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GENERAL STATEMENT

Our institute was established 40 years ago as a center for comprehensive genetics research. Outstanding accomplishments in the advancement of genetics research 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, seven years ago the institute was reorganized into a National Inter-university Research Institute. With the foundation in 1988, of the Graduate University for Advanced Studies consisting of seven inter-university research institutes, our institute has admitted a number of graduate students every year in the Department of Genetics of the Graduate School of Life Science.

Recent rapid progress in the field of 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 changing demands. Our institute houses the DNA Data Bank of Japan (DDBJ), one of the three banks in the world that gather, store and distribute information on DNA sequences. DDBJ will continue to act as a regional center for such activities. It is obvious, however, that this and other actions taken by the institute, are far from sufficient. With suggestions and guidance from people in and outside the institute, I would like to lead the institute into a more fruitful and progressive future.

Regarding personnel changes in the past year, Prof. T. Ohta, Head of the Department of Population Genetics, was appointed as vice-director in June, while Prof. Y. Kuroda (Laboratory of Phenogenetics) and Associate Professors K. Tutikawa (Laboratory of Evolutionary Genetics) and S. Iyama (Genetic Stock Research Center) retired at the end of March, Drs. S. Hayashi and Y. Ugawa joined us as research members in the DNA Research Center, where Drs. T. Ikemura and T. Gojobori were promoted to professors and Dr. Y. Tateno to associate professor.

It is a pleasure to note that Prof. A. Ishihama was honored by the Mochida Memorial Award for Scientific Research for his contribution to the development of molecular studies on the regulation of gene expression.

[Signature]
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MORISHIMA, Hiroko, Professor, National Institute of Genetics

Adviser
MORIWAKI, Daigoro; Honorary member, National Institute of Genetics
PROJECTS OF RESEARCH FOR 1990

1. DEPARTMENT OF MOLECULAR GENETICS

Laboratory of Molecular Genetics

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

Laboratory of Mutagenesis

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

Laboratory of Nucleic Acid Chemistry

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)
Studies on bacterial plasmids and transposons (OHTSUBO)

3. DEPARTMENT OF ONTOGENETICS

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

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

Laboratory of Physiological Genetics
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)
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)
Genetic studies of speciation in rice (SANO and HIRANO)
Studies on plant gene expression (HIRANO and SANO)
Bio-archaeological analysis of plant remains (SATO and NAKAMURA)

**Laboratory of Applied Genetics**

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

6. RESEARCH FACILITIES

**Genetic Stock Research Center**

Theoretical studies in 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)
Studies on genes in HLA locus (MATSUMOTO and IKEMURA)
Computer analysis of DNA sequences (GOJOBORI and UGAWA)
Construction of DNA sequence database (GOJOBORI, UGAWA, HAYASHIDA, TATENO and MIYAZAWA)
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, HAYASHI and UEDA)
Molecular genetics of development of *Caenorhabditis elegans* (KOHARA)
Radioisotope Center

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

Experimental Farm

Molecular genetics of plant development (NAKAMURA)
Genetical analysis of physiological traits in plants (NAKAMURA)
RESEARCH ACTIVITIES IN 1990

I. MOLECULAR GENETICS

The Promoter Selectivity of Escherichia coli RNA Polymerase, I.
Involvement of the C-Terminal Region of α Subunit in Recognition of cAMP-CRP-Dependent Promoters

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

The RNA polymerase of E. coli is composed of four different subunits. The core enzyme with the subunit structure α2ββ′ is fully active in RNA polymerization, whereas the binding of one of the various species of σ subunits is required for specific initiation at promoters. Subunit α is composed of 329 amino acid residues, and its major role has been proposed to promote assembly of the two large subunits, β and β′, into enzyme complexes (reviewed in Ishihama, A. (1981) Adv. Biophys. 14, 1-35). In fact, in a temperature-sensitive E. coli strain carrying the rpoA112 mutation in the gene encoding the α subunit, the subunit assembly is blocked at an early step (Kawakami, K. and Ishihama, A. (1980) Biochemistry 19, 3491-3495). Through sequence analysis of PCR-amplified DNA, the rpoA112 mutation was found to cause a substitution of Cys for Arg at amino acid residue 45, indicating that the N-terminal region of α plays an important role in subunit assembly (Igarashi, K. et al. (1990) Nucleic Acids Res. 18, 5945-5948).

To test this possibility, we constructed a set of C-terminal deletion mutations of the rpoA gene. C-Terminal truncated α subunits 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-core complexes, while those truncated at residues 176 or 150 did not form such complexes (Igarashi, K., Fujita, N. and Ishihama, A. (1991) J. Mol. Biol.* On leave of absence from Institute of Cell and Molecular Biology, University of Edinburgh, Edinburgh, UK.
Studies of *E. coli* strains producing the same set of truncated α subunits showed that the α-296, α-256 and α-235 species can even form pseudo-holoenzymes *in vivo* (Hayward, R., Igarashi, K. and Ishihama, A. (1991) *J. Mol. Biol.*, in press). However, both *rpoA*101 (Arg-to-Cys substitution mutation at position 191) and *rpoA*112 could not be complemented by the expression of any one of these truncated α subunits, implying that the C-terminal region plays a yet unidentified essential role in transcription.

We then overproduced the C-terminal deletion derivatives lacking 73 or 94 amino acid residues, assembled *in vitro* into enzyme molecules, and tested for biological functions associated with the reconstituted enzymes. To our surprise, the reconstituted core enzymes were as active in poly (A)·poly (dT)-dependent poly(A) synthesis as the native core enzyme. Furthermore, with the addition of σ70, these core enzymes were reconstituted into holoenzymes, which exhibited transcription activity at the lacUV5, *trp*, and *rplJ* promoters as well as the native holoenzyme. The mutant holoenzymes were, however, unable to catalyze cAMP-CRP activated transcription from the *lac* and *uxuAB* promoters (Igarashi, K. and Ishihama, A. (1991) *Cell* 65, 1015–1022). These results demonstrate that the C-terminal region of the α subunit plays an essential role in recognition of the promoters which require cAMP-CRP. The simplest interpretation would be that the C-terminal region of the α subunit provides a contact site for CRP, which binds at a site upstream of the promoter −35 signal on the promoters examined.

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**The Promoter Selectivity of *Escherichia coli* RNA Polymerase, II. Comparison of Three Assay Systems of 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 holoenzyme (Eσ70) are composed of two conserved hexanucleotide sequences, TTGACA and TATAAT, which are located at 35 and 10 bp (base pairs), respectively, upstream of the transcription initiation site. Previously, we developed the *in vitro* mixed transcription assay system to determine two parameters governing promoter strength, *i.e.*, binding affinity to RNA polymerase (parameter I) and rate of open complex formation (parameter
II) (for a review see Ishihama, A. (1988) Trends Genet. 4, 282–288). To reveal the role of individual bases within these two regions, we constructed a set of single base substituted variant promoters derived from the lacUV5 promoter, and determined their promoter strengths using the mixed transcription system (Kobayashi, M. et al. (1990) Nucleic Acids Res. 18, 7367–7372).

Since the abortive initiation assay is widely used for measurement of promoter strength, we also compared these two in vitro assay systems of promoter strength using a set of variant promoters with all possible substitutions within the promoter −35 region. No significant difference was observed in the promoter strengths determined by the two independent methods.

Next, we attempted to measure promoter strength in vivo. For this purpose, we inserted DNA fragments carrying the variant promoters into plasmid pMS4342 at a site between the ara promoter and the lacZ gene. Promoter strength in vivo was determined by measuring β-galactosidase activity in the absence of arabinose. The results indicate that: i) the promoter containing the consensus sequence TTGACA at the −35 region was the strongest in both in vitro and in vivo assays; and ii) promoter strengths measured in vivo were generally in good agreement with those measured in vitro, especially with the values of parameter I. Thus, at least for the promoters examined, the strength in vivo appears to correlate with binding affinity to RNA polymerase.

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

Miwako Ozaki, Nobuyuki Fujita, Akira Wada* and Akira Ishihama

In exponentially growing cells of E. coli, the synthesis of core enzyme subunits, α, β and β', of RNA polymerase is coordinated and under growth rate-dependent control (for reviews see Ishihama and Fukuda (1980) Mol. Cell. Biochem. 31, 489–496; Ishihama (1981) Adv. Biophys. 14, 1–35). This coordination is, however, disrupted in stationary-phase cells and, as a result, the newly synthesized subunits are degraded without being assembled,
even though RNA polymerase is one of the metabolically stable proteins in exponentially growing cells. Last year, we reported that, during growth transition of *E. coli* culture from exponential growing to stationary phase, the pre-existing RNA polymerase is modified into at least three different forms, which can be separated by phosphocellulose column chromatography (for details see Ozaki, M. *et al.* (1991) *Mol. Gen. Genet.*, in press).

In order to determine the altered functions associated with the stationary-phase RNA polymerases, we examined the promoter selectivity for these modified RNA polymerases using the *in vitro* mixed transcription system and our collection of natural and synthetic *E. coli* promoters. Results indicated that each of the three RNA polymerases carries different promoter selectivity from that of the regular holoenzyme (*Eσ* 70) from exponentially growing cells.

As an attempt to identify the structural basis of the observed functional modulation, we carried out holoenzyme reconstitution experiments from the core enzyme and *σ* 70 subunit, which were isolated from each enzyme form. The hybrid enzymes from the exponential phase *σ* 70 and the stationary-phase core enzyme showed essentially the same promoter recognition properties as those of the stationary-phase holoenzymes. This indicates that the core enzyme is modified during growth transition from exponential to stationary phase. Modification of RNA polymerase might be a major mechanism for the global control of gene transcription at least during growth phase transition.

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

Manabu Nakayama, Nobuyuki Fujita, Syozo OsaWA* and Akira Ishihama

The promoters of *Micrococcus luteus*, a bacterium whose chromosomal DNA has a high G+C content (74%), diverge from the consensus pro-okaryotic 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 and examined their promoter recognition properties in the *in vitro* mixed tran-
scription system using promoter collections from both organisms.

The sets of *E. coli* and *M. luteus* promoters recognized by two purified RNA polymerases were found to overlap partly. Some *E. coli* promoters were correctly transcribed by the *M. luteus* RNA polymerase as well as the *E. coli* enzyme. To identify the structural unit involved in promoter recognition, we purified the *M. luteus* σ subunit with a molecular weight of about 60 kDa (Nakayama, M. et al. (1991) *J. Biol. Chem.* **266**, 2911–2916). With the addition of either *M. luteus* or *E. coli* core enzyme, it was reconstituted into holoenzymes. Likewise, the *M. luteus* core enzyme was reconstituted into a hybrid holoenzyme through the addition of the *E. coli* σ\(^{50}\) subunit. Both hybrid holoenzymes were, however, able to initiate transcription only from a set of promoters which were recognized by both of the native holoenzymes. These observations indicate that not only the σ subunit but also the core enzyme participates in promoter selection.

In addition to the σ\(^{60}\) subunit, we found a minor species of *M. luteus* σ subunit which differs from the major species in both molecular size and promoter selectivity.

**Cloning and Sequence Determination of the *Schizosaccharomyces pombe* rpb1 Gene Encoding the Largest Subunit of RNA Polymerase II**

Yoshinao Azuma, Masahiro Yamagishi, Rei Ueshima and Akira Ishihama

Extensive studies are being carried out on cis-acting DNA signals and trans-acting protein factors involved in transcription regulation in eukaryotes. By contrast, little is known on the basic machinery of RNA synthesis, except that three types of nuclear RNA polymerases, I, II, and III, exist, each being composed of more than 10 different subunits. In order to get detailed knowledge of the structure and function of RNA polymerases, we started cloning and DNA sequencing of the *Schizosaccharomyces pombe* genes encoding the subunits of RNA polymerase II. This year, we cloned both the genomic DNA and cDNA coding for the largest subunit using the corresponding gene, RPB1, of *Saccharomyces cerevisiae* as a cross-hybridization probe, and determined the complete sequence of genomic DNA and some of the PCR-amplified cDNA (Azuma, Y. et al. (1991) *Nucleic Acids Res.* **19**, 461–468).

The predicted protein coding sequence, interrupted by six introns in the
genomic clone, encodes a polypeptide of 1,752 amino acid residues in length with the molecular weight of 194 kDa. The polypeptide contains the eight structural domains conserved among the largest subunits of RNA polymerases from other eukaryotes. Both the metal binding motif in the N-terminal proximal first domain and the DNA binding motif in the third domain exist in the *S. pombe* protein. The C-terminal domain (CTD) is composed of 29 repetitions of the heptapeptide motif, Tyr-Ser-Pro-Thr-Ser-Pro-Ser.

Northern analysis of RNA from exponentially growing cells showed that the size of the *rpb1* transcript is about 5.6 kb in length. The sites of transcription initiation and polyadenylation were determined by primer extension experiments and cDNA sequencing. In a gene disruption experiment and Southern analyses of total *S. pombe* DNA, the *rpb1* gene was identified as an essential gene consisting of a single copy on the *S. pombe* chromosome.

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**The Promoter Selectivity of Influenza Virus RNA Polymerase: in vitro and in vivo Analysis of the Promoter Structure**

Kunitoshi YAMANAKA*, Kyosuke NAGATA and Akira ISHIHAMA

The genome of influenza virus consists of eight single-stranded RNA segments of negative polarity. Both 5'- and 3'-terminal sequences of 12–13 nucleotides in length, which are complementary to each other, are conserved among the eight segments and various virus strains. In virus particles, RNA polymerase (a complex of three P proteins, PB1, PB2 and PA) binds to each RNA segment at the stem of a panhandle-like structure (Honda, A. *et al.* (1987) *J. Biochem.* **102**, 1241-1249), while the nucleoprotein (NP) binds to viral RNA every 15–20 nucleotides (Yamanaka, M. *et al.* (1990) *J. Biol. Chem.* **256**, 11151-11155), altogether forming ribonucleoprotein (RNP) cores. In agreement with this structural model, transcription *in vitro* catalyzed by RNP cores is initiated from the 3' terminus of each RNA segment (Honda, A. *et al.* (1986) *J. Biol. Chem.* **261**, 5987–5991). These observations suggested that the promoter for transcription includes the 3'-terminal conserved sequence of viral RNA.

Previous *in vitro* studies using isolated RNA polymerase and synthetic

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model RNA templates showed that the 3' terminal conserved sequence is indeed needed for promoter activity (Parvin, J. et al. (1989) J. Virol. 63, 5142-5152). Mutations in this region more or less result in the reduction of promoter activity in vitro.

To examine the structure needed for in vivo promoter function, we established a transfection system of reconstituted RNP cores. For this purpose, we first made cDNA for segment 8 RNA, replaced the coding region for the nonstructural protein (NS) with the chloramphenicol acetyltransferase (CAT) gene, and inserted the resulting recombinant gene at a site downstream from the promoter for T7 RNA polymerase. The antisense CAT RNA was synthesized in vitro by transcribing this DNA with T7 RNA polymerase, and reconstituted into model RNP cores by mixing with viral proteins, RNA polymerase and NP, which were isolated from purified RNP cores by CsCl centrifugation. Transfection of HeLa cells with this reconstituted CAT RNP in the presence of the helper ribonucleoprotein cores led to significant expression of the recombinant CAT gene as detected by measuring plus-strand CAT mRNA using a combination of reverse transcription and RCR, and also by immunostaining of the CAT protein using monospecific antibodies (Yamanaka, K. et al. (1991) Proc. Natl. Acad. Sci. USA, in press). This transfection system was used for analysis of the promoter structure for influenza viral RNA polymerase.

**Mechanism of Growth Inhibition of Influenza Virus by the Mouse Mx Gene**

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

The inbred mouse strain A2G is resistant to infection by 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 of the Mx protein, however, remains known.

In order to reveal the action mechanism of the Mx protein, we examined the effect of Mx protein, induced in primary cultures of A2G mouse embryos

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by interferon treatment, on each step of the growth cycle of influenza virus. The absorption of viruses, the uncoating of envelope and membrane, and the transport of viral RNA-protein cores from cytoplasm to nuclei all were found to take place in the presence of the Mx protein as well as in its absence. In contrast, transcription of viral genes, translation of viral mRNAs, and the production of infectious viruses were reduced by more than 30 fold. These results indicate that the primary inhibition site for the interferon-induced Mx protein is at the step of viral mRNA synthesis.

To examine in detail the mechanism of the Mx protein action, we purified the Mx protein by successive chromatography on DEAE-cellulose, phosphocellulose, protein-pak 300 and SP-TOYOPEARL columns from both cell extract from *Escherichia coli* expressing the Mx cDNA, and liver extract from A2G mouse previously treated with poly(I)·poly(C) for interferon induction. The purified Mx protein showed GTP-binding activity with GTPase activity. A mutation in the nucleotide binding motif reduced the activities of both GTP binding and GTPase.

Expression of Human Thymidylate Synthase Minigenes:
Enhancer-like Activity in Intron 1

Sumiko Kaneda, Nobuyuki Horie, Keiichi Takeishi, Dai Ayusawa
and Takeshi Seno

Thymidylate synthase (TS) is a housekeeping enzyme responsible for the formation of thymidylic acid in the de novo pathway. The expression of the TS gene is cell-cycle regulated. The human TS gene is about 16 kilo bases in length and composed of seven exons and six introns. In order to locate the essential sequence responsible for normal expression of the gene, we constructed a series of minigenes, consisting of about 4 kilo bases of the 5'-flanking sequence, 1.7 kilo bases of the 3'-flanking sequence, and all the exons with or without the intron at normal positions. The activities of minigenes were determined by transfecting each of them into TS-negative thymidine auxotrophic mouse cell mutant FSthy21 and measuring the frequency of TS-positive stable transformants.

The minigene pmHTS1, which has intron 1 showed a high transforming activity compared to that of pcHTS1, a human TS cDNA containing the SV40 promoter region. However, the minigene pmHTS0 which lacks all the introns or pmHTS2 which has only intron 2 showed activity one-tenth
and one-hundredth the level of that of pmHTS1, respectively. These results suggested that intron 1 contains an enhancer-like sequence. The enhancer activity was located in the 5’ half of intron 1 and was active regardless of their position and direction. Notably, this enhancer has promoter specificity. The CAT activity was stimulated by the construct with the 5’ half of intron 1 and 5’ flanking sequence of the TS gene, whereas the activity was not stimulated with the SV40 promoter. This observation indicates that the 5’ half of intron 1 and 5’ promoter region stimulate the transcription of TS gene in a cooperative manner.

Cell-Cycle Directed Regulation of Human Thymidylate Synthase Gene

Atsushi Takayanagi, Sumiko Kaneda, Dai Ayusawa and Takeshi Seno

In order to locate the regulatory sequences responsible for cell-cycle dependent expression of the human thymidylate synthase (TS) gene, we constructed a series of minigenes lacking some or all introns, and related chimeric genes partially replaced by corresponding SV40 promoter sequences or 3’ regulatory sequences in the Okayama-Berg expression vector. The constructs were introduced into a TS-negative mutant of rat 3Y1 cells to establish stable transformant lines.

Each transformant line was synchronized in the G0/G1 phase by serum starvation, and then stimulated to traverse into the S phase by the addition of serum. The level of TS mRNAs was compared between cells in G0/G1 and those in S phases by Northern hybridization analysis with a fragment of human TS cDNA as a probe. The results showed that both genomic 5’-flanking sequences and intron 1 were responsible for the S phase-specific increase of the mRNA. Each of the sequence alone was able to stimulate the expression of the TS gene at S phase but to a lesser extent. The rate of transcription of each transformant line was almost the same for the two phases as measured by a nuclear run-off experiment. Therefore, the cell-cycle dependent expression of the TS gene is regulated posttranscriptionally.
Mechanism of DNA Degradation during Thymidylate Stress in Mammalian Cells

Fumiaki Yamao, Nobuyuki Imai and Takeshi Seno

Thymidylate stress is known to cause rapid cell death, so called thymineless death. In mammalian cells chromosomal DNA degradation occurs during this stress, resulting in DNA fragments ranging from 50 to 200 kb in length. Previous studies suggest that the DNA cleavage is coupled with DNA replication. In order to investigate the molecular basis of the DNA degradation, temperature-sensitive growth mutants of mouse mammary carcinoma cell line FM3A were isolated by selecting thymineless death resistsants at high temperature (39.5°C). Among the ts mutants we screened those whose chromosomal DNA is not degradable under thymidylate stress even at a permissive temperature (33.5°C). Consequently, four mutants were established and classified into two complementing groups by cell fusion analysis. The mutated gene in one complementation group was identified as that for ubiquitin-activating enzyme E1 since mutants were not complemented with the authentic E1 mutant. Previous analysis of E1 mutants of mouse cell lines suggested that ubiquitination is somehow involved in DNA synthesis and cell cycle regulation of the S/G2 phase. Thus, it could be supposed that dynamic regulation of the chromatin structure during DNA replication is coupled with ubiquitination of some nuclear protein(s) which may be affiliated with chromatin structure of replication machinery. The chromatin associated with the ubiquitinated protein(s) would be sensitive to nucleolytic activities during its replication under thymidylate stress, resulting in the instability of the chromosomal DNA.

Ubiquitin-Activating Enzyme as a Determinant of Cell-Cycle Progression

Dai Ayusawa, Sumiko Kaneda and Takeshi Seno

Ubiquitin is a conserved, small protein found in all eukaryotes. The covalent ligation of ubiquitin to various cellular target proteins is implicated in various cellular functions. Ubiquitin-activating enzyme E1 catalyzes the first step of sequential transfer of ubiquitin to target proteins. However, it is unclear how each target protein is involved in a particular biological function, especially in terms of cell-cycle progression, due in part
to lack of genetic analysis.

Human ubiquitin-activating enzyme E1 was cloned by genetic complementation of a temperature-sensitive (ts) mouse FM3A cell mutant FS20 which has a thermolabile E1 enzyme, is defective in DNA synthesis, and arrested in the S phase upon shift-up in temperature. The cloned E1 full-length cDNA (3,428bp) in a mammalian expression vector was able to transform FS20 to non-ts cells with a frequency of $10^{-3}$ to $10^{-4}$ per surviving cell. The determined nucleotide sequence of the cDNA encodes a putative protein of 108kDa, the amino acid sequence of which was confirmed in part by direct sequencing of the peptide fragments obtained from the purified human E1 protein. With cell-cell hybridization, FS20 did not complement with the ts85 mutant which has been characterized as a G2-specific cell-cycle mutant and to have the thermolabile E1 enzyme. Thus, enzyme E1 appears to play a multiple role in cell-cycle regulation.

**DNA Supercoiling and Eukaryotic Transcription—Cause and Effect**

Susumu HIROSE and Tsutomu OHTA

Both underwinding and overwinding of the DNA double helix induce twisting and coiling of the helix unless the DNA strands can rotate freely. The coils thus formed are termed negative and positive supercoils, respectively. An enzyme that changes the topological form of DNA is called topoisomerase. There are two types of topoisomerase in eukaryotes: DNA topoisomerase I and II. Both of these enzymes can relax negative and positive supercoils but neither can introduce supercoils into relaxed DNA.

Though chromatin DNA in eukaryotic cells is topologically underwound, bulk DNA is not under superhelical tension because the supercoils are constrained in histone-DNA interactions. This does not necessarily exclude the possibility that a small fraction of the chromatin has unconstrained supercoils. Recent progress in the field of DNA topology highlights a linkage between unconstrained supercoils and eukaryotic transcription: supercoiling affects transcription and transcription can cause supercoiling. For details, see *Cell Struct. Funct.* 15, 133–135, 1990.
Negative Supercoiling of DNA with Eukaryotic DNA Topoisomerase II and Dextran Sulfate

Kohichi OKADA, Tsutomu OHTA and Susumu HIROSE

In the presence of a molar excess of eukaryotic DNA topoisomerase II and an appropriate concentration of dextran sulfate, relaxed closed circular DNA is converted to a negatively supercoiled form. The reaction is dependent on ATP. Neither adenosine 5'-[β,γ-imido]triphosphate nor adenosine 5'-[γ-thio] triphosphate can substitute for ATP. The negative supercoils formed are relaxed with the subsequent addition of DNA topoisomerase I to the supercoiling reaction mixture. Covalent closure of a nicked circular DNA in the presence of DNA topoisomerase II and dextran sulfate but in the absence of ATP, causes a small decrease in the linking number. These results suggest that when appropriate concentrations of dextran sulfate are present, the binding of a molar excess of eukaryotic DNA topoisomerase II constrains a small number of negative supercoils in DNA, which in turn generates unconstrained negative supercoils at the expense of ATP. For details, see J. Biochem. 109, 365-369, 1991.

Core Promoter of the Mouse Myelin Basic Protein Gene Governs Brain-Specific Transcription in Vitro

Taka-aki TAMURA*, Kohsuke SUMITA*, Susumu HIROSE and Katsuhiko MIKOSHIBA*

The core promoter of the mouse myelin basic protein (MBP) gene from -36 to +12 was preferentially transcribed in brain nuclear extracts. Both the TATA at -34 and downstream elements to +12, were required for efficient, accurate and brain-specific transcription. From brain and liver nuclear extracts, we have partially purified the general transcription factor TFIID. The partially purified fractions contained TATA element binding factors of the MBP promoter as well as adenovirus major late promoter (MLP). The tissue-derived TFIID was functionally exchangeable for the HeLa TFIID, and directed transcription from the MLP. Surprisingly, brain THIID activated transcription from the MBP core promoter while liver TFIID did so to a much lesser extent. Exchange of the TATA-containing short DNA stretch to the MBP core promoter for a corresponding

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region of the mouse albumin promoter or MLP abolished the brain specificity. We found that several tissue-specific promoters other than MBP, such as mouse neurofilament and human α-1-antitrypsin promoters were also transcribed much more efficiently with brain and liver TFIID, respectively. We suggest that different tissues contain functionally non-equivalent TFIID or TFIID-like activities. For details, see *EMBO J.* 9, 3101–3108, 1990.

**Identification and Purification of a *Bombyx mori* Homologue of FTZ-F1**

Hitoshi Ueda and Susumu Hirose

Extracts from embryos and from the posterior silk glands of the silkworm, *Bombyx mori* contain a sequence specific DNA binding factor termed BmFTZ-F1. The factor binds to the recognition site of FTZ-F1, a positive regulator of the *fushi tarazu* gene in *Drosophila melanogaster* (Ueda, H., *et al.*, 1990, *Genes Dev.* 4, 624–635). BmFTZ-F1 and FTZ-F1 share the same methylation interference patterns, the same chromatographic behaviors and similar protease digestion profiles. Anti-FTZ-F1 cross-reacts with BmFTZ-F1. These results indicate that BmFTZ-F1 is a *B. mori* homologue of FTZ-F1. The mobility of the factor-DNA complex formed in the silk gland extract changes depending on developmental stages. Purification of BmFTZ-F1 to an almost homogeneous state reveals that the factor is 73 kd protein. For details, see *Nucleic Acids Res.* 18, 7229–7234, 1990.

**Search for Target Genes of *Drosophila* Homeotic Genes**

Shigeo Hayashi and Susumu Hirose

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

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

Shigeo Hayashi, Allan Shirras and Susumu Hirose

One of the enhancer trap lines #2605 has studied in detail. β-galactosidase is expressed in stripes in every segment during mid embryogenesis and later in the anterior spiracle. A lethal gene was identified which is closely associated with the P-element insert and was mapped cytologically to polyten band 35C~D. Loss of function mutations affect segmentation of the larva and the adult abdomen. We are currently cloning the gene named fleabug (flg) in collaboration with Dr. Allan Shirras at Lancaster University. So far, ~60 kb of genomic DNA flanking the P-element insert has been cloned. Mapping of the flg mutation and flg transcript are in progress.

**Identification and Characterization of a Gene strawberry which is Required for Organization of Sensory Organs**

Shigeo Hayashi, Jiro Watanabe and Susumu Hirose

We have identified a new lethal gene strawberry located in the left arm of the third chromosome. A loss of function mutation of the gene affects the assembly of cells composing the larval sense organ in the antenno-maxillary complex and the adult compound eye. In the larva, duplication and loss of the sense organ is observed. In the adult, fusion of several ommatidias in the compound eye occurred. Presumably the gene controls proper cell division and differentiation of cells in external sense organs. About 17 kb genomic DNA flanking the gene has been cloned by virtue of a P-element plasmid rescue. We are currently cloning the cDNA of the gene.
Demonstration of Extrinsic DNA from Immune Complexes in the Plasma of a Patient with Systemic Lupus Erythematosus

Kunihiko Terada*, Eiji Okuhara*, Yoshihiko Kawarada* and Susumu Hirose

Antigen DNA isolated from immune complexes, present in the plasma of three patients with active systemic lupus erythematosus, was cloned and sequenced. One clone, designated pKS7, was found to have a region homologous with that of the \textit{E. coli} metK gene, and another, designated pKS8, had a region homologous with a sequence including the replication origin of bacteriophage \textit{f1}. A gel retardation assay revealed that pKS7 and pKS8 interacted with the patient's IgG fraction to form immune complexes. The affinity-purified antigen DNA proved to originate from bacteria or a bacteriophage. For details, see \textit{Biochem. Biophys. Res. Commun.} 174, 323–330, 1991.

\textit{In vitro} Recombination System Using Nuclear Extract from Mouse Testis cells

Tsuyoshi Koide, Tomoko Sagai, Toshihiko Shiroishi and Kazuo Moriwaki

Homologous recombination within the mouse MHC region during meiosis is not random, but occurs preferentially at restricted sites, so-called recombinational hotspots. So far, four different hotspots have been identified in this region. The molecular mechanism responsible for the high frequency of recombination at the hotspots is not clear. Further study of site-specific recombination at the molecular level requires an efficient assay system. If an \textit{in vitro} assay system is established, it can be used to search for protein factors which may interact with the hotspots and to elucidate target DNA sequence. Thus, we are attempting to develop an \textit{in vitro} recombination system. To evaluate the recombination frequency, we constructed plasmids, termed pBPPlacRB, which contain an indicator gene, \textit{lacZ}, located between two identical DNA fragments harboring the recombinational hotspot identified between the Ab3 and Ab2 genes in the MHC. A similar plasmid, named pBPPlacCB, containing two different DNA fragments, one harboring the hotspot and the other irrelevant to the hotspot

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sequence, was also prepared as a control construct. We used nuclear extracts from mouse testis cells to evaluate the homologous recombination frequency between two DNA fragments in vitro. After incubation with the nuclear extracts, the plasmids were used for transformation of E. coli deficient in lacZ activity. Plasmids with recombination between two DNA fragments loop out the indicator lacZ gene. The recombinant and non-recombinant plasmids can be easily scored on the basis of color of colonies. As a result, approximately 10% of colonies transformed by pBPlacRB plasmids were scored as lacZ negative. More than 90% of them were revealed to be recombinants between two homologous DNA fragments. In contrast, the control plasmid construct, pBPlacCB, exhibited a very low frequency of lacZ negative colonies. Only 0.3% of colonies were scored as lacZ-. These results indicated that this assay system is efficient for evaluating homologous recombination frequency in vitro. At present, we are preparing other plasmid constructs in order to test the feasibility of using this system to reproduce the site-specificity of recombination is observed in in vivo within the mouse MHC region.

Gene Expression at the wx Locus of Rice (Oryza Sativa)
Hiro-Yuki Hirano and Yoshio Sano

The wx locus controls amylose synthesis in rice endosperm. The amylose content in total starch greatly affects the quality of rice. Mutants at the wx locus are easily screened by seed phenotype and the genotype of the locus is determined by the I2/KI staining of the pollens. Thus the gene at the wx locus is one of the best genes for molecular genetic research among the genes of higher plants.

We cloned the wx+ gene from rice and determined the entire sequence of the coding region. The deduced amino acid sequence indicated a putative transit peptide of 77 amino acids and a mature protein region of 532 amino acids. The putative transit peptide, which may be essential for transporting the product of the wx+ gene (wx+ protein) into the amyloplast, has some characteristic features similar to those of the transit peptide associated with proteins to be transported into the chloroplast.

An antiserum against the rice wx+ protein (anti-WXP serum) was prepared and the expression of the wx locus was examined using DNA cloned from the wx locus and the anti-WXP serum. The wx+ gene was expressed spe-
specifically in only the endosperm and pollen, although the amount of the \( wx^+ \) protein in pollen was about 2% of that in the endosperm. The \( wx^+ \) gene was active at the early stage and middle stages but almost inactive at the late stage of seed development. During active transcription of the gene, the \( wx^+ \) protein and total starch appear to accumulate linearly. The level of the \( wx^+ \) protein in plants grown at semi-low temperatures (18°C) during seed maturation was higher than that of plants grown at normal temperatures (28°C). The endosperm grown at 18°C contained an elevated level of amylose content. This suggests that the low quality of rice grown at low temperature in the northern area of Japan is due to a higher amylose content, which is caused by the enhancement of \( wx^+ \) gene expression.

Cloning and Characterization of Restriction Fragments Homologous to B-1 Sequences in Cytoplasmic Male Sterile Rice

Masa-Aki Yamada

Cytoplasmic male sterility (\( cms \)) has been reported in various higher plants. Two supercoiled circular DNAs, B-1 and B-2, were found in the mitochondria of a cytoplasmic male sterile strain in rice, and cloned into plasmid pUC 12. Using these as probes, it was previously reported that hybridization with restriction fragments revealed the presence of various sequences homologous to B-1 and B-2 in mitochondrial DNA and in nuclear DNA, both in the \( cms \) strain and in the normal strain (Nawa et al., 1987).

DNA fragments of length 5.1 kb homologous to B-1, produced by digestion with \( 
EcoRI \) and \( Hind \) III of the mitochondrial DNA from the \( cms \) strain, were respectively cloned into plasmid pUC 13. Digestion by \( Hinc \) II of the 5.1 kb \( EcoRI \) fragment (EM5–113) produced two fragments, of length 1.6 kb homologous to B-1 and of length 3.5 kb fragment (EM5–113–1) not homologous to B-1. Digestion by \( EcoRI \) of the 5.1 kb \( Hind \) III fragment (HM5–32) produced two fragments, of length 0.4 kb homologous to B-1 and of length 4.7 kb not homologous to it.

To test for contaminations of nuclear DNA into mitochondrial DNA during the preparations of the mitochondrial fraction, digests with \( EcoRI \) of DNAs from the nuclear fraction and the mitochondrial fraction of the \( cms \) strain were analyzed by Southern hybridization with EM5–113–1 fragments, which were not homologous to B-1. Sequences homologous to EM5–113–1 fragments were present in the digests with the \( EcoRI \) of both nuclear
DNA and mitochondrial DNA, but in the nuclear DNA were present at lower levels. In the digests with EcoRI of DNAs from the nuclear fraction and the mitochondrial fraction of callus cells, additionally cultured in shaking culture for 2 months, sequences homologous to EM5-113-1 were present in both DNAs, but at lower levels in the nuclear DNA, while in the case of sequences homologous to B-1, a weakly labeled band was detected in nuclear DNA but undetectable in mitochondrial DNA.

These results suggested that the EM5-113 fragments detected in the mitochondrial DNA were not contaminations of the nuclear DNA, and the sequences homologous to B-1 in the mitochondrial genome DNA varied in the state of callus cells cultured.
Upon infection, the single-stranded DNA of bacteriophage f1 is converted to double-stranded replicative form (RF) through synthesis of the minus strand, which is carried out by the combined action of RNA polymerase and DNA polymerase III of the host. The (−) strand origin has been defined as the region of single-stranded DNA that is protected by RNA polymerase against nuclease digestion in the presence of *E. coli* single strand binding protein (SSB). The protected region contains two hairpin structures [B] and [C] (Gray et al. (1978) *Proc. Natl. Acad. Sci. USA.* 75, 50-53). An RNA primer is synthesized from a site downstream (in terms of the template DNA strand) of the hairpin [C] by RNA polymerase (Geider et al. (1978) *Proc. Natl. Acad. Sci. USA.* 75, 645-649). However, this

![Fig. 1. Mutations of the minus strand origin.](image-url)
Table 1. Activities of mutant origins

<table>
<thead>
<tr>
<th></th>
<th>in vivo</th>
<th>in vitro</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Conversion to RF</td>
<td>(−) ori plasmid replication</td>
</tr>
<tr>
<td>wild-type</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>R368</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>R377</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>R410</td>
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<td>R411</td>
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<td>R420</td>
<td>−</td>
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<tr>
<td>R421</td>
<td>+</td>
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</tr>
</tbody>
</table>

region does not contain any sequences similar to the consensus sequence for transcriptional promoters.

In order to determine which sequence(s) is essential for the origin function, we constructed a number of deletion and insertion mutants in hairpins [B] and [C] (Fig. 1). The mutants were examined for activity of the origin. We used three assays: (1) rate of conversion of 32P-labeled phage DNA to double-stranded RF upon infection, (2) novel in vivo system of plasmid replication that is dependent on the function of the phage (−) strand origin, and (3) an in vitro reaction of primer synthesis, which consists of viral single-stranded DNA, E. coli RNA polymerase and E. coli SSB.

The results of all three assays were consistent with each other (Table 1), and suggested that [1] deletion of regions in the 3' end of hairpin [C], which contain the initiation point of the primer RNA, does not seriously affect the (−) strand origin function, [2] the entire region of hairpin [B] is essential, and [3] the distance between hairpins [B] and [C] is important.

We thank Drs. A. Ishihama, K. Igarashi, N. Shimamoto, and K. Takahashi (National Institute of Genetics) for providing us with purified RNA polymerase and SSB.
SOS Induction in *E. coli* by Infection with Mutant Filamentous Phages that are Defective in Complementary Strand Synthesis

Nahoko Higashitani, Atsushi Higashitani and Kensuke Horiuchi

SOS responses in *E. coli* are controlled by a complex circuitry involving the RecA and LexA proteins. When DNA in a cell is damaged, an inducing signal is generated, which activates the RecA protein. The activated RecA protein causes proteolytic cleavage of the LexA protein, and this leads to derepression of the SOS genes, which are repressed by the LexA protein. *In vitro* studies have revealed that the RecA protein is activated when it forms a ternary complex with single-stranded DNA and ATP (Craig, N. L. *et al.* (1980) *Nature* **283**, 26-30). *In vivo*, however, it has remained unclear what constitutes the inducing signal.

The f1 mutant phage R377 lacks the entire region of the minus strand origin, and thus is defective in complementary strand synthesis (see the accompanying report). We found that *E. coli* cells infected with R377 formed filaments, while those infected with wild-type f1 did not. It is known that *E. coli* cells exposed to SOS-inducing treatment continue to divide, but are inhibited from septating, and thus form filaments (Gottesman, S. *et al.* (1981) *J. Bacteriol.* **148**, 265-273). Whether R377 infection causes the SOS induction was studied using an *E. coli* test strain that carried a lacZ gene under the control of a promoter of an SOS-responsive gene (dinD). The activity of β-galactosidase in this strain increased by infection with R377, but not with the wild-type f1 phage. To further confirm this point we measured the level of RecA protein in extracts of phage-infected cells using the anti-RecA antibody. The results of Western blotting experiments showed that R377, but not the wild-type f1, induced synthesis of the 40 kDa RecA protein. The activated RecA mediates proteolytic cleavage of the lambda repressor, which in turn induces growth of lambda increased by infection of lambda lysogens by R377.

Another (-) ori deletion mutant, M13ΔE101, has been described by Kim *et al.* (1981) *Proc. Natl. Acad. Sci. USA.* **78**, 6768-6788). They have also constructed M13Gori101, in which the (-) strand origin of G4, another single-stranded DNA phage, was inserted into M13ΔE101. We found that infection with M13ΔE101 results in the SOS induction in *E. coli*, while infection with M13Gori101 did not.

From these results we conclude that infection of *E. coli* cells with mutant
filamentous phages which are defective in the origin of minus strand synthesis induces the SOS functions. This observation strongly suggests that single-stranded DNA by itself induces the SOS response in vivo.

Replication of Filamentous Phage f1: Relationship between DNA Topology and the Nicking Activity of the Initiator Protein (gpII)

Atushi Higashitani and Kensuke Horiuchi

Gene II protein (gpII), which is coded for by the filamentous phage f1 genome, is a multifunctional protein that participates in DNA replication at a number of levels. It introduces a single-strand break at a specific site on the plus strand of negatively supercoiled replicative-form (RF-I) (Meyer et al. (1979) Nature 278, 365-367). The 3'hydroxyl end of the nick serves as the primer for initiation of plus strand rolling circle replication (Gilbert & Dressler (1968) C.S.H. Symp. Quant. Biol. 32, 473-484). gpII functions also at a step beyond nicking; unwinding of origin DNA at the initiation step (Geider et al. (1982) J. Biol. Chem. 257, 6488-6493), and cleavage and circularization of the displaced single strand at the termination step (Harth et al. (1981) Eur. J. Biochem. 119, 663-668). When incubated with gpII in vitro, approximately 60% of RF I molecules are nicked to yield RF II, while the other 40% are converted to a relaxed, closed form (RF IV) as the result of nicking and joining (Meyer & Geider (1979) J. Biol. Chem. 254, 12642-12646). Thus, gpII has a sequence-specific topoisomerase activity.

In vivo studies have shown that E. coli IHF (integration host factor) stimulates filamentous phage DNA replication approximately 30-fold through its action on the replication enhancer sequence (Dotto et al. (1984) J. Mol. Biol. 172, 507-521; Johnston & Ray (1984) J. Mol. Biol. 177, 685-700; Greenstein et al. (1988) Proc. Natl. Acad. Sci. USA. 85, 6262-6266). Several enhancer independent mutants of gpII have been isolated, which allow wild-type levels of replication in the absence of either the enhancer sequence or IHF (Dotto & Zinder (1984) Nature 311, 279-280; Kim & Ray (1985) J. Virol. 53, 871-878). Two of these mutants were mapped at codon 40 and 73, respectively. Both M40I (Met40→Ile) and G73A (Gly73→Ala) mutations increase the co-operativity with which the protein binds to the origin to form a functional complex for the nicking reaction (complex II: Greenstein & Horiuchi (1990) J. Mol. Biol. 221, 91-101; Higashitani

In this study, in order to determine the level of negative-supercoil of RF I required for the nicking reaction, a series of RF-topoisomers with different negative-superhelical densities was prepared and used as substrates. The results shown in Fig. 1 demonstrate that the in vitro nicking reaction by the wild-type gpII requires a high level of RF I superhelicity, while an enhancer-independent mutant version, G73A, does not require such a high level of supercoiling. When substrate DNA has a negative-superhelical density of 0.025, which corresponds to the value estimated for the in vivo superhelical density in E. coli (Bliska & Cozzarelli (1987) J. Mol. Biol. 194, 205–218), the wild-type gpII converted about 35% of the substrate DNA to RF II and 65% to RF IV. The G73A mutant gpII converted this substrate to a mixture of 80% RF II and 20% RFIV under the same experimental conditions (Fig. 1). When the substrate consisted of completely relaxed circles, G73A gpII still converted about 35% of the molecules to RF II. The wild-type gpII converted only 8% of this substrate to RF II (Fig. 1). Furthermore, the G73A gpII showed an approximately 5-fold greater nicking activity compared to the wild-type gpII on a linear DNA fragment containing the origin.

The nicking activity of gpII on purified f1 DNA was not affected by the addition of IHF, regardless of the superhelical density of the substrate. How do the IHF protein and the enhancer sequence enhance the replication of f1 DNA in vivo? One possibility is that IHF may reduce the requirement

![Fig. 1. Reduced superhelicity requirement in the nicking activity of the mutant gpII.](image-url)
for negative superhelicity, as has been suggested for the strand transfer reaction of the phage Mu in vitro (Surette & Chaconas (1989) J. Biol. Chem. 264, 3028–3034). The only known step in f1 DNA replication that requires superhelicity is the initial nicking reaction by gpII. Stimulation of f1 DNA replication in vivo by the replication enhancer sequence and IHF is well established, but the conventional in vitro replication system for phage DNA is not affected by either the enhancer sequence or IHF. A possible explanation for this would be that there is a factor(s) in the cell which is necessary for manifestation of the IHF effect and that the factor(s) is missing in the in vitro system. Such a factor(s) can be an IHF-dependent stimulator of replication or an inhibitor that can be nullified by IHF. Identification of the factor(s) requires further study.

### Cloning and Nucleotide Sequence of the ispA Gene Responsible for Farnesyl Diphosphate Synthase Activity in Escherichia coli

Shingo Fujisaki*, Hiroshi Hara, Yukinobu Nishimura, Kensuke Horiuchi and Tokuzo Nishino**

*Escherichia coli* ispA strains have temperature-sensitive farnesyl diphosphate (FPP) synthase [EC 2.5.1.1] activity, and the defective gene is located at about min 10 on the chromosome. The wild-type *ispA* gene was subcloