pnd*, indicating that the phenotype of egg states in silkworm lines having both genes is absolutely under the control of the *npnd* gene.

In short, the *npnd* gene (system) controls the nature of multivoltinism and is also highly responsible for seasonal adaptations. A number of researchers have done extensive investigate to clarfy the diapause phenomenon and consequently they have accumulated much valuable information on this subject. Among other things, the photoperiod or daylength has been shown to be the most prominent factor in various physical variables as an indicator of seasonal change. Accordingly, it seems more likely that a function of the gene *npnd* takes part in a series of integrations of various environmental information including the photoperiod. The present interpretation appears to be consistent with some reports which have been shown a connection between brain and the determination of egg-states (Fukuda, 1951; Morophoshi, 1972, '76).

Genetic Studies on the Diapause Phenomenon in *Bombyx* 2. A Possible Role of the Central Nerve Center System on Adaptation

Akio MURAKAMI

The photoperiod changes more drastically in the temperate zone than in the tropics. Daylength increases gradually from the winter solstice to summer solstice in the temperate zone, while the situation is reversed

from the summer solstice to the winter solstice. At both vernal and autumnal equinoxes, daylength becomes 12 hours. In short, the photoperiod is slightly changing day by day.

Considering this, it can be assumed that embryos of *Bombyx* recongnize such a trend: embryos have some function for precisely measuring time differences creating a biological (or circadian) clock. It seems likely that they may store the received information as changes of environmental conditions for about 24 hours in their cerevral nerve center system (CNCS) or brain, to infer time differences in daylength for two consecutive days. Surprisingly enough, in *Bombyx*, for the egg-state conversion of the tropical multivoltine races from the nondiapause type to a diapause one in the climate in Japan, it takes about 1.5 to 2.0 months from the input of the environmental information to the outcome or secretion of diapause factor (DF) in the pupal body cavity, so that it is necessary for long term information preservation. It is also reasonable to say that the *Bombyx* CNCS may have the ability to integrate various kinds of external information or physical stimulations, and store and transmit the adjusted information through the larval period to the mid-stage pupae. Some instructions from the pupal CNCS are dispatched to the SG which synthesizes and secrets DF into the body cavity. DF is synthesized in all silkworm races. Thus, the embryo CNCS which has already received information differ according to the quality of photoperiod, temperature, etc., and transmits the integrated information through the larval CNCS to the mid-stage pupal CNCS which gives some instructions to SG to secret DF which decides finally the fate of the egg-state: during embryonic development some CNCS which have received information on short daylength and/or low temperature or the both promote the secretion of DF, while those which have received long daylength and/or high temperature information, inhibit the secretion. Of course, timing of the endocrinological event is absolutely under the control of the inherent developmental programme of *Bombyx*. In any case, the time course in a series of neural transmission passways of information appears to require a remarkably long duration, suggesting that this process comprises a certain type of mechanism for information storage, a long term memory. It is of interest to understand how the operation of such a storage system in *Bombyx*. It is well known that in *Bombyx* the most popular egg incubation method for obtaining a completely converted egg-state with a voltinism opposite to the originally determined one (from

diapause to nondiapause type, and *vice versa*) is treatment of the egg by keeping for several days under relevant incubation conditions. This suggests that the modification of voltinisms may take part in certain confirmational events.

To sum up, the conversion process of egg-state for the voltinism takes place in three steps: first is the reception of environmental information in certain sensory organs, second is information integration, storage and transmission in CNCS, and third is the endocrinological performance in pupal SG, as the result of orders of environmental information or stimulation.

Genetic Studies on the Diapause Phenomenon in *Bombyx*
3. Artificial Modification of the Diapause State in Temperate Races to Nondiapause Type

Akoi MURAKAMI

In the preceding section, it was stated that egg-states in the tropical multivoltine races convert easily from the genetically determined nondiapause type to a diapause type in the process of seasons under natural climatic conditions in Japan. One of the Chinese bivoltine silkworm races, the Daizo strains (this is regarded as multivoltine races in India) and a few other strains of either Chinese or Japanese races partly resume a 2nd generation after the first spring generation in Japan. However, in general, most of the bivoltine races do not enter a 2nd generation during the year. Univoltine strains do not repeat a summer generation after the spring generation as far we know. Thus, it becomes clear that the conversion from a nondiapause state to a diapuse one in tropical multivoltine races is produced during the process of seasonal change, while the temperate univoltine as well as bivoltine races, except in a few cases, never change their genetically determined diapause state to a nondiapause one under natural conditions. Accordingly, it seems more likely that the former diapause state is a facultative type and the latter an obligatory type, suggesting that there is different fundamental mechanism for the induction of diapause states between the tropical and temperate races.

It is of interest to note that as is well known in *Bombyx*, the diapause state in the temperate uni- and bivoltine races can be highly changed after artificial treatment of eggs (or embryos) under short daylength or complete

darkness and low temperature incubation regimes for several days during the latter half of embryonic development, contrary to environmental (or photoperiodic) conditions which spontaneously induce diapause eggs from nondiapause ones during seasomal changes in Japan. This fact clearly indicates the basic mechanism concerned in the temperate races is unlike that for tropical races. In this respect, egg incubation conditions of short daylength and low temerature or a combination of both are regarded as diapause preventing stimuli by Lees (1955). It is necessary to select biological materials (or strains) for studies on the diapause phenomenon as an arresting agent or factor of embryogenesis in *Bombyx*.

An example of chemical modifications in the diapausing state of the temperate race compared to nondiapausing ones is that when one day old eggs are immersed in acidious solutions including hot dilute HCl for a few minutes, they turn into almost complete nondiapausing embryos. Also, dechorination of diapausing eggs alters their original state to that of non-diapausing eggs and embryonic development resumes immediately to some extent in *in vitro* artificial culture media as well as saline (e. g., Ohtsuki *et al.*, 1975). A similar phenomenon is also observed in spontaneously occurring-parthenogenetic diapausing eggs (Murakami *et al.*, 1987). In addition, various methods including electricity spark, picking, *etc.*, are also to some extent effective in breaking the diapausing state and resumption of embryonic development. From this evidence, it can be said that a slight modulation of a certain controlling principle(s) for the determination of diapause state is enough to completely change eggs state to an opposite one.

In short, the nature of already genetically determined egg-state or voltinism in any voltinistic races of the *Bombyx* silkworm is easily convertible to the opposite egg-state regardless of in both natural and artificial conditions.

The Mutagenic Activity of a Carcinostatic Drug, Neocarzinostatin*, in Germ Cells of *Bombyx Mori* L.

Akoi MURAKAMI

A carcinostatic agent, Neocarcinostatine (NC) [$C_{462}H_{708}N_{130}O_{156}S_4$; m.w. 10707.29] is a product of *Streptomyces carzinostaticus* var F-41 and is composed of 109 amino acids and two -S-S- bonds. This acid polypeptide

* This drug was purchased from Yamanouchi Pharmaceutical Co., Tokyo.

drug with a high molecular weight induces gene mutation or DNA damage in certain stocks of *E. coli* (WP_2, etc.) and an unscheduled DNA synthesis in human leucocytes. There has been no report as to mutagenic effects of NC on germ cells of higher organisms. A preliminary investigation was carried out to determine whether NC has genetic toxicity for germ cells of treated silkworm (*B. mori*). Three different doses, 2, 4 and 6 ng per capita of NC dissolved in 0.025 ml saline were injected into the body cavity of 8-day-old F_1 hybrid pupae from a cross between wild-type strain C108 females and Aojuku males. The germ cells at this stage of *Bombyx* pupae are mature sperm and prophase I oocytes for males and females, respectively. The incidence of mutations was determined by the specfic locus method using egg-color genes, *pe* (5–0.0) and *re* (5–31.7).

The results obtained indicate that in pupal mature sperm none of doses of NC induced specific locus mutation, either whole-body (complete) or fractional-body (mosaic) type, while in prophase I oocytes similar doses of NC were found to induce a significant oncrease in the number of mutants in relationship to dosage. Most mutants detected were fractional-body types, while the incidence of whole-body type was somewhat higher than that of the corresponding control.

The mutagenic effect of NC in *Bombyx* pupae appears to be the same as that of acridine compounds (Murakami, 1972, '73), suggesting that the mechanism by which acridine compounds induce recessive visible mutations could be, in part, applied to that of NC: both prophase I oocytes and mitotic cleavage nuclei are susceptible to NC mutagenesis, but mature sperm with condensed and/or paracrystallized genetic materials are not. This cellular difference in NC mutagenesis may be due to differential DNA conformations in germ cells rather tahn sex differential metabolic activity in the pupal body including gonad, between sexes. Nevertheless, it should be recognized that pupal prophase I oocytes in vitellogenesis prior to the completion of chorion formation are apt to incorporate the drug into the cells in contrast to mature sperm, regardless of metabolic activity.

The preponderance of mosaic mutations in NC-treated female pupae may be interpreted as an indication that the accumulated NC in oocytes may act as a mutagen on mitotic cleavage (DNA replication) nuclei as observed in mutagenesis with acridine compounds. A possible explanation for the occurrence of a small number of complete mutations in female pupae is that this type of mutant may be caused by two independent mu-

tagenic events of NC on the zygotic cell as well as a dingle event on the ovum. Of course, other mechanisms (such as an induced recombination-like event between paired homologous DNA) cannot be excluded as possibilities.

In the present experiment, high molecular weight NC or its metabolite(s) were only mutagenic for oocytes treated during vitellogenesis before the completion of chorion formation in the female pupae. Hence it is likely that a certain physical interaction with DNA bases such as an intercalation is a necessary, but not a sufficient condition, for NC mutagenesis. At any rate, there is a possibility that a carcinostatic drug, NC, is able to induce recessive visible mutations in *Bombyx* germ cells.

Egg Permeability and Development of Embryo in *Drosophila*

Kiyoshi MINATO

In general, it has been shown that insect eggs, even when dechorionated, are impermeable to aqueous solutions. Limbourg and Zalokar ('73) reported the existence to some extent of permeability (uptake of uridine) only in freshly laid eggs (within 2hr at 25°C) in *Drosophila*, and made the eggs at various stages permeable to aqueous solution with octane treatment. Those eggs became very sensitive to the quality of incubation media, and therefore died early or developed abonrmally when incubated in inadequately prepared aqueous media. However, we found that freshly laid eggs themselves are also permeable enough without treatment and die or develop abnormally when incubated in aqueous media.

In preliminary experiments in which we searched for better incubation conditions for microscopical observation of eggs in *Drosophila melanogaster*, we sometimes got many un-hatched eggs when eggs were dechorionated and incubated in aqueous media. But if these eggs were incubated in non-aqueous media such as paraffin oil, this situtation was never seen. Moreover, in aqueous incubation, it is found that the younger the used eggs were, the more un-hatched eggs were produced. When the eggs older than the syncytial blastoderm stage at the start of incubation were used, they developed normally and few un-hatched eggs were seen.

The development of freshly laid eggs incubated in aqueous media were observed. Eggs incubated in distilled water soon became opaque, showing no signs of development such as nuclear cleavage or migration to the egg

surface. After several hours there were swelling possibly due to the hypotonicity of the medium. Incubated in 0.9% NaCl, eggs developed to a later stage and formed cellular blastoderms in limited areas of the eggs (mostly at the posterior third to half), which, after several hours, caused teratogenic embryos an opaque and amorphous area in the anterior and a more transparent bag-like structure made of cell layers in the posterior. These embryos somewhat shrank and hence detached from the vitelline membrane at the anterior and posterior ends. When incubated in 0.75% NaCl, embryos showed the least degree of developmental anomaly: eggs sometimes developed nearly to the sac-like midgut stage though they had poorly developed organs, and they moved under the vitelline membrane though they were rather small compared to the normal embryos and never hatched.

Thus, the extent and features of developmental abnormalities observed above appeared to depend upon the tonicity of the media used (hence, 0.75% NaCl seems to be nearly isotonic). This relationship suggests that freshly laid eggs of *Drosophila* are permeable enough at least for water to die early or develop abormally when incubated in aqueous media except in carefully prepared media.

Axial and Lateral Head Formation in Hydra

Hiroshi SHIMIZU, Tsutomu SUGIYAMA and Yasuji SAWADA

Axial and lateral head formation in hydra was investigated. Hydra produces two basic types of head-like structures. One is regeneration of a new head after removal of the original head. This type of head formation occurs at the most apical end and in the same direction as the apico-basal axis of the original animal. The other is bud formation in an asexually growing animal. This type of head formation occurs in the budding zone and in a direction which is perpendicular to the axis of the parental polyp. The relatonships btween these two types of head structure formation were examined using the "mirror-image" grafting procedure described in this report last year.

The grafting was done as follows; Head was removed from polyps by amputation with a sharp surgical knife. A nylon fishline was then pushed through the gastric cavity of two decapitated polyps in such a manner as to make the wound opening of the two polyps face and cover each other.

The fishline was removed after 3–5 hours, and the two polyps grafted together symmetrically in a mirror-image fashion by this operation were then kept under normal culture conditions for seven days.

Grafted polyps produced two different types of structures at the graft junction. One was a ring of tentacles which formed around the circumference of the graft junction. These grafted polyps later separated from each other in some cases, producing two independent normal regenerates eventually. Thus, tentacle ring formation can be regarded as a variation of normal head regeneration caused by the grafting.

The other type of structure formed was a "lateral head". Initially a small protrusion appeared at one (occasionally two) point of the circumference of the graft junction. It grew in size gradually, and formed a hypostome and tentacles around it at the tip. This process was similar to normal budding, except for one important aspect. A normal bud forms a foot and detaches from the parent, whereas a lateral head forms no foot and remains attached to the graft.

The ratio of grafts forming a tentacle ring and a lateral head varied greatly depending on the sites of amputation and grafting on the original polyps. As the site moved from a more apical to a more distal position, the ratio of tentacle ring formation decreased, whereas the ration of lateral head formation increased.

These observations show that the capacity to form a tentacle ring (head regeneration) forms a gradient from head to foot, whereas the capacity to form a lateral head (budding) forms a gradient in the opposite direction. This suggests that the two head-type structures (head regeneration and budding) are regulated by two different, but presumably closely related, mechanisms.

Attempts are currently being made to formulate a new theoretical model for hydra pattern formation. In the new model, nonlinear elements of chemical reaction-diffusion (Gierer and Meinhardt, 1972) and non-linear elements of "active boundary" produced by injury effect (MacWilliams, 1982) will be integrated to simultaneously account for axial and lateral head formation in hydra.

Two Way Controls of Interstitial Stem Cell Proliferation 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 2 types of nerve cells and 4 types of nematocytes under normal growth conditions. When the I-cell level is lowered artificially (e. g. by hydroxyurea treatment), I-cells grow faster at the beginning by increasing the self-renewal probability but later grow slowly, eventually attaining the normal growth rate (Bode, *et al.*, 1976). These results indicate that there is a homeostatic control mechanism for maintaining the I-cell level.

In the present study, I examined control mechanisms involved in the homeostasis of hydra stem cells. The methods employed were to introduce a small number of I-cells into an epithelial host which lacked all the cell types in an I-cell lineage (stem cells, their differentiation intermediates and products; nerves and nematocytes). First, the distal half of an epithelial polyp and the proximal half of a normal polyp were axially grafted allowing I-cells to migrate from proximal to distal regions. After 24 hr, the distal half was isolated to regenerate. During the first 3 days of regeneration, essentially no migrated I-cells increased in number. Instead they produced a large number of nerve cells. The number of I-cells started to increase rapidly thereafter. When epithelial and normal tissues were kept in contact, I-cells increased continuously in number (note that I-cell migration occurs only during the first 24 hr and then ceases). These results suggest that some component necessary for I-cell growth is missing in epithelial tissue and that the component is a product of nerve cells which either directly affect I-cell proliferation or negatively control nerve cell differentiation, thus indirectly affecting the rate of I-cell proliferation. Second, a small piece of normal tissue was laterally transplanted to an epithelial polyp. By doing so, an animal with a small number of I-cells and nerve cells (and some other cell types as well) was created. Under these conditions, I-cells grew faster during the first 10 days by increasing the self-renewal probability, but later the growth rate leveled off, reaching a normal rate. These results together with those described above indicate that two different mechanisms are involved in the homeostatic control of I-cells. (1) When enough nerve cells are present, the rate of I-cell growth depends on the I-cell density.

Namely, a negative feedback signal emanates from I-cells themselves to control the growth rate of I-cells. (2) In the absence of nerve cells, the self-renewal probability of I-cells decreases, thus favoring nerve cells differentiation, since the signal necessary for I-cell growth or a negative feedback signal for nerve cell differentiation is missing.

Purification and Structure Analysis of an Autocrine Growth Factor in Conditioned Medium Obtained from Primary Cultures of Scleral Fibroblasts of the Chick Embryo

Miki FUJIOKA, Yoshie OHIYA, Kazuo WATANABE
and Nobuo SHIMAMOTO

The time course of cell proliferation and cell differentiation may be determined by environment including growth factors. Most simply, local concentrations of growth factors could control a spatial distribution of cells. One candidate for such a situation is the scleral fibroblast of the chick embryo which forms a thin layer around the eye balls. As the first step to check this hypothesis, we purified a related growth factor.

We found that the cells secrete autocrine growth factors. One factor was purified from conditioned medium collected from growing-phase cultures by DEAE-Sepharose column chromatography and non-denaturing polyacrylamide gel electrophoresis. The specific activity was increased 1,100-fold through purification. The purificed growth factor was heat-stable, and resistant to acidic or basic treatment. Removal of the glycoside chain did not alter activity but reduction by dithiothreitol or digestion by trysin hampered activity. These characteristics are similar to those of the plateletderived growth factor (PDGF) but its molecular weight, 32 kd, is much larger than that of PDGF. Thus this factor might be form a new family of growth factors. The primary sequence was determined for 20 amino acids at N terminal and the cDNA is now being screened.

Assembly of Connectin (Titin) in Relation to Myosin and α-Actinin in Cultured Cardiac Myocytes

Yutaka SHIMADA

By using polyclonal and monoclonal antibodies against connectin (titin) which stains the A-I junctional area and the A band domain (polyclonal

anti-connectin and monoclonal 4C9) and the I band domain (monoclonal SM1), the developmental relationship of this elastic protein with sarcomeric proteins, especially myosin and α-actinin, was examined in embryonic chick cardiac myocytes *in vitro* under fluorescence microscopy. During premyofibril stages, I-Z-I proteins were detected first (α-actinin dots and diffuse actin [phalloidin and anti-troponin C] staining), and later in these areas connectin and myosin dots appeared with nearly identical distribution. Somewhat later, phalloidin-positive nonstriated fibrils were observed in a straight course. They were aleays reactive with antibodies against α-actinin and troponin C, but unreactive or only weakly reactive with anti-connectin and anti-myosin. Initally, α-actinin dots were aligned these fibrils but did not form striations. As they aggregated to form Z bands, connectin and myosin came to exhibit typical striations ("doublets" and A bands, respectively). No difference in the staining pattern was observed with two hinds of monoclonal antibodies against different domains of connectin filaments (4C9 and SM1) at early phases. As myosin staining showed clear A bands, connectin epitopes became arranged in polarized positions. We conclude that primitive I-Z-I complexes appear prior to the assembly of connectin and myosin filaments, and then connectin filaments developing intimateyl and corrdinately with myosin become associated with the α-actinin lines. Thus, it appears that the putative elastic protein connectin, plays some role in integrating myosin filaments with the preexisting I-Z-I brushes. The occasional absence of connectin and the absence of A bands between the two Z bands, beyond both of which clear sarcomeres have been formed, indicate that connectin is not a preformed scaffold for myofibrils, at which sarcomeric proteins accumulate.

Translational Control of Troponin C Gene Expression in Chicken Skeletal Muscles

Yutaka SHIMADA

The expression of the cardiac/slow troponin C (C/STnC) gene in develop-cardiac and skeletal muscles was analyzed with cDNA probes and an antibody specific for C/STnC. Northern blot, S1 nuclease protection assay and Western blot analyses showed that both the C/STnC mRNA and the C/STnC proteins were expressed in the ventriclar muscle of specimens from embryo to the adult. However, in the breast muscle, although C/

STnC mRNA and the C/STnC protein were present in the embryo, the synthesis of the C/STnC protein decreased as development proceeded. The C/STnC protein was not found at detectable levels in the adult breast muscle in spite of the presence of C/STnC mRNA. In vitro translation analysis indicated that C/STnC mRNA transcribed in the adult breast muscle was translatable. These results suggest that functional C/STnC mRNA was translationally inactive in the adult breast muscle and that the expression of C/STnC gene was controlled at the level of translation in the adult breast muscle. Thus, it appears that translational control plays an important role in tissue-specific and developmentally regulated isoform changes in troponin C.

V. CYTOGENETICS

Effect of H-2 Complex on Morphological Abnormality of Mouse Spermatozoa

Kazuo MORIWAKI and Akihiko MITA

Genes governing the morphological characteristics of sperm head seem to be located on several chromosomes including No. 17 and Y in mouse. The possible relevance of the H-2 complex on chromosome 17 to the morphological abnormality of spermatozoa has already been demonstrated (Suh, Styrna & Moriwaki. Genet. Res. Camb., 53: 17, 1989). To confirm the previous findings and to locate more precisely such a gene in the H-2 complex, the frequency of spermatozoa with abnormal head shape was scorted in 43 B10.H-2 congenic strains. The precentage of head abnormality ranges from 1% to 50%. Thirty three strains including B10, B10.A, B10.A (2R), B10.A (3R), B10.A (4R), B10.AKM, B10.BR, B10.D2, B10.G, B10.HTT, B10.PL (73NS), B10.RIII (71NS), B10.S, B10.MOL-ANJ, B10. MOL-MSM, B10.MOL-OHM, B10.MOL-OKB, B10.MOL-SGR, B10. MOL-TEN2, and B10.M-YNG, exhibited the abnormality at frequencies between 5% and 30%. Significantly higher frequencies in the abnormality were observed in the following 3 strains: 30.3% in B10.A (5R), 50.5% in B10.M and 32.2% in B10.MOL-TEN1. On the other hand, lower frequencies of less than 5% were observed in B10.DA (80NS), B10.GD, B10.Y, B10.HTG and B10.Po.

As far as the present survey is concerned, those strains carrying the d or q haplotype in the H-2K gene show relatively lower frequency of sperm abnormality. Both of the strains with H-2K^{f} exhibited considerably a higher frequency of abnormal sperm. Results obtained from B10.A (3R) and B10.A (5R) are something puzzling, since both strains have almost the same genic constitution in the H-2 complex and in their genetic background as well. Further analyses of the origin of their Y chromosomes and the B10 genetic background is necessary.

A New Variant of the Mouse β-globin Gene, Hbb^q, in Northwestern China

Tsuyoshi KAWASHIMA Nobumoto MIYASHITA, Toshihiko SHIROISHI,
Wang Chong YAN*, Wang Cheng HUAI* and Kazuo MORIWAKI

We studied the allelic frequency of the hemoglobin beta chain gene (*Hbb*) of wild mice, *Mus musculus*, mostly in Asia. The wild populations in the northern part of China, Korea and the central part of Japan exhibited an almost monomorphic distribution of *Hbb*^{p}. The southern part of Asia shows a lower frequency of *Hbb*^{p} and a predominant distribution of *Hbb*^{d} (Minezawa *et al.*, 1979; Miyashita *et al.*, 1985). In the process of the survey of *Hbb* polymorphism in China, we found another allele of *Hbb*, named *Hbb*^{q}. It is distributed in a rather wider area in northwestern China. From the electrophoretic pattern on cellulose acetate membrane, *Hbb*^{q} was assumed to be an intragenic recombinat between *Hbb*^{s} and *Hbb*^{p}. But recent analysis on polyacrylamide gel electrophoresis suggests the possibility that the *Hbb*^{q} is a completely new allele at both the *b1* and *b2* loci. Molecular analysis of *b1* and *b2* genes in *Hbb*^{q} is now in progress using southernblotting with *Hbb* DNA probes.

In vitro Development of Preimplantation Embryos Derived from Inter Subspecific Hybrids between *Mus musculus molossinus* and *M. m. domesticus*

Michiko NIWA, Noboru WAKASUGI** and Kazuo MORIWAKI

Japanese wild mice, *Mus musculus molossinus*, are genetically remote from laboratory mice which are derived predominantly from European wild mice *M. m. domesticus*. Developmental abnormality was observed in preimplantation embryos from F2 hybrids between the MOM strain (one of the inbred strain derived from Japanese wild mice) and the C57BL/6 strain (B6; an inbred strain of laboratory mice) (Niwa, M. and Wakasugi, N. 1990, *Zool. Sci.* 7: 209). We examined the *in vitro* development of preimplantation embryos from hybrids between C3H/He (C3H) and M. MOL-MSM (MSM) and between SK/Cam (SK) and MSM. C3H is a laboratory inbred strain. SK and MSM strains are inbred strains derived

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Table 1. Developmental ability of embryos obtained on Day 1 of pregnancy

crosses ♀×♂	No. of ♀♀ examined	No. of embryos[a] collected (means±sem)	No. of embryos that develoed to blastocysts during 4 days in culture
C3H×C3H	7	60 (8.6±1.0)	57 (95.0%)
C3H×MSM	8	69 (8.6±0.5)	68 (98.6)
MSM×MSM	16	76 (4.8±0.2)	75 (98.7)
MSM×C3H	1	5	5 (100.0)
(C3H×MSM)F1×C3H	1	9	9 (100.0)
(C3H×MSM)F2×C3H	18	124 (6.9±0.5)	105 (84.7)
C3H×(C3H×MSM)F2	9	89 (9.9±0.9)	86 (96.9)
SK×SK	6	45 (9.8±0.7)	14 (31.1)
MSM×MSM	16	76 (4.8±0.2)	75 (98.7)
(SK×MSM)F1×SK	8	41 (5.1±0.5)	39 (95.1)
SK×MSM)F1×MSM	7	30 (4.3±0.7)	30 (100.0)
(SK×MSM)F2×SK	14	75 (5.4±0.6)	53 (70.7)
(SK×MSM)F2×MSM	20	114 (5.7±0.4)	80 (70.2)
B10×(SK×MSM)F2	9	78 (8.7±0.4)	70 (89.7)
(B6×C3H)F1×C3H	9	89 (9.9±0.9)	89 (100.0)
(B6×C3H)F2×C3H	11	112 (10.2±1.0)	111 (99.1)
(B6×C3H)F2×B6	10	80 (8.0±0.7)	75 (93.8)

[a] Morphologically normal 2- to 6-cell embryos were counted

from *M. m. domesticus* wild mice and Japanese wild mice, respectively. We also observed the embryos of hybrids between B6 and C3H as a control. The results are shown in table 1. The SK strain showed a low level of developmental ability in preimplantation embryos. F1 hybrids from all crosses showed a high embryonic survival rate. A very low level of abnormality was observed in embryos sired by F2 males. On the contrary, some

embryos from F2 females between C3H and MSM (15.3%) and between SK and MSM (29.8%) died before the blastocyst stage. These F2 females were divided into two types on the basis of the proportion of embryos that developed to blastocysts: one type showing a higher rate of survival embryos and the other a lower rate. Almost all embryos derived from F2 females between B6 and C3H developed to blastocysts. These results suggest that embryonic mortality probably occurred during the preimplantation development of F2 females between *M. m. molossinus* and *M. m. domesticus* and that the abnormality rate depends on the strain combination in genetic crosses.

Studies of Adrenal Function in Mouse Development by Transgene.

Hideo GOTOH Toshihiko SHIROISH and Kazuo MORIWAKI

A chromosome of H-2 *aw18* haplotype has a deletion in the H-2 class III region spanning both the gene for fourth component of complement (C4) and the gene for the active form of steroid 21-hydroxylase (21-OHase). Because of the lack of 21-OHase activity, *aw18* homozygous mice are unable to synthesize any adrenal steroids, neither glucocorticoids nor mineralocorticoids. So far, we have found several developmental abnormalities associated with the *aw18* haplotype.

(1) Neonatal lethality: Homozygotes for the *aw18* haplotype die within two weeks after birth. Administration of adrenal homogenate to supplement adrenal steroids rescued the *aw18* homozygote. The cause of the lethality is assumed to be the defect in adrenal steroid synthesis.

(2) Adrenal organogenesis: Histological abnormalities were observed in whole adrenal glands of *aw18* homozygotes. These abnormalities are known to arise at the mid-gestation period, when medurally cells migrate into cortical anlage. The cell-to-cell interaction during adrenal organogenesis seems to be abnormal in these mice.

(3) Embryonic malformation: In fetuses from intercrosses of *aw18*/+ heterozygotes, several kinds of severe-type malformations were observed with high frequency.

We are now introducing a genomic DNA clone of murine 21-OHase gene into the *aw18* mice. If the exogenous gene is experssed in adrenal glands *in vivo*, the *aw18* homozygotes will be rescued. These transgenic mice will become powerful tools for studying unknown functions of adrenal

steroids in mouse development.

Antigenic Polymorphism of a Serum Protein Found in a Strain Derived from Indonesian Wild Mice (*Mus musculus castaneus*)

Yoshi-nobu HARADA, Cece SUMANTRI*, Takeshi TOMITA*
and Kazuo MORIWAKI

M. Cas-Bgr 1 is a strain derived from Indonesian wild mice which have been classified into one subspecies, *Mus musculus castaneus*. Antigenic specificities of the M. Cas-Bgr 1 strain were studied by gel precipitation analyses with alloantisera produced by reciprocal alloimmunizations with the BALB/c strain. A specific precipitation line, which migrated between and regions of serum globulins, was observed by immunoelectrophoretic analysis with BALB/c anti-M. Cas-Bgr 1 serum. This precipitation line must be a serum protein whose antigenic polymorphism has not been reported previously. Hereafter, the serum protein will be called alpha-protein-3A (phenotype symbol: APH-3A). None of the M. Cas-Bgr 1 mice immunized with BALB/c serum produced a specific antiserum that gave clear precipitation lines on immunoelectrophoretic analysis. A putative counterpart for APH-3A was not observed in BALB/c serum.

A genetic analysis of APH-3A was carried out on F_1, F_2 and backcross progeny produced by crosses of M. Cas-Bgr 1 and BALB/c strains. The results suggested that APH-3A was controlled by an autosomal dominant gene. Therefore, the locus controlling the APH-3A was tentatively called

Table 1. List of mouse strains used for serological survey of APH-3A in this study.

APH-3A-positive	M. Cas-Bgr 1
APH-3A-negative	A/WySnJ, AKR/J, BALB/c, BALB/cAnN, CBA/Ms, CBA/J CBA/CaHN, CE/J, C3H/He, C57BL/6J, C57BL/10J, C57L/J, C58L/J, CS, DBA/1J, DBA/2J, DCR/c, DDK, DDN, DM, DNI, HTG, HTH, HTI, HRS/J, ITES, IIITES, IQI, IVC-E, IXBL, KR, MA/MyJ, NC, OZA, OZB, Ozc, Ozd, OZF, P/J, PONY, RFM, RIIIs/J, SIIIB, SJL/J, SM/J, SWR/J, TF/GnLe, WB/ReJ-W, ZCA, 129/Sv, 129/J

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alphaprotein-3 (gene symbol: *Aph-3*) and alleles for APH-3A-positive and APH-3A-negative were named *Aph-3a* and *Aph-3b*, respectively.

A serological survey of APH-3A was conducted on 51 laboratory strains but no strain including BALB/c had the APH-3A.

DNA Sequeuce Comparison in the D-loop Region of Mitochondrial DNA within and Among Subspecies of the Species *Mus musculus*

Hiromichi YONEKAWA, Nobumoto MIYASHITA and Kazuo MORIWAKI

Based on mtDNA haplotype analysis with restriction enzymes, we previously proposed the hypothesis that Japanese wild mouse *Mus musculus molossinus* is a hybrid 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, we carried out two lines of research; an extensive survey of wild mice in neighboring countries of Japan, especially China, using restriction enzymes with which we could identify subspecies, and the development of a new efficient method for base sequence comparison of intersting region(s) on mtDNA. The objective of the former research was to clarify subspecies boundaries between *M. m. musculus* and *M. m. castaneus* in Japan and China. The latter's objective was to confirm whether there are subspecies-specific base substitutions on the mtDNA sequences. If there are, we can examine more precisely the molecular evolution of mtDNA during the subspeciation of *Mus musculus*.

For the second objective, we sequenced four entire mtDNA by collaboration in Dr. K. Fischer Lindahl of Howard Hughes Medical Institute in Dallas (Fischer Lindahl *et al.* (1989) Cold Spring Harbor Symp. Quant. Biol. **54**: 563). In comparing these four mtDNA sequences, we found that there are at least three highly polymorphic regions in mouse mtDNA; in particular the replication origin of heavy stand (D-loop region) is the highest. Based on this result, we selected this region as the first target of sequence comparison, since we can expect the greatest opportunities of finding base substitutions in this region when we survey the mouse subspecies with monomorphic mtDNA. To do so, we introducted the polymerase chain reaction (PCR) method into the research. To carry out DNA sequencing experiments efficiently, we made six sequencing primers which have common sequences among *Mus musculus*, *Rattus norvegicus* and *Rattus rattus*. We have finished seven sequences of the D-loop region

so far and found there are two clusters of highly polymorphic sites in the region. We also found that some substituted bases are shared by mice belonging to the same subspecies, and thus they look like subspecies-specific substitutions, suggesting that we can identify subspecies by checking these substitutions. The D-loop region seems to have higher frequencies of transversions and insertions/additions than other regions.

VI. MUTAGENESIS AND RADIATION GENETICS

Genetic Analysis of Pulmonary Adenoma Susceptibility (*Pas*) in Mice

Nobumoto MIYASHITA and Kazuo MORIWAKI

We estimated the number of genes which affect urethane-induced pulmonary adenoma in crosses between adenoma-sensitive A/WySnJ and resistant B10.A/SgSnJ strains of mice. As these strains carry an identical *H-2* haplotype ($H\text{-}2^a$), the effect of the *H-2* complex on induction of pulmonary adenoma can be disregarded. The mice were sacrificed in the 5th month after a single subcutaneous injection of 1.5 mg/g urethane/g body weight. After the lungs were fixed in ethanol/formaldehyde (9: 1), the number of adenoma foci was assessed.

The B10.A strain developed a lower multiplicity of pulmonary adenomas, 1.70 ± 0.23/mouse. In contrast, the A/Wy strain developed a greater multiplicity, 27.15 ± 1.02/mouse. (A/Wy×B10.A)F1 mice exhibited tumors similar to A/Wy parents. The mean number of tumors averaged 25.23 ± 0.79/mouse. In a backcross with B10.A, a distinct bimodal distribution was shown and the numbers of animals in resistant ($n=54$) and sensitive ($n=42$) groups were nearly the same. This result shows that the difference between those strains was due to a single locus (*Pas-1*) with susceptibility dominant. We studied the linkage of this putative *Pas-1* locus with 13 polymorphic loci between the parental strains. No significant linkage was detected.

VII. POPULATION GENETICS

Role of Gene Duplication in Evolution

Tomoko OHTA

It is now known that many multigene and supergene families exist in eukaryote genomes: multigene families with uniform copy members, like genes for ribosomal RNA, those with variable members like immunoglobulin genes, and supergene families such as those for various growth factors and hormone receptors. Many such examples indicate that gene duplication and subsequent differentiation are extremely important in organismal evolution. In particular, gene duplication could well have been the primary mechanism for the evolution of complexity in higher organisms. Population genetic models for the origin of gene families with diverse functions are presented, in which natural selection favors those genomes with more useful mutants in duplicated genes. Since any gene has a certain probability of degenerating through mutation, success versus failure in acquiring a new gene by duplication may be expressed as the ratio of probabilities of spreading of useful versus detrimental mutations in redundant gene copies. Also examined are the effects of gene duplication on evolution by compensatory advantageous mutations. Results of the analyses show that both natural selection and random drift are important in the origin of gene families. In addition, interactions between molecular mechanisms such as unequal crossingover and gene conversion, and selection or drift have been found to have a large effect on evolution by gene duplication. For details, see *Genome* **31**, 304–310.

The Mutational Load of a Multigene Family with Uniform Members

Tomoko OHTA

The mutational load of a multigene family with uniform members was studied through computer simulations. Two models of selection, truncation and exponential fitness, were examined, using a simple model of gene conversion. It was found that the load is much smaller than the Haldane-

Muller prediction under truncation selection, and that it becomes approximately equal to the value calculated by the formula, $nv\ (1\text{-}q)/(m\text{-}nq)$, where n is the copy number, v is the rate of detrimental mutation per gene copy, m is the truncation point in terms of the number of detrimental genes eliminated, and q is the equilibrium frequency of detrimental mutation. However equilibrium frequency could not be analytically obtained. For the exponential fitness model, the load is close to the Haldane-Muller value. When there is no gene conversion, the load becomes larger than in cases with conversion both for the truncation and the exponential fitness models. Thus, gene conversion or other mechanisms that are responsible for the contraction—expansion of mutants on chromosomes, help in eliminating deleterious mutations occurring in multigene families. For details, see *Genet. Res.* **53**, 141–145.

Time for Spreading of Compensatory Mutations under Gene Duplication

Tomoko OHTA

Evolution by compensatory mutations is accelerated by gene duplication because selective constraint is relaxed by gene redundancy. A mutation is called compensatory if it corrects the effect of an earlier deleterious mutation. Without duplication, Kimura has shown that the time for spreading of compensatory mutations is greatly reduced by the tight linkage between the two chromosomal sites of mutations. In this report, the time for spreading with gene duplication was studied using the diffusion equation method of Kimura, together with computer simulations. It was shown that, when $2Nv_-$ is much less than unity, the time for spreading is greatly shortened by gene duplication as compared with the case in which there is complete linkage between the two sites of mutations, where $2N$ is the effective population size (haploid) and v_- is the rate of compensatory mutations. However, if $2Nv_- > 1$, gene duplication is not effective for accelerating evolution by such mutations. For details, see *Genetics* **123**, 579–584.

Gene Genealogy in Three Related Populations: Consistency Probability between Gene and Population Trees

Naoyuki TAKAHATA

A genealogical relationship among genes at a locus (gene tree) sampled from three related populations was examined with special reference to population relatedness (population tree). A phylogenetically informative event in a gene tree constructed from nucleotide differences consists of interspecific coalescences of genes in each of which two genes sampled from different populations are descended from a common ancestor. The consistency probability between gene and population trees in which they are topologically identical was formulated in terms of interspecific coalescences. It was found that the consistency probability thus derived substantially increases as the sample size of genes increases, unless the divergence time of populations is very long compared to population size. Hence, there are cases where large samples at a locus are very useful in inferring a population tree. (See *Genetics* **122**: 957–966, 1989 for details.)

Stochastic Models for Molecular Clocks

Naoyuki TAKAHATA

Compared with the rate of evolution at the phenotypic level, the rate of molecular evolution is fairly constant in time as well as among diverse lineages. The most commonly used statistical model to describe such constancy (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 have shown that the rate of molecular evolution is considerably over-dispersed which has called into question the validity of using a simple Poisson process. Several efforts have been made to develop more realistic models of molecular evolution. Here I reviewed three such statistical models based upon doubly stochastic, cluster, and renewal processes. Although these models do not immediately specify what the mechanisms of molecular evolution might be, they do make qualitatively different predictions and give some insight into their inference. The main concern is whether or not the neutral theory of molecular evolution can explain,

without major modifications, highly overdispersed molecular clocks. (See pp. 433–444 in Proceedings of the 47th Session, Vol L III (3) Bulletin of the International Statistical Institute, Paris, 1989, FRANCE for details.)

A Population Genetic Model of Selection that Maintains Specific Trinucleotides at a Specific Location

Hidenori TACHIDA

The periodic appearance of specific trinucleotides along the DNA sequence have been reported in chicken core DNA, and it has been suggested that the phenomenon is related to the supercoiling of DNA around nucleosomes. A population genetic model was constructed in which selection is operating to maintain specific trinucleotides at a specific location on the DNA sequence. Assuming low mutation rates, equilibrium probabilities of the appearances of respective trinucleotides were computed. Vague patterns appeared if the product of the effective size and the selection coefficient was 0.1–2.0. The genetic load and substitution rates were also computed. When the model was applied to the chicken DNA data, the product of the effective size and the selection coefficient was estimated to be 0.1–0.2. With this intensity of selection, the substitution rate was hardly different from that in cases without selection. However, the genetic load became fairly large. Considering the large number of times that DNA coils about nucleosomes, the number of trinucleotides sites must be very large, and thus the total load might be too large. This suggests the existence of epistasis among sites to reduce the total load if selection is responsible for this periodic pattern observed in chicken core DNA. The details will be published in *J. Mol. Evol.*

A Study on Fixation Probabilities in a Subdivided Population with Varying Environments

Hidenori TACHIDA

The probability with which a mutant gene appearing in a population is ultimately fixed is an important quantity in evolutionary studies. Kimura derived a general formula to compute the fixation probability in random mating populations (Kimura, M., 1964, *J. Appl. Probab.* **1**: 177–232). This was extended by Maruyama to structured populations (Maruyama,

T., 1970, *Genet. Res.* **15**: 221–226). There it was shown that in haploid organisms the fixation probability is the same as that in a panmictic population if selection is operating uniformly over subpopulations. In this study, fixation probability was studied in a subdivided population in which selection pressure was different from one subpopulation to another. A comparison with the fixation probability in a corresponding panmictic population, in which selection coefficients are the averages of those in subpopulations, was made. When selection was very weak, such that the product of the selection coefficient and the size of subpopulations were much less than one or when the migration rate was very large, there was no difference between the fixation probability in a subdivided population and that in a corresponding panmictic population. Next we considered the case in which the migration rate is small and selection is not weak. In the case of two subpopulations, the fixation probability can be approximately computed if the migration rate is very small. It was found that the fixation probability in a subdivided population was larger than that in a corresponding panmictic population if selection favored one allele in one subpopulation and favored the other in the other subpopulation. A simulation study is now in progress to determine the validity of this approximation. Also cases with more than two subpopulations will be studied using this approximation.

Theoretical Studies on DNA Polymorphism and Statistical Method for Testing the Neutral Mutation Hypothesis

Fumio Tajima

A large amount of genetic variation at the DNA level is maintained in natural populations. The amount of genetic variation is determined by mutation, recombination, natural selection, random genetic drift, population structure, and so on, and can be measured by the average number, $\hat{k}$, of (pairwise) nucleotide differences and the number, S, of segregating sites in a sample of DNA sequences.

When the amount of DNA polymorphism is determined solely by mutation and random genetic drift, the expectations of these numbers are given by $E(\hat{k})=M$ and $E(S)=aM$, where $M=4Nv$, N is the effective population size, v is the mutation rate per generation per DNA sequence, $a=\sum_{i=1}^{n-1}(1/i)$, and n is the sample size, so that we have the following relationship:

$$E(\hat{k})=E(S)/a \qquad (1)$$

When the population is panmictic and the population size is constant over a long period of time, we can test the neutral mutation hypothesis by examining whether or not Equation (1) holds. It can be shown that $\hat{k}$ tends to be larger than S/a if natural selection which increases genetic variation, is operating, whereas $\hat{k}$ tends to be smaller than S/a if natural selection which reduces genetic variation, is operating. I have developed a statistical method for testing the neutral mutation hypothesis by using the relationship between $\hat{k}$ and S. Applying this statistical method to DNA sequences sampled from natural populations of *Drosophila melanogaster*, it has been shown that large deletions/insertions (longer than 100 pb), which may be caused by transposable elements, are deleterious. For details, see *Genetics* **123**: 585–595.

When the population is not panmictic, the relationship between $\hat{k}$ and S is quite complicated and Equation (1) does not necessarily hold. I examined this relationship by using the two-subpopulation model and obtained the following results: (i) $E(\hat{k})$ is independent of the migration rate. (ii) $E(S)$ is independent of the migration rate when two or three DNA sequences are randomly sampled from the same subpopulation, but depends on the migration rate when more than three DNA sequences are sampled. (iii) The population subdivision can increase the amount of genetic variation at the DNA level even in a subpopulation in some cases. For details, see *Genetics* **123**: 229–240.

The size of population often changes drastically, and the change in population size might affect the amount of genetic variation maintained in natural populations. I studied the expected number, $E(S)$, of segregating sites and the expectation, $E(\hat{k})$, of the average number of (pairwise) nucleotide differences among DNA sequences sampled from a population which is not in equilibrium. The results obtained indicate that, when the population size has changed drastically, S is influenced by the size of the current population more strongly than $\hat{k}$, while $\hat{k}$ is affected by the size of the original population more sharply than S. The results also indicate that $\hat{k}$ is affected by a population bottleneck more strongly than S. For details, see *Genetics* **121**: 597–601.

The Neutral Theory of Molecular Evolution and the World View of the Neutralists

Motoo KIMURA

The main tenet of the neutral theory is that the great majority of evolutionary changes at the molecular level are caused not by Darwinian selection but by random fixation of selectively neutral (or very nearly neutral) alleles through random sampling drift under continued mutation pressure. The theory also asserts that the majority of protein and DNA polymorphisms are selectively neutral, and that they are maintained in the species by mutational input balanced by random extinction rather than by "balancing selection." The neutral theory is based on simple assumptions. This enabled us to develop mathematical theories (using the diffusion equation method) that can treat these phenomena in quantitative terms and that permit theory to be tested against actual observations. Although the neutral theory has been severely criticized by the neo-Darwinian establishment, supporting evidence has accumulated over the last 20 years. In particular, the recent burst of DNA sequence data has helped to strengthen the theory a great deal. I believe that the neutral theory triggered reexamination of the traditional "synthetic theory of evolution."

In this work, I review the present status of the neutral theory, including discussions of such topics as "molecular evolutionary clock," very high evolutionary rates observed in RNA viruses, a deviant coding system found in *Mycoplasm* together, with the concept of mutation-driven neutral evolution, and the origin of life. I also present a world view based on the conception of what I call "survival of the luckiest." For details, see *Genome* **31**: 24–31.

A Shifting Balance Type Model for the Origin of Cultural Transmission

Kenichi AOKI

Most selectively advantageous traits that are culturally transmitted probably cannot be genetically determined. On the other hand, the capacity for culture is undoubtedly a genetically determined trait, which may have evolved because of associated advantages. Conditions for the spread of a gene for cultural transmission based on a haploid sexual model of

this hypothesis. Two genotypes, communicators and non-communicators, were assumed, and communicators were further distinguished by the presence or absence of the trait. Only cultural transmission from parent to child was considered. In a large panmictic population, initial spread of the gene for cultural transmission is facilitated if both mother and father contribute to the education of their offspring. If only the mother or father does, a two-fold advantage is required for the culturally transmitted trait. Cultural transmission may also evolve without unduly strong selection in a subdivided population, like that of some primates, where a shifting balance type process can occur. By assuming that the groups synchronously undergo reduction to the same finite size at a point in the life cycle, it is possible to define probability distribution of the numbers of the three types among groups. Numerical results obtained by iterating this probability distribution revealed a dependence on group size and migration rate that can be interpreted in terms of the three phases of the shifting balance process. The independent rediscovery rate, defined in analogy with the mutation rate, was also shown to be an important parameter.

VIII. EVOLUTIONARY GENETICS

Pattern of Nucleotide Substitutions and Its Implications for the Immunological Diversity of Human Immunodeficiency Virus

Takashi Gojobori, Etsuko N. Moriyama and Nobuaki Shimizu

Human immunodeficiency virus (HIV) exhibits immunological hypervariability, which has been an obstacle to the successful production of effective anti-HIV vaccines. With the aim of finding characteristics of the mechanism which generates the immunological diversity of the envelope protein, we estimated patterns of nucleotide and amino acid substitutions in the *env* gene of HIVs. The results obtained showed that nucleotide changes between A and G are predominant compared to those between other nucleotides. Since this feature is consistent with the pattern of nucleotide substitution of other retroviral genes but is quite different from those of most eukaryotic genes, a high rate of nucleotide substitution between A and G appears to be specific for retroviruses including HIVs. Moreover, the nucleotide substitutions between A and G at the first and second positions of a codon tend to cause changes in hydrophilic amino acids much more frequently than in hydrophobic amino acids. Since epitopes on the *env* protein of HIVs consist mainly of hydrophilic amino acids, a high rate of nucleotide substitution between A and G can markedly alter the antigenicities of epitopes on the *env* protein without affecting the distribution of electric charges. These specific patterns of nucleotide substitutions may play an important role in generating the immunological hypervariability of the *env* protein in HIVs. For details, see *FEBS Letters* **250**: 591–595, 1989.

Evolutionary Relationships between Human and Simian Immunodeficiency Viruses

Takashi Gojobori, Etsuko N. Moriyama, Hajime Tsujimoto and Masanori Hayami

To elucidate the evolutionary origin of human and simian immunodeficiency viruses (HIVs and SIVs), we determined the nucleotide sequence of

the complete genome of a novel simian lentivirus, SIV_{mnd}, isolated from a wild-caught mandrill in Africa (Gabon). We compared the nucleotide sequence of the *pol* region (the regions of reverse transcriptase and endonuclease) of SIV_{mnd} with those of other primate and ungulate lentiviruses. Phylogenetic trees constructed, showed that HIVs can be classified into two groups, HIV-1 and HIV-2 groups. It also showed that two types of SIVs (SIV_{mac} isolated from rhesus macaques and SIV_{sm} isolated from a sooty mangabey) were closely related to the HIV-2 group. However, SIV_{agm} isolated from an African green monkey and SIV_{mnd} were distantly related to HIVs and form the third and fourth groups. The results indicate that these four viral groups might have diverged from a common ancestor at almost the same time before the spread of AIDS in humans. For details, see *Nature* **341**: 539–541, 1989.

Rates of Nucleotide Substitution for Drosophila Species

Etsuko N. MORIYAMA and Takashi GOJOBORI

To examine the relationship between evolutionary rates and generation time, we estimated the rates of nucleotide substitution for Drosophila species. We computed the numbers of synonymous (silent) substitutions between the species compared, using the nucleotide sequences of 14 functional genes and a pseudogene for a total of 13 Drosophila species. The results obtained showed that the rate of synonymous substitution for Drosophila is two to five times higher than those for mammals and 1.5 to three times higher than those for rodents. Such a high rate for Drosophila can be explained by the shorter generation time of this organism. Furthermore, the rate of synonymous substitution for Drosophila is estimated to be $(10.8\text{–}20.2)\times 10^{-9}$/site/year, which varies with the gene examined. In particular, *Adh*, *Hsp82*, *Ubx* and *Rp49* have rather high rates, while *Xdh*, *pcp*, *Gart*, *en*, *per*, 3′ORF of *Adh* and *beta-2 tubulin* have rather low ones. On the other hand, the rate of nucleotide substitution for the pseudogene of *Adh* was estimated to be about two times higher than that of synonymous substitution for the functional *Adh*. Therefore, the variability of evolutionary rates for Drosophila genes may be caused by a difference in the degrees of functional constraints against synonymous substitutions. For details, see *Genetics* **122**: s41, 1989 and *J. Mol. Evol.* **28**: 391–397, 1989.

Evolutionary Origin of Kringle Structure in Apolipoprotein (a)

K. Ikeo, K. Takahashi and T. Gojobori

A group of serine proteases is involved in the system of blood coagulation and fibrinolysis. These serine proteases have "kringle" domains, each of which represents a conspicuous secondary structure formed by three pairs of intrachain disulfide bonds. The number of kringle domains varies with the type of proteins. Both urokinase and coagulation factor XII contain one kringle. Tissue-type plasminogen activator and prothrombin have two kringle domains. Plasminogen has five. Recently, 38 kringle structures have been found in human apolipoprotein (a) (apo(a)), which is a LDL-like plasma protein. To estimate the evolutionary origin of these kringle structures, we constructed a phylogenetic tree using of all the available sequences of kringle domains. This tree showed that the plasminogen might originally have duplicated to become its present form and is the ancestral gene of apolipoprotein (a). This ancestral molecule of apolipoprotein (a) may have consisted of the fourth and fifth plasminogen-type kringles and a protease domain. Thereafter, the fourth plasminogen-type kringle underwent multiple duplications, resulting in 38 kringles in human apolipoprotein (a). In particular, the latest duplication of the kringle domains is considered to have occurred several million years ago. For details, see *Thromb Haemostas* **62**: 345.

Molecular Evolution of Hepadnaviruses

Yasuo Ina, Etsuro Orito*, Masashi Mizokami*,
Etsuko N. Moriyama and Takashi Gojobori

Hepatitis B virus (HBV) is the etiological agent of a hepatitis that affects an estimated 200 million people worldwide. HBV is a member of the hepadnavirus family that includes woodchuck hepatitis virus (WHV), ground squirrel hepatitis virus (GSHV) and duck hepatitis B virus (DHBV).

To examine whether the evolution of the hepadnavirus family is host-dependent, we constructed phylogenetic trees using 18 nucleotide sequences. The trees obtained show that all 12 strains of HBV can be classified into four subgroups that are not compatible with conventional (serological)

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subtypes. We estimated the rate of synonymous (silent) substitution of HBV for the P region to be 4.57×10^{-5} per site per year. This value is about 10^4 times higher than that of a host genome but 10^{-2} that of the retroviral genes. Applying this rate to the phylogenetic tree, we estimated that DHBV diverged from a common ancestral virus about 30,000 years ago, that WHV and GSHV diverged about 10,000 years ago, and that HBV diverged within the last 3,000 years. Because these divergence times of the viruses are much more recent than those of the host species, it suggests that the hepadnavirus family evolved independently of host-species divergence. For details, see *Proc. Natl. Acad. Sci. USA* **89**: 7059–7062.

A Method for Molecular Phylogeny Construction by Direct Use of Nucleotide Sequence Data

Yoshio TATENO

In estimating a molecular phylogenetic tree (or molecular tree), there are several reasons for the view that nucleotide sequence and amino acid sequence data are more informative than a distance matrix which may be obtained from the sequence data but not otherwise. First, consider a common site in a set of nucleotide sequences of particular homologous genes from various species in question. If the site is occupied by a series of nucleotides which consist of at least two different kinds with the same one present at least two times, the site is called an "informative" site, which makes it possible to divide the series of nucleotides into two or more subsets. In this sense, there are as many possible pieces of evolutionary information as the number of the sites in a homologous gene. Second, a pattern of evolutionary change of nucleotides can be hypothesized on the basis of actual sequence data. For example, transition and transversion can be distinguished from each other, and their ratio can be estimated. Third, such evolutionary changes as insertions and deletions can sometimes be taken into account when inferring the evolutionary relationships of species or genes in question. Fourth, in the process of tree estimation, an evolutionary distance between a pair of nucleotide sequences can be estimated by using any of the proposed methods whose basis for the nucleotide substitution pattern is in accordance with that of the pair. Those four points also hold, to a great extent, for amino acid sequence data. One cannot make use of any of the four significant features, if one is to estimate a mo-

lecular tree from a distance matrix.

In 1981 Felsenstein developed the maximum likelihood (ML) method to estimate a molecular tree from a set of nucleotide sequence data. This method has become popular among molecular evolutionists, mainly because its scientific background is endorsed by statistics. No other method has such a strong backbone. The ML method is, however, accompanied with at least four problems or drawbacks. First, there has been no guarantee that a single maximal (not maximum) point uniquely exists in the parameter range on the likelihood surface. If there are two or more maximal points there, the ML method does not necessarily give the maximum likelihood estimate. This problem has been partially solved by us (see our report in the previous issue, pp. 95–96, No. 39, 1988). Second, as you might think, "informative" sites sometimes conflict with one another, so that the true evolutionary relationships are smudged by the conflicting implications. This may be resolved by increasing the number of sites to be considered. Third, because the method depends heavily on a probabilistic model, its performance may be too sensitive to make the method useful in practice. The second and third problems will be discussed in the next report below. Fourth, the ML method takes uncompromisingly large amounts of computer execution time until it produces the results. This is true even when only eight or so species or OTUs (operational taxonomic units) are involved. There is so far no feasible way to relax this computational burden, which still restricts the method to a quite limited range of uses.

It is known that the number of tree topologies increases steeply, as the number of OTUs gradually increases: the number is 34,459,425 for 10 OTUs and 654,729,075 for 11 OTUs, for example. If we consider this together with the fact that nucleotide substitution in evolution is a stochastic process, we realize that molecular tree estimation is a probabilistic work. Thus it is not reasonable for most methods to give only one tree without any statistical criterion.

Reflecting on such situations, I developed a molecular tree estimation method. This method estimates a molecular tree and ancestral sequences simultaneously from nucleotide sequence data on the basis of parsimony and sequence homology. I named it the stepwise ancestral sequence (SAS) method. It produces plural trees for a set of nucleotide sequence data in descending order of magnitude of parsimony. The final tree may

be chosen from them by considering various lines of related evidence. I think that biological evidence must take precedence over any others.

It is noted that there are two principles which govern molecular tree estimation; likelihood and parsimony. The two principles have been considered and treated to be mutually exclusive. Although a method developed on parsimony is statistically less endorsed than one on likelihood like Felsenstein's, the former takes much less execution time than the latter. This means that the former can estimate a molecular tree of 100 or more OTUs within a reasonable amount of computer time. The problem then is which is better in estimating the correct tree in reality. Statistics does not necessarily lead to reality or to the correct tree. This problem has not yet been solved or worked out thoroughly, and remains to be studied in greater depth.

The properties and performance of the SAS method were then examined through computer experiments, in which the evolution of a hypothetical gene along a model molecular tree of six OTUs was simulated, and produced six descendant genes at the terminals of the tree. The SAS method was then applied to the descendant genes to produce plural estimates to the tree. This was repeated 100 times. The results show that the correct tree is found among the plural trees with a probability of about 90%. It is also shown that the method satisfactorily estimates the ancestral sequences. Those results are of course model dependent, and interpreted as such. For details, see *J. Mol. Evol.* **30**: 85–93, 1990.

Robustness of Maximum Likelihood Tree Estimation against Different Patterns of Base Substitutions

Kaoru FUKAMI* and Yoshio TATENO

As noted in the previous report above, Felsenstein's maximum likelihood (ML) method intrinsically depends on a probabilistic model of nucleotide (or base) substitutions, so that its performance may be quite sensitive to the model. The model in this case is related to the transition versus transversion ratio and the relative contents of the four bases. It is also expected that the performance is affected by the conflict between "informative" sites which is inevitable, as long as we assume that each nucleotide site evolves independently. Note that the ML method does not work without

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this assumption.

To study those problems, we first statistically evaluated the robustness of the ML method in the estimation of molecular trees against different base substitution patterns, including Jukes and Cantor's (1969), which does not distinguish between transition and transversion, and is the simples ever proposed. Namely, we conducted computer simulations, in which we could set up various evolutionary models of a hypothetical gene, and define a true tree to which an estimated tree from the ML method was to be compared. In this case, two sizes of the gene were considered to see the effect of the number of sites in a gene on performance; 300 and 1500 nucleotides were used. The rates of substitution per site for the two cases kept the same. The results show that topology estimation by the ML method is considerably robust against different ratios of transition to transversion and different GC contents, but branch length estimation is not so. When the simplest model is used, the method is resistant to the GC contents, but rather sensitive to the ratio of transition to transversion.

We then applied the ML method with different substitution patterns to sequence data on the *tax* gene from T-cell leukemia viruses, whose evolutionary process must have been more complicated than that of the hypothetical gene. The results indicate that the simplest model is as useful as more complicated ones for making inferences about molecular phylogeny of the viruses.

On the other hand, comparison of the results between the 300 and 1500 nucleotides cases reveals that the performance is much better in the latter case than in the former. This is mainly due to the fact that sampling variance is reduced with enlarging gene size. (*J. Mol. Evol.* (in press)).

Publication from Genetic Resources Section, Genetic Stocks Research Center

Shin-ya IYAMA and Yoshio TATENO

This section is in charge of collection, compilation, and publication of information on experimental organisms preserved in universities and research institutes in Japan. In 1990 a catalog for laboratory rats was published and distributed to researchers. This was made possible in collaboration with a group of concerned researchers and the section. It is noteworthy that this catalog lists strain names under a new designation which

were discussed and formulated by both groups. The groups will propose that the designation be used worldwide, because, unlike laboratory mice, no consistent designation has been established for laboratory rats.

We have cooperated with the Department of Cytogenetics and researchers outside our institute to develop an image database system for ants. This includes not only text information about their characteristics but also photo-image information on the organism taken by professionals. Thus the system gives both pictures and text on the display screen of a personal computer as the results of retrieval. It is needless to say that pictures are more convincing than text in a database for identification and classification of organisms like the present one. For this reason, we anticipate that image databases like ours will become popular and have a large impact on research and development in various areas. We have developed a prototype of the database system which includes the two types of information for 100 ant species.

This section is also in cooperation with the Rice Genetics Cooperative for the publication of *Rice Genetics Newsletter*, which is an annually issued international journal on rice genetics studies. The sixth volume was published and distributed worldwide in 1990.

IX. HUMAN GENETICS

Characterization of the Telomeres and Sub-Telomeric DNA Sequeuces of Human Chromosomes

Takashi IMAMURA, Hitoshi NAKASHIMA, Asao FUJIYAMA, and Tomoko HASEGAWA

The chromosome termini, or telomeres, of a variety of unicellular organisms have been characterized in some detail. They consist of a series of simple tandemly repeated sequeces that are G-rich on one strand, and C-rich on the other. It has become clear over the past few years that unusual structural properties and modes of replication are associated with these terminal repeats. These features may account for some of the special properties of telomeres, such as the ability to resist fusion to other telomeres and to chromosome breaks and random rearrangements.

Human telomeres are similar to those of simple organisms. It has been shown that the telomere erpeat of $(TTGGGG)_n$ from *Tetrahymena thermophila* will cross hybridize with human telomeres, and that the cloned *Arabidopsis* telomere repeat $(TTAGGGG)_n$ also recognized human telomeres. Human telomeres have repeating units of (TTAGGG), equivalent to those of the trypanosome telomere. We have now used a series of synthetic oligonucleotides and restriction enzymes to clone directly this sequence from human chromosome DNA, mainly by using a polymerase chain reaction. The present and the prvious reports suggest, though it is not proven yet, that human telomeres are not uniform throughout their length, comprising some of multiple, different, but related tandem motifs. In somatic tissue cells, the major, most distal, component consists of 5–10 kb of repeats that must be very similar to the sequence $(TTAGGG)_n$. A $(TTAGGG)_4$ probe can now be used to identify cloned telomeric DNA fragments derived from both ends of each human chromosome. Study is now being directed towards cloning and sequencing of the sub-telomeric single copy fragments adjacent to these repeating units, which will prove to be invaluable genetic markers for completing the linkage map of the human genome and for characterizing chromosome rearrangements involving telomeres, some of which may have been stabilized during evolution.

Genetic Polymorphisms of Gene Conversion within the Duplicated Human α-Globin Loci

Hitoshi NAKASHIMA, Asao FUJIYAMA, Shunjiro KAGIYAMA
and Takashi IMAMURA

The identification of individuals possessing one or three adult α-globin genes on a single chromosome, instead of the normal duplicated α-globin genes, provides strong genetic evidence for intergenic recombination mechanisms, such as unequal crossover and gene conversion, in the evolution of human multigene families. Additional evidence that this tandem arrangement of homologous α-globin gene sequences promotes unequal recombination is the production of deletions, which are indistinguishable from those found in human α-thalassemia genes, upon propagation of the cloned α-globin gene DNA in *Escherichia coli*. Although the duplicated human α-globin genes encode identical polypeptides, previous studies have established that the $\alpha 1$ and $\alpha 2$ genes are not identical at the DNA level. This finding could be inferred by the alignment of restriction maps of the α loci residing on the one-, two-, and three-gene chromosomes. We report herein the first estimate by DNA analysis of the frequency of sequence rearrangements within α-globin loci in Japan.

Of the 645 Japanese subjects studied, we identified 10 individuals heterozygous for a chromosome with the triplicated α-globin loci. The frequency of the triple α-loci was 0.008 in this population, while that of the single α-locus, i. e., α-thalassemia 2 gene, might be lower than 0.0008. Analysis of haplotypes using particular RsaI site polymorphism in the α-globin gene complex strongly suggests that the triple $\alpha\alpha\alpha$ loci may have had multiple origins in this population. Of particular interest is that the finding is strikingly different in Melanesia, where people are exposed to different malaria endemicities. The much higher prevalence of deletional α-thalassemia gene associated with endemicites of malaria in different parts of the Melenesian Islands, as compared to the triple $\alpha\alpha\alpha$ genotype, indicates that the former genotype confers an apparently selective advantage with respect to malaria.

Duplication of a gene is the first step toward the evolution of the multigene family. Although it seems clear that hemoglobin abnormalities will not be a major cause of disease in Japan, the extensive survey in this country could promise to provide an important insight into the origin, dissemination,

and persistence of mutations affecting a very important protein, a better insight than can come from countries where selective factors may be coloring the picture much more strongly. Thus far, the situation in Japan suggests that genetic recombinations within the duplicated α-globin genes responsible for the numerical variations of copies of the α-globin gene encountered here do not confer a marked selective advantage, but are approximately neutral and owe their low frequency to a simple mutation process and genetic drift that occurred in a relatively small number of founders in this population.

A Common Chinese β-Thalassemia Mutation Found in a Japanese Family

Yuji NARITOMI, Hitoshi NAKASHIMA, Masaaki KAGIMOTO,
Yasushi NAITO, Eisuke YOKOTA
and Takashi IMAMURA

We have identified the substitution of a thymine for a cytosine at nucleotide position 654 in the second intron of the β-globin gene that causes β-thalassemia in a Japanese family. This mutation has until now been found to occur rather frequently only in patients of Chinese origin but has rarely been found in other ethnic groups. The molecular basis for the thalassemia syndrome of these patients can be explatined by aberrant m-RNA splicing in IVS-2.

Beta-thalassemia is a heterogeneous inherited disorder of β-globin gene expression and one of the most common single gene defects. Although various mutations have been identified in defective β-globin genes, a limited number of specific mutation are prevalent in a given ethnic group in which the thalassemia occurs rather frequently. The β-thalassemia gene in this Japanese family and those in Chinese may be of the same origin as they are both framework 1 genes. The spread of the mutation to the presnt day geographical pattern can be explained on the basis of population migration. Analysis of haplotypes of restriction enzyme site polymorphisms supports this assumption.

Intraspecific Nucleotide Sequence Differences in the Major Noncoding Region of Human Mitochondrial DNA

Satoshi HORAI and Kenji HAYASAKA

Nucleotide sequences in the major noncoding region of human mitochondrial DNA (mtDNA) from 95 human placentas were determined. These sequences include a 482 base-pair (bp) long region encompassing most of the D-loop forming region. Comparison of thses sequences with those previously determined has revealed remarkable features of nucleotide substitutions and insertion/deletion events. The average nucleotide diversity among the sequences is estimated as 1.45%, which is three to four-fold higher than the corresponding value estimated from restriction-enzyme analysis of the whole mtDNA genome. A hypervariable region was also defined. In this 14 bp region, 17 different sequences were detected. More than 97% of the base changes are transitions. A nonrandom distribution of nucleotide substitutions and sequence length variations was also noted. Phylogenetic analysis indicates that diversity among Negroids is much larger than that among Caucasoids or Mongoloids. In fact, part of the Negroids first diverged from the rest of the individuals in the phylogenetic tree. A striking finding in the phylogenetic analysis was that Mongoloids can be separated into two distinct groups. A divergence of part of the Mongoloids follows the earliest divergence of part of the Negroids. The remainder of the Mongoloids subsequently diverged together with the Caucasoids. This observation confirmed our earlier study which clearly demonstrated the existence, through restriction enzyme analysis, of two distinct groups in the Japanese.

DNA Amplification from Ancient Human Skeletal Remains and Their Sequence Analysis

Satoshi HORAI, Kenji HAYASAKA, Kumiko MURAYAMA,
Noriyuki WATE*, Hiroko KOIKE*
and Nobuyuki NAKAI**

The development of a polymerase chain reaction (PCR) makes it possible to amplify the DNA of an aimed region from a very small amount. By

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applying this technique, we tried to amplify mtDNA from 22 samples of human bones derived from different eras. In the first PCR we were able to amplify DNA only in the positive control. However, when we took a portion of the first PCR product and performed a second 30-cycle PCR, we were able to amplify DNA in some samples of bone extracts. Although we could amplify DNA in the second PCR for the majority of samples, it was impossible to amplify DNA in some samples, which is probably due to the condition of the preserved bones. However, we succeeded in amplifity mtDNA from the oldest one of them, a skull named Urawa-1, excavated at a depth of five meters in Urawa city in 1988. Using collagen which was simultaneously isolated from this skull, the absolute age of Urawa-1 was estimated at 5790+120 years B. P. through the direct detection of ^{14}C using accelerator mass spectrometry. This age is equivalent to the first term of the Jomon-period in Japanese prehistory.

We determined a 190-bp nucleotide sequences from the second PCR product of Urawa-1 by the direct sequence method. The nucleotide sequence from Urawa-1 was compared with those from 101 contemporary humans from three major racial groups and six additional sequences determined for individuals from Southeast Asia. In comparing the sequence from Urawa-1 with those from the 107 individuals, we found complete identity in two Southeast Asians (a Malay and an Indonesian) out of the 16 non-Japanese Asians. However, we didn't observe an identical sequence to Urawa-1 among the 61 contemporary Japanese. Specifically, 14 Japanese differed at one position and 8 Japanese differed at two positions. Furthermore, 38 Japanese differed at three to eight positions even in this short region. These observations indicate that this ancestor of Japanese who presumably lived in the central part of Japan about 6000 years ago had a common origin with some contemporary Southeast Asians.

Restriction enzyme analysis revealed that the Japanese population could be separated into at least two distinct groups: the group with a smaller frequency (Group I) first diverged from the other group with a larger frequency (Group II). This was also confirmed by our recent sequence analysis of the major noncoding region for Mongoloids including non-Japanese Asians. In this connection the ancient Japanese speciman (Urawa-1) belongs to one of the two groups (Group II) of modern Japanese. Part of the people who migrated from the continent during the period two to three thousand years ago may be representative of the other group (Group

I) of modern Japanese, because the sequences from Group I individuals showed three to eight nucleotide differences in the 190-bp region in comparison with that from Urawa-1. Although our findings were obtained through the determination of nucleotide sequences from only one ancient individual this archaeological and molecular genetical study gives a new source of perspective on the evolutionary history of human populations. For details, see Proc. *Japan Acad.* **65B**: 229–233, 1989.

Heteroplasmies and Polymorphisms in the Major Noncoding Region of Mitochondrial DNA in Japanese Monkeys Associated with the Tandem Repeated Sequences

Kenji HAYASAKA, Takafumi ISHIDA* and Satoshi HORAI

We analyzed polymorphisms in the major noncoding region of mitochondrial DNAs (mtDNAs) in 100 Japanese monkeys derived from 12 local populations. Ninety of the 100 samples were analyzed using DNA amplified by a polymerase chain reaction. We found individuals which were heteroplasmic for mtDNAs in two populations. These heteroplasmies were associated with length polymorphisms which resulted from the presence of tandem repeated segments in the noncoding region. We classified the mtDNA of the 100 Japanese monkeys into six types. One type was observed in seven populations and another in two populations, while the remaining four types were observed only one population each. In 11 out of the 12 populations, only one type of mtDNA was observed. These findings support the maternal inheritance of mtDNAs and the life history of Japanese monkeys.

Mitochondrial DNA Mutation in a Family with Leber's Hereditary Optic Neuropathy

Makoto YONEDA**, Shoji TSUJI**, Toyoaki YAMAUCHI**,
Takashi INUZUKA**, Tadashi MIYATAKE**,
Satoshi HORAI and Takayuki OZAWA***

Leber's hereditary optic neuropathy is characterized by optic neuropathy

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and cardiac arrhythmia. The age of onset ranges from adolescence to late adulthood. Maternal transmission in some pedigrees suggested a mutation in mitochondrial DNA (mtDNA). Sequencing of 85% of the mtDNA from a black patient with this disease revealed 25 base substitutions, compared with normal mtDNA (the Cambridge sequence). Of these substitutions one—a G-to-A transition at nucleotide position 11778 in the Cambridge sequence, converting the 340th arginine to histidine in NADH dehydrogenase subunit 4—has been suggested as the mutation correlated to Leber's hereditary optic neuropathy because the 340th arginine is highly conserved among various species. The mutation removes an *Sfa*NI restriction site, which has also been found in a few North American and European pedigrees.

We studied leucocyte mtDNA *Sfa*NI restriction fragment length polymorphisms (RFLP) in a Japanese family with Leber's hereditary optic neuropathy and found the identical mutation only in maternal members, including two affected patients. The loss of a 0.92 kbp fragment and a 0.68 kbp one, and the appearance of a 1.6 kbp fragment, were observed only in the maternal members, including the patients. Restriction mapping with other enzymes ruled out deletion or rearrangement of mtDNA. Nucleotide sequence analysis of polymerase chain reaction products in patients' mtDNA revealed a G-to-A conversion identical to that reported by Wallace *et al.*

This mutation was found in two patients with the disease and was maternally inherited through three generations. It has been demonstrated in patients of differrent ethnic origin and, taken together, these reports strongly support the hypothesis that the G-to-A mutation at position 11778 is the cause of Leber's hereditary optic neuropathy. For details, see *Lancet* **i**: 1076–1077, 1989.

Specific Amplification of Deleted Mitochondrial DNA from a Myopathic Patient and Analysis of Deleted Region with S_1 Nuclease

Tomoko TANAKA-YAMAMOTO*, Masashi TANAKA*, Kinji OHNO*, Wataru SATO*, Satoshi HORAI and Takayuki OZAWA*

Heteroplasmy of the normal-sized and the deleted mitochondrial genome has been observed in mitochondrial myopathy. The deleted region of the

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genome in the skeletal muscle of a patient was analyzed both by the conventional Southern blot method and by the novel method of employing a combination of polymerase chain reaction and S_1 nuclease digestion. The results obtained by these methods were compared. Southern hybridization using various mitochondrial DNA fragments localized the deletion from at least position 9020 to 14955, but regions of uncertainty of 1 kb remained on both ends of the deletion. Using the polymerase chain reaction, a fragment from the deleted genome was specifically amplified by choosing a pair of primers surrounding the deletion, and two fragments adjacent to the start and end of the deletion were amplified from the normal-sized genome. S_1 nuclease analysis of the heteroduplexes formed among these fragments demonstrated that the deletion extended from position 8650+50 to 15660+60. This methods does not require radioisotopes and, moreover, can determine the deleted region within 5 h, in contrast to the 2 days required by the conventional Southern blot analysis. These results indicate that the novel method is faster and more accurate than the conventional method for determination of the deleted region of the genome. For details, see *BBA* **1009**: 151–155, 1989.

Increased Level of Mitochondrial Gene Expression in Polyps of Familial Polyposis Coli Patients

Akiko YAMAMOTO*, Satoshi HORAI, and Yasuhito YUASA**

To elucidate the mechanisms of polyp formation in familial polyposis coli patients, cDNA clones, expressed differently between normal colonic mucosae and polyps, were screened. Three polyp-derived cDNA clones showed increased levels of transcription under neoplastic conditions. Among them, clone P6 was determined to be derived from the mtDNA encoding parts of subunit 1 of NADH dehydrogenase and 16S rRNA. The numbers of copies and restriction enzyme patterns of the mtDNA did not vary in either normal or neoplastic tissues, and a level of different mitochondrial mRNA was increased in the same proportion, suggesting a change in the rate of synthesis and/or degradation of the mtRNA. No cDNA clone which was expressed higher in normal colonic mucosae than in polyps, was isolated from the cDNA library derived from normal mucosae. For details, see *Biochem. Biophy. Res. Commun.* **159**(3): 1100–1106, 1989.

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X. APPLIED GENETICS

Association between *Pox-1* Variation and Seed Productivity Potential in Wild Rice

Hiroko MORISHIMA

Alleles at an isozyme locus *Pox-1* in rice are differentially distributed in cultivated species and their wild relatives. Populations of seed propagating taxa (*Oryza sativa*, *O. glaberrima*, annual type *O. rufipogon*, *O. breviligulata*) are nearly monomorphic with an allele called 2A, while those of vegetatively propagating ones (perennial type *O. rufipogon*, *O. longistaminata*) carry another allele called 4A at high frequency. This association between allozyme variation and propagating system was consistently found over broad areas at the species level as well as at the ecotype level.

Asian common wild rice *O. rufipogon* tends to be differentiated into two ecotypes; perennial and annual types which differ not only in longevity but also in many other life-history traits. A cross between an annual and a perennial strain of *O. rufipogon* which carry $Pox\text{-}1^{2A}$ and $Pox\text{-}1^{4A}$ in homozygous state, respectively, was extensively studied to elucidate the genetic mechanism of association between *Pox-1* variation and life-history. 1) In F_3, homozygotes with $Pox\text{-}1^{2A}$ showed higher reproductive allocation, shorter plant stature, earlier flowering and shorter anther, which are all characteristics of annual life history, than those with $Pox\text{-}1^{4A}$. 2) When the hybrid population was selected for early flowering, allele 2A increased. When it was naturally grown for three years under conditions favorable for vegetative propagation, allele 4A increased. 3) To check whether this association is due to pleiotropy of *Pox-1*, isogenic lines were made through selfing for six generations holding the *Pox-1* locus heterozgous. In the isogenic pairs thus prepared, the association between allozyme and characters mostly disappeared.

The above results suggest that quantitative trait loci for these life-history traits exist, and that some of them are clustered on a chromosome segment marked by *Pox-1*. Their linkages, however, are not tight judging from the experiments with isogenic lines. There must be certain selective interactions between those genes including *Pox-1* to maintain this gene block in nature.

Variation in Resistance to Bacterial Leaf Blight between and within Wild Rice Populations

C. HAMAMATSU, Y. I. SATO and H. MORISHIMA

Host-pathogen interaction in a natural ecosystem, when compated with that in an agroecosystem, provides a perspective on the evolutionary dynamics of plant-pathogen systems. The present study deals with inter- and intra-populaltional variability in response to bacterial leaf blight caused by *Xanthomonas campestris pv. oryzae* in the wild rice, *Oryza rufipogon*. Forty-two strains of wild rice (each representing a natural population) collected from various regions of Asia were examined regarding response to infection by five pathogen races isolated in Japan. Mulitvariate analysis of the data revealed variation in general resistance, and variation in race-specific resistance. Variation patterns obtained, however, did not show distinct relationship to geographical distribution nor to ecotypic differentiation (perennial *vs.* annual type). Some Chinese strains showed race-specific responses.

To assess intra-populational variation in respones to the pathogen, three wild rice populations from Thailand (one annual and two perennial types) were studied together with two populations of Chinese primitive cultivars. In each population, 30 to 40 lines derived from randomly chosen mother plants growing in their natural habitats were examined by injection method and clip-inoculation using three Japanese pathogen races. Wild rices as well as land races were found to preserve a large amount of intra-populational genetic variability in response to the pathogen races. To the pathogen races used in the present study, wild rices were generally resistant though land races were susceptible. The amount of intra-populational variability in resistance to this desease did not seem to be related to genic diversity estimated from isozyme variations.

Responses to three different pathogen races were compared among lines in three wild populations. In most cases, intensities of infection (lesion length of leaves) were positively correlated between different races within-population, indicating that race-nonspecific resistance possibly governed by polygenes is mainly responsible for intensity of infection. In addition, the existence of race-specific resistance governed by a major gene was suggested in an annual population NE4, judging from discontinous variation in response to race, T7133. This was also confirmed by injection method.

Further, two populations NE4 and CP20, each consisting of 30 lines, were compared as to genetic structure of population in resistance to a pathogen race, T7133. Analysis of variance indicated that between-line variation (heterogeneity in the field) was larger in the annual population NE4, while within-line variation (heterozygosity in the field) was larger in the perennial population CP20. This could be interpreted by differences in their breeding systems.

To understand the complex pattern of host-pathogen interactions, use of more pathogen races, particularly those prevailing in areas from which study-populations were sampled is needed.

Genetics of Life-history Trait Associations in Wild Rice from Thailand

P. BARBIER* and H. MORISHIMA

In natural populations of wild rice *Oryza rufipogon*, life-history traits (such as the age at flowering, the components of seed productivity, and investment in vegetative growth and asexual reproduction) as well as characteristics of the mating system, are found tightly intercorrelated. The resulting trait-associations correspond to differentiation between annuals and perennials occurring within this species. Intra-populational genetic variability of most of these traits seems, however, to be low. In order to determine which trait associations have a genetic basis, a series of crosses were made between individuals issued from one annual and several perennial populations located in Thailand. These perennial populations slightly differ by their degree of relatedness to the annual population used, as judged from the genetic distances calculated from isozyme data and by their sequence data at some nuclear genes.

F_2 and F_3 progeny of four annual × perennial crosses with the plants from the most closely related perennial population were grown in a glasshouse and various life-history traits were measured to examine their correlation pattern. In particular, the existence of negative correlations between fitness components characterizing the annual *vs.* perennial habit, was tested.

In contrast to the situation in natural populations, only a few correlations between traits were found in the progeny of crosses. A negative correlation between the number of days to flowering and the number of

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panicles (component of fecundity) was consistently found in F_2 and F_3. This correlation, which probably has a genetic or developmental basis, may have played a role in the evolution of early reproducing, high yielders (such as the annual type). However, only slight evidence could be found for the existence of antagonistic pleiotropy between components of fecundity and traits related to post-reproductive survival. F_2 progeny of other crosses are under investigation.

A New Hypothesis on the Parentage and Dissemination of Japanese Rice

Yo-Ichiro SATO

The parentage of Japanese rice, and the origin of early-heading cultivars distributed in the northeastern part of Japan were discussed from the viewpoint of genetics. Native cultivars in Japan and neighbouring countries were examined to survey the distribution of genes for hybrid weakness, F_2 chlorosis and black hull-color which segregate only in crosses between distantly-related cultivars. It was found that Japanese native cultivars are polymorphic in the loci examined, suggesting that Japanese rice has plural origins. The geographical distribution pattern of the gene for hybrid weakness suggests two paths of dissemination; one was from mainland China ("keng" type) and another was from tropical islands. Modern Japanese rice is considered to be formed through a natural hybridization between these two groups.

Early-heading progeny are expected to appear theoretically in hybrids between two late-heading *japonicas*. In fact, part of F_1 plants derived from a cross between "keng" and Philippino upland cultivar headed as early as early-heading cultivars in the northeastern part of Japan. The origin of early-heading cultivars can be well explained under the present hypothesis. The previous hypothesis which assumed an early-heading mutation, however, is not supported by recent finding that rice cultivation had begun in the northern Tohoku area already in the early Yayoi period. For details, see Archaeology and Natural Sciences Vol. 22.

Toward a Rationale of Genetic System in the Flowering Response of the Rice Plant to Photoperiod

Yoshio SANO

Plants utilize a precise and predictable photoperiod for determining the time of floral initiation so as to accomplish their life cycle during a favorable period. Although one of the major genes controlling photoperiod sensitivity, Se_1 (or Lm^u), has been genetically well characterized, our present knowledge is still limited for concerning diversified flowering responses to photoperiods in rice. A problem in genetic studies of rice often comes from distorted segregations which occur in crosses between ecotypes and/or distantly related taxa. The author has worked on genetic differentiation between distantly related taxa of rice and examined the genic differences after introducing alien chromosomal segments carrying a given gene into a genetic tester of rice. The Se_1 locus was also successfully introduced from various accessions of rice including wild forms. During this procedure, two additional genetic factors were found which drastically change the heading time in the presence of Se_1. Their precise chromosomal locations and their gene actions are under investigation and this information will be published elsewhere. The discovery of these two genetic elements might offer an opportunity for better understanding of the complex regulatory system in photoperiod sensitivity.

a) Suppressor gene for Se_1

An Indica type, *Oryza sativa*, Peiku (Acc108) is photoperiod insensitive and it comes to heading about a week earlier than T65 with $Se_1{}^+/Se_1{}^+$. The genotype of Acc108 has been shown to be Se_1/Se_1 since a large chromosomal segent from *wx* to Se_1 was successfully introduced into Taichung 65 from Acc108S_1 (g) utilizing a segment containing the S_1 gene (hybrid sterility from *O. glaberrima*) as a recombination suppressor around the *wx* locus. The resultant plant came to heading two to three weeks later than T65 and as late as T65Se_1 (g). The F_1 plants between T65 and Acc 108S_1 (g) suggested that Acc108 possesses a dominant suppressor (s) for Se_1. To extract and examine the suppressor gene, early flowering segregants from selfed progenies were backrossed with T65Se_1 (g). BC_1F_2 and BC_2F_2 segregants gave a ratio of 1: 2: 1 regarding flowering time. The F_3 lines also showed that the early flowering plant is homozygous for the dominant supperssor. The suppressor gene was tentatively designated

Su(t)-Se_1.

b) Enhancer gene for Se_1

A wild progenitor (*O. rufipogon*) is strongly photoperiod sensitive and it produced no panicle during the summer season under natural conditions in Mishima. The chromosomal segment from *Wx* to Se_1 was also introduced from W593 (*O. rufipogon* from Malaysia) to T65*wx* (a near-isogenic line of Taichung 65 with *wx*). The segment was successfully introduced without the destruction of the segment since S_6 (gamete eliminator) is tightly liked with Se_1 and most of the hybrid derivatives tend to carry Se_1. Unexpectedly, the homozygote for *Wx* and Se_1 which was derived from BC_7F_2 produced no panicle in Mishima while the homozygote for *wx* and Se_1 always headed as early as the T65Se_1 (g) strain. This suggests that BC_7F_1 is heterozygous for an enhancer gene which dramatically delayed heading in the presence of Se_1 and that the enhancer gene is tightly linked to *Wx*. The data from BC_8F_1, BC_7F_2 and BC_7F_3 consistently supported these assumptions. The enhancer gene was tentatively designated *En*(t)-Se_1 and the gene order was estimated to be *wx*-*En*(t)-Se_1-Se_1-S_6.

The present results confirmed that there are at least two genetic elements which negatively and positively regulate the experssion of Se_1. Although the genic interaction remains to be studied, the present study revealed that investigations of differential regulation at the Se_1 might elucidate further information about diversified photoperiod sensitivity in rice.

A Gene Complex Responsible for Seed Shedding and Spreading Panicle Found in Wild Rice Species

Mitsugu EIGUCHI and Yoshio SANO

Generally, cultivated crops readily interbreed with their wild progenitors when they coexist under natural conditions and produce fertile progenies, revealing their close relationships. This holds true for Asian cultivated and wild forms of rice, giving rise to difficulties in distinguishing them in some instances. The only reliable morphological trait which distinguishes wild forms from cultivated forms is seed shedding. Wild rice forms show uniformaly, a high degree of seed shedding, suggesting a strong natural selection for seed shedding in wild species. On the other hand, cultivated forms show a wide range of intensities in seed shedding but generally it is a weak degree of seed shedding, if any. This strongly suggests that the

genetic changes which occurred during the formation of various rice cultivars were not simple. We decided to study on the genetics of seed shedding in order to examine their genetic changes in relation to the evolution from wild to cultivated rice forms.

The materials used were three strains of *Oryza rufipogon* (a wild progenitor of *O. sativa*, W107 as an annual form and W149 and W573 as perennial forms) and a strain of *O. glumaepatula* (W1192), which showed seed shedding. A genetic stock (Taichung 65 carrying genetic markers of *g* and *lg*, designated T65*g lg*) was used as a cultivated form without seed shedding, in these crossing experiments. Degree of seed shedding as described here means seeds shedding naturally after ripening as observed in wild rice forms. Such a high degree of seed shedding is never observed in cultivated forms. In addition, spreading panicle which is characteristic of wild forms and seems to be important for seed dispersal under natural conditions, was also examined in segregating papulations.

The F_1 hybrids between T65*g lg* and wild forms all showed seed shedding and spreading panicle like wild forms, which suggested that the two traits are controlled by dominant genes. The F_2 segregations between T65*g lg* and *O. rufipogon* revealed that seed shedding is controlled by two dominant genes and sperading panicle by one. The confirmation of the two dominant genes for seed shedding supports the previous finding by Kadam (1936) that wild rice carries two dominant genes responsible for seed shedding. One of them is known to be loosely linked with la (lazy habit, the 8th likage group). In addition, the other dominant gene responsible for spreading panicle (designated Spr_3 (t) tentatively) seems to be linked with both *lg* and *Ph* in the 2nd linkage group. The F_1 plants of T65*g lg*×W107 and T65*g lg*×W1192 were backcrossed with T65*wx* in order to introduce alien genes responsible for the traits into the *sativa* genetic background. In each generation, plants showing seed shedding and spreading panicle were used for further crossings. The high frequencies of plants with both traits in each backcross generation suggested a tight linkage between the genes controlling the two traits although such a linkage was not detected in the F_2 generation.

The near-isogenic lines carrying two dominant genes, each of which control seed shedding and spreading panicle, were selected from BC_6 (×W107) and BC_8 (×W1192) generations. F_2 and F_3 data showed support for the finding that each line carries two dominant genes and that the two

genes for seed shedding and spreading panicle were tightly linked with a recombination value of about 2% in both cases. This indicates that wild rice species generally carry a gene complex which is responsible for seed dispersal. The order of the two dominant genes from W1192 was also estimated as *lg*-Spr_3(t)-Sh_3-*Ph*, suggesting that the gene complex is preserved in distantly related wild rice taxa. Accordingly, two steps of mutation are needed for the establishment of the gene complex found in cultivated forms. At the time the gene complex was established during domestication, it seems to have been readily maintained in cultivated forms by selecting nonshattering and compact panicles, a typical syndrome resulting from automatic selection due to planting harvested seed.

Extraction and Amplification of a Chloroplast DNA Fragment from a Single Ancient Rice Seed

Ikuo NAKAMURA and Yo-Ichiro SATO

If DNA extracted from ancient rice seeds can be analyzed, the scope of the phylogenic view of rice would certainly be broadened. PCR enables us to amplify DNA from museum specimens and archaeological finds (Higuchi *et al.* 1984, Paabo 1985, Golenberg *et al.* 1990). Our purpose was to investigate whether a chloroplast DNA fragment could be amplified from a single ancient rice seed found in a Japanese archaeological excavation (1,200 years old). We were able to successfully extract 50–100 ng of DNA from a single ancient rice seed. This was half the amount of DNA extractable from a single living seed using the same extraction procedure. The DNA fragments from the ancient seed were recovered as 15–20 kbp fragments and were a little smaller than intact DNA. Since the ancient seeds were sunk in water-saturated soil, water must have prevented the ancient DNA from their strand cut and modification by oxidation. This is why the DNA was preserved in extremely good condition.

We were able to amplify the rice chloroplast sequence, a 1.3 kbp fragment covering *trn*Q, *psb*K, *psb*I and *trn*S genes corresponding to 1% of total rice chloroplast DNA, from the ancient DNA through a polymerase chain reaction. The 1.3 kbp chloroplast fragment of living leaves was hybridized with 1.3 kbp fragments amplified from ancient seeds as well as from living seeds. Restriction analysis showed that these 1.3 kbp fragments were correctly amplified from rice chloroplast DNA. The DNA analysis of

ancient rice seed described can greatly contribute to the investigation of the origin and varietal diversification of rice cultivars.

Anomalous 3: 4: 1 Segregation of α- and β-Subunits of Seed Conglycinin in Mutant Soybean

Ikuo NAKAMURA, Hiroya ODANAKA and Norihiko KAIZUMA

A soybean mutant (*dm/dm*) lacks the α- and β-subunits of β-conglycinin in seed storage protein and is a lethal during the early stage of germination. This mutant was selected from progenies of a γ-ray irradiated soybean cultivar; Wase-suzunari (Odanaka and Kaizuma 1988). Seed proteins were extracted from an anti-hilum portion of seed from progeny derived from selfed heterozygous plants and were subjected to SDS-polyacrylamide gel electrophoresis. These two subunits were simultaneously segregated at the ratio of 3 (+/+): 4 (+/*dm*): 1 (*dm/dm*). This segregation ratio has not been observed in any higher animals and plants. As the α- and β-subunits are coded by at least two genes, respectively (Harada *et al* 1989) and a weak genetic likage was reported between the α- and β-subunit genes (Tsukada *et al.* 1986), a relatively large chromosomal region covering these genes might be lost in the mutant and such a deleterious chromosome might be involved in the anomalous segregation.

Our hypothesis to explain this anomalous Mendelian segregation is that some marker gene aberrantly segregates at 3: 1 during the meiosis of female or male gamatecytes while normal meiotic segregation (1: 1) occurs during another gamategenesis in the same flower of the heterozygous plant. If this were true, the resulting segregation of the marker gene would be 3: 4: 1 in the progeny. Many researchers reported aberrant 3: 1 (6: 2) segregation in yeasts and lower fungi known as gene conversion (Szostak *et al* 1983). The frequencies of gene conversion in these organisms is quite low. However the deletion of the two subunits was stably inherited at a 3: 4: 1 ratio in this soybean mutant. This suggests that the aberrant 3: 1 segregation also stably occurs during miosis specific to only one-side gamategenesis. We are now investigating this phenomena at the molecular level.

Construction of a Targeting Signal for Plant Mitochondria

Ikuo NAKAMURA, Junko KYOZUKA and Ko SHIMAMOTO

An artificial DNA sequence (306 bp) coding a targeting signal (97 amino acids) for plant mitochondria was constructed. The β-subunit of F_1-ATPase in *Nicotiana plumbaginifolia.* is encoded by a nuclear gene and is localized in the mitochondria (Bountry and Chua 1985). Four pairs (A, B, C and D) of oligonucleotides were designed, based on the amino acid sequences, including the targeting signal of this protein, and unique restriction sites with unchanged amino acid sequences were arranged at both ends of each fragment to link these fragments. A total of eight oligonucleotides (76- to 89-mers) were synthesized by DNA synthesizer. Each pair of oligonucleotides was annealed and ligated into a pUC type vector. Then, the cloned fragments were sequenced and correctly synthesized fragments were linked in turn (A-B-C-D). Some deletion clones were made by the exonuclease III digestion from the 3′ end of this fragment, because the signal processing site of F_1-ATPase β-subunit was not known. These synthesized and deleted DNA fragments were ligated with a reporter gene (β-glucuronidase) and then truncated genes were integrated into a plant expression vector. The targeting function of the synthesized signals for plant mitochondria is being assayed.

On the Conditions Causing the Periphery Effect in Genotype Distribution within Plant Populations

Katsuei YONEZAWA

Plant individuals do not change their positions and tend to mate with neighbouring individuals because of limited dispersion of pollen. In addition, many plant species do selfing with a fairly high rate. Genotypes in a plant population therefore are expected to distribute very unevenly, forming patches of various sizes and shapes. One type of unevenness in genotype distribution, the difference of periphery area from central area, i. e., periphery effect, has not been extensively investigated. A greater understanding of periphery effect is necessary for accomplishing our knowledge on plant populations because every plant population in reality has boundaries. This subject is important also for defining the optimum mehtod of sampling from plant populations for both scientific surveys and

practical purposes such as geneitc resource collection.

Genotype distribution in a population is determined by various breeding and life-history characteristics (e. g., longevity, overlap of generations, flowering date and duration, mating system, distance and frequency of dispersion of pollen and seeds, etc.), and heterogeneities in macro- and micro-environmental conditions. Among these factors, dispersion of pollen and seeds and the selfing rate of plants seem to be most fundamental, and are now being investigated using Monte Carlo computer simulation. The periphery effect (PE) was measured by a ratio, (Patchiness of periphery area- Patchiness of central area)÷(Patchiness of central area). The computations obtained hitherto showed that the periphery effect occurs most prominently not in the absence of but in the presence of some degree of pollen dispersion, roughly, under a dispersion of 2–5 in units of distance between adjacently neighbouring individuals. The contribution of seed dispersion could not be perceived as clearly, but a dispersion larger than 2 seemed to suppress development of the periphery effect. The influence of selfing rate also remains to be confirmed through further computations, but a trend could be observed in which a more pronounced periphery effect occurs with a lower rate of selfing. It may tentatively be suggested that the periphery effect best develops in the presence of a moderated rather than highly limited dispersion of pollen. In plant populations with a strongly restricted gene flow, homozygosity and patchiness of homozygotes progress rapidly, but to the same extent in both central and boundary areas, and as a result, no differences occur between the two areas.

Computer Simulated Evaluation of the Hybrid Weakness Gene System as a Means of Preventing Genetic Contamination of Rice Cultivars

Katsuei YONEZAWA, Yo-Ichiro SATO, Tetsuro NOMURA*
and Hiroko MORISHIMA

The effectiveness of hybrid-weakness caused by a complementary interaction of two dominant genes, *Hwc-1* and *Hwc-2*, in suppressing the spread of the red-grain gene into ordinary white-grained rice cultivars was investigated based on the Monte Carlo computer simulation method. It was shown that the weakness genes, capable of reducing the fitness of the carrier by 80 percent or more, work quite effectively to suppress the genetic

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contamnination of white-grained cultivars due to the inflow of pollens from red-grained cultivars. The complementary weakness genes, however, do little to restrain the spread of the red-grain gene once the gene has been incorporated into the population in the form of red-grained progeny produced due to imperfect lethality of the initial hybrid between red- and white-grained cultivars. The weakness genes also have no or little effect, when contamination was initiated by the inflow of seeds or seedlings from red-grained cultivars. In these situations, removal (selection) of red-grained plants is the only method for exterminating the red-grain gene. The removal of red-grained plants needs not to be intensive if carried out continually. The spread of the red-grain gene after initial contamination is suppressed if the red-grain gene and weakness genes are linked. The suppressing effect of the linkage, however, is not substantial when the outcrossing rate is around or smaller than 0.01 as assumable in most rice cultivars. For details, see *Plant Breeding* **104**: 241–247 (1990).

XI. DATABASE

Activities of the DNA Data Bank of Japan

Sanzo MIYAZAWA and Hidenori HAYASHIDA

A primary task of the DDBJ is, of course, DNA sequence collection. However, in addition to that, we have a wide range of activities; 1) DNA data collection and data entry in collaboration with other databanks, 2) data distribution, including the secondary distribution of the GenBank and EMBL databases in Japan, 3) development of research tools for sequence analysis, and 4) the regular publication of newsletters to inform people of activities of the DDBJ. The DDBJ computer system is open to researchers for data submission and for providing online access to DNA and related databases. In the following, I will briefly report this year's activities of the DDBJ. For details, see "DDBJ News Letter No. 8, 1989" and "No. 9, 1990".

1) Data Entry and Management

Sanzo MIYAZAWA and Hidenori HAYASHIDA

Our data collection began in December, 1986 and is carried out in collaboration with the GenBank and the EMBL Data Library. The collaboration includes projects on designing a new feature table and rebuilding a DNA database. DDBJ has charge of mainly scanning journals published in Japan, and before, data directly submitted by authors had been forwarded to either GenBank or EMBL according to journal split. However, DDBJ started locally processing all direct submissions in October 1989; Direct data submissions which the EMBL Data Library is supposed to process are still forwarded to the EMBL at this stage.

Since we released the first version of the DDBJ database in July, 1987, our database has been released every half year; version 5, which included 679,378 bases in 395 entries, was released in July, 1989, and version 6 including 841,236 bases in 496 entries was released in January, 1990. The DDBJ collected about 305 kb in the year from January 1989 to January 1990. This amount of data is almost equal to 1.25 times the data collected last year, but only 1/40 of the total DNA sequences collected in the world.

However, this may be reasonable, if the number of staff of the DDBJ is compared with those of GenBank and the EMBL Data Library. Each release included a coding sequence database and a peptide sequence database that were extracted and translated from the original DNA sequence database, and files of journal index, accession number index, short directory, and data submission form.

2) FLAT Database and Sequence Analysis System for DNA and Proteins: Release 1.2.

Sanzo MIYAZAWA

We have been developing a search and retrieval system for flat file databases in order to provide simple tools for using DNA and protein sequence databases. This system called FLAT consists of primitives most of which perform a single operation and work as filters in the UNIX system. Its first version, 1.0β, was released in 1988. The most recent version, 1.2, was released in October, 1989. New features added to this release include functions of (1) electronic mail database server and (2) automatic update of databases according to new data sent by e-mail. One can search and retrieve DNA and protein sequence databases by sending search/retrieval commands by electronic mail to the specific address through Junet, Usenet, Internet, and Bitnet. Available databases include PIR, SwissProt and PRF as well as DDBJ, EMBL, and GenBank databases. The EMBL and GenBank databases are updated twice a day by adding new entries which are daily sent by e-mail from those databanks. The DDBJ database is updated as well. Functions provided by the server include keyword search on definition lines, search by author name, journal name and accession number, and entry retrieval by specifying entry names or accession numbers. This FLAT search/retrieval system for sequence databases is designed to be portable among UNIX systems which are available for a wide range of computers from super to micro computers.

For details, see "DNA Data Bank of Japan: present status and future plans" by Sanzo Miyazawa in "*Computers and DNA, SFI Studies in the Sciences of Complexity*, Eds. G. Bell and T. Marr (Reading MA: Addison-Wesley), vol. VII, pp. 47–61, 1989".

Basigin, a New, Broadly Distributed Member of the Immunoglobulin Superfamily, Has Strong Homology with Both the Immunoglobulin V Domain and the β-Chain of Major Histocompatibility Complex Class II Antigen

Teruo MIYAUCHI*, Takuro KANEKURA*, Akihiro YAMAOKA*, Masayuki OZAWA*, Sanzo MIYAZAWA and Takashi MURAMATSU*

Lotus tetragonolobus agglutinin (LTA) binds preferentially to early embryonic cells in the mouse. The affinity-purified antibody raised against LTA receptors from embryonal carcinoma cells was used to screen a λgtll expression library of F9 embryonal carcinoma cells, resulting in detection of a cDNA clone specifying a new glycoprotein termed "basigin". The glycoprotein has been suggested as being a transmembrane one, and was found to be a new member of the immunoglobulin (Ig) superfamily. The molecular weight of basigin was largely in the range between 43,000 and 66,000, while that of the peptide portion with a putative signal sequence was inferred to be about 30,000. Significant levels of basigin mRNA were detected not only in embryonal carcinoma cells, but also in mouse embryos at 9–15 days of gestation and in various organs of the adult mouse. The Ig-like domain of basigin is unique, since it has strong homology to both the β-chain of the major histocompatibility class II antigen and the Ig V domain. The number of amino acids between the two conserved cysteine residues is intermediate between those of the Ig V and C domains. Therefore, basigin is an interesting protein in connection with the molecular evolution of the superfamily.

For details, see "*J. Biochem.* **107**, 316–323, 1990".

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ABSTRACTS OF DIARY FOR 1989

Biological Symposium

287th	meeting	Jan. 5	Genome scanning method for cloning uncharacterized genes: cloning and characterization of P^{vn} from mutant mouse (Yoichi Gondo)
288th	—	Jan. 12	1. Current genetic research in R. O. C. Taiwan 2. Computerized numerical assignment for the nucleotide sequence of T24 human bladder carcinoma oncogene (I-Hung Pan)
289th	—	Mar. 20	Physical mapping of the *C. elegans* genome (Alan Coulson)
290th	—	Mar. 20	High resolution genetic maps of mouse chromosomes 14 and 17 with special emphasis on the *t* complex and the disorganization mutation (Joe Nadeau)
291st	—	Mar. 24	*P* elements in Drosophila (William R. Engels)
292nd	—	May 12	Genomic imprinting in the mouse and possible example in man (Bruce M. Cattanach)
293rd	—	May 17	Evolutionary aspects of codon usage (Michael Bulmer)
294th(A)	—	June 1	Reverse transcriptase and retrovirus-like elements in the prokaryotes (Masayori Inoue)
294th(B)	—	June 1	M-protein of influenza virus: Antigenic analysis and intracelluar localization with monoclonal antibodies (Doris Bucher)
295th	—	Aug. 25	Analysis and prediction of protein structure (William R. Taylor)
296th	—	Aug. 28	Non-Mendelian inheritance and evolution

			of maternal characters (Russell Lande)
297th	—	Oct. 2	G+C% mosaic structures of the genomes of higher vertebrates and plants (Giorgio Bernardi)
298th	—	Oct. 26	Eukaryotic transcription factors and mechanisms (Robert Roeder)
299th	—	Nov. 20	Regulation of growth hormone gene expression (Christopher Lefever)
300th	—	Nov. 22	Studies of mechanisms of DNA transposition reactions (Kiyoshi Mizuuchi)
301st	—	Dec. 4	Microdissection and microcloning of human banded chromosome (Uwe Classen)
302nd	—	Dec. 8	Activation of a silent GC-rich promoter in an intron caused by deletion of the original promoter (Makoto Taketo)

Mishima Geneticists' Club

351st	meeting	Feb. 1	A test of the neutral theory based on DNA polymorphisms (Fumio Tajima)
352nd	—	Feb. 27	Physiological functions of the c-mos oncogene at the beginning of meiosis (Naoyuki Sagata)
353rd	—	May 2	Physiological roles of interferons: Analysis using transgenic mice (Masahide Asano)
354th	—	May 24	What determines the composition and levels of anticodons in the cells? (Fumiaki Yamao)
355th	—	June 2	Evolution of surface antigen genes under balancing selection (Akira Sasaki)
			Restriction map polymorphisms of X chromosome genes in *Drosophila melanogaster* (Naohiko Miyashita)
356th	—	June 5	Mouse gentic lethality caused by defect of steroid biosynthesis (Hideo Gotoh)
357th	—	June 28	Control mechanisms of eukaryotic transcription by regulatory proteins (Masami Horikoshi)
358th	—	July 25	Regulation of expression of the *Drosophila*

			even skipped gene (Tada-atsu Goto)
359th	—	Aug. 30	Future directions of the protein sequence database (David G. George)
360th	—	Sept. 7	Analysis of proteins involved in genetic recombination in mouse spermatogonia (Atsushi Higashitani)
			Function of C-terminal region of *E. coli recA* protein (Satoshi Tateishi)
361st	—	Sept. 9	Isozymes in fish (Yoshihisa Fujio)
			Some topics concerned with chromosome research (Junichi Furuyama)
			Development of muscle cells (Yutaka Shimada)
362nd	—	Nov. 13	Plasticity of DNA conformation around the Drosophila alchohol dehydrogenease gene (Kaoru Miyahara)
363rd	—	Nov. 16	Genetics of chemosensory identification (Kunio Yamazaki)
364th	—	Dec. 4	Regulation of gene expression by *Drosophila Anthennapedia* gene product (Shigeo Hayashi)

FOREIGN VISITORS IN 1989

April 9, 1984–	Pascale Barbier, Université des Sciences et Techniques du Languedoc, Montpéllier, France
August 3, 1987–March 31, 1989	Ling Hua Tang, Institute of Food Crops, Jiangsu Academy of Agricultural Sciences, China
December 21, 1987–March 20, 1989	Xiao Mei Wu, Lanzhou Institute of Biological Product, Ministry of Public Health, China
December 21, 1987–March 20, 1989	He Zhao, Lanzhou Institute of Biological Product, Ministry of Public Health, China
Septemer 1, 1988–	Guan Cheng Sun, Sericultural Research Institute, Chinese Academy of Agricultural Sciences, China
January 7, 1989–	Yong Hong Wang, Lanzhou Institute of Biological Product, Ministry of Public Health, China
January 12	I-Hung Pan, National Taiwan University, Taiwan
March 2–May 30, 1989	Djoho T. Iskander, Bandung Institute of Technology, Indonesia
March 6–16	William B. Provine, Cornell University, U. S. A.
March 20	Joe Nadeau, The Jachson Laboratory, U. S. A.
March 20–22	Alan Coulson, Laboratory of Molecular Biology, U. K.
March 20–22	Dieter Söll, Yale University, U. S. A.
March 21–28	William R. Engels, University of Wisconsin, U. S. A.
March 23	Cheng Huai Wang, Lanzhou Institute of Biological Product, Ministry of Public Health, China
April 7	E. Liseecki, RERF, Hiroshima
April 10–	Fengshan Wang, Tianjin Institute of Industrial Hygiene and Occupational Diseases, China
May 12	Bruce M. Cattanach, MRC Radiobiology Unit, U. K.
May 16–17	Michael Bulmer, University of Oxford, U. K.
May 30–June 2	Doris Bucher, New York Medical College, U. S. A.
June 1–2	Masayori Inouye, University of Medicine & Dentistry of New Jersey, U. S. A.
June 15	A. T. Bachin, Cambridge, U. K.

June 15	C. Queen, Palo Alto, CA, U. S. A.
June 21–23	Graham Cameron, EMBL Data Library, Heidelberg, Germany
June 21–23	Patricia Kahn, EMBL Data Library, Heidelberg, Germany
Jnue 21–23	David Hazledine, EMBL Data Library, Heidelberg, Germany
June 21–23	Jane Peterson, GenBank, NIH, Bethesda, U. S. A.
Jnue 21–23	Julie Ryals, GenBank, Mountain View, CA, U. S. A.
June 21–23	David Benton, GenBank, Mountain View, CA, U. S. A.
June 21–23	Christian Burks, Los Alamos National Laboratory, U. S. A.
June 21–23	Paul Gilna, GenBank, Los Alamos National Laboraatory, U. S. A.
June 21–23	Michael Cinkosky, GenBank, Los Alamos National Laboratory, U. S. A.
June 29	Anne Androuais, CNRS, France
July 19	S. N. Raina, University of Delhi, India
July 19	A. V. Shurkhal, N. I. Vavilov Institute of General Genetics, U. S. S. R.
July 19	Douglas Soltis, Washington State Universiry, U. S. A.
July 19	Pamela Soltis, Washington State Universiry, U. S. A.
July 19	Steven B. Browles, University of Georgia, U. S. A.
July 19	William F. Grant, McGill University, Canada
July 26	N. Schuppl, N. I. Vavilov Institure of General Genetics, U. S. S. R. Academy of Sciences, U. S. S. R.
July 26	V. Kolubelov, Institute of Bioorganic Chemistry U. S. S. R. Academy of Sciences, U. S. S. R.
August 25–26	William R. Taylor, National Institute for Medical Research, U. K.
August 28–29	Russell Lande, University of Chicago, U. S. A.
August 30	David G. George, Georgetown University, U. S. A.
October 2	Giorgio Bernardi, Institut Jacques Monod, France
October 20	Hubert Hug, University of Zurich, Switzerland
October 26	Robert Roeder, The Rockefeller University, U. S. A.
November 16	Akkas Uddin Armed, Bangladesh Rice Research

	Institute, Bangladesh
November 16	Peng Junhua, Crop Research Institute, Sichuan Academy of Agricultural Sciences, China
November 16	Hamed E. El-Hassawi, Seeds Production, Department, Behera Governorate, Egypt
November 16	Gogineni S. V. Prasad, Plant Breeding Division, Directorate of Rice Research, India
November 16	Gholamabbas Kianoush, Manzandaran Agricultural Research Center, Iran
November 16	Mat H. B. Abdullah, Farmers Association, Bakat Baru, Kemubu Agricultural Development Authority, Malaysia
November 16	Rosa E. S. Infantes, Enterprise of Rice Commercialization, Peru
November 16	Primitivo M. Esteban, Jr., Cagayan State University, Philippines
November 16	Sawat Somsa-ard, Department of Agriculture, Thailand
November 20	Christopher Lefevere, Cedars-Sinai Medical Center, U. S. A.
November 22	Kiyoshi Mizuuchi, National Institutes of Health, U. S. A.
December 4	U. Claussen, University of Erlangen, Germany
December 8	Makoto Taketo, The Jackson Laboratory, U. S. A.

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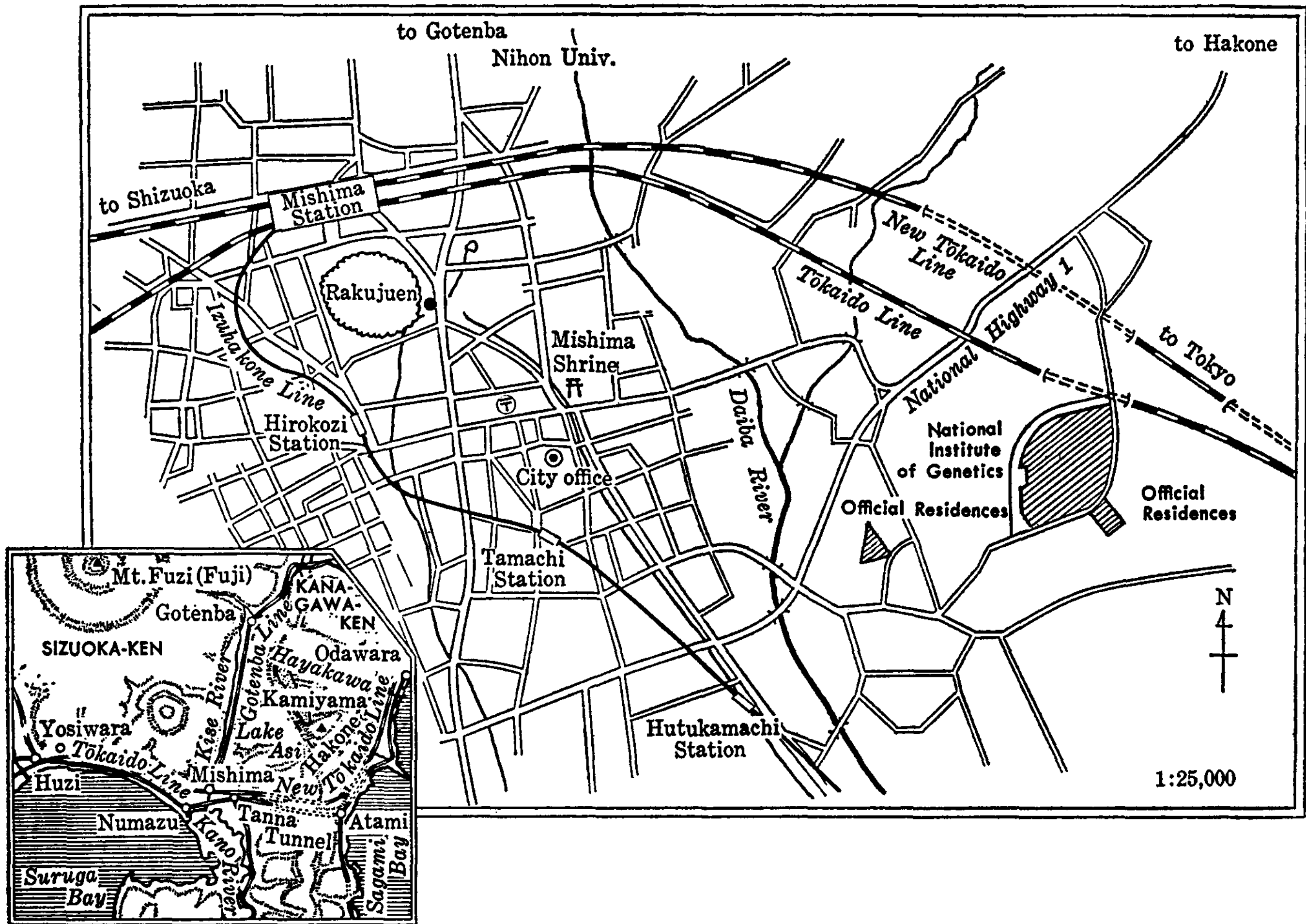

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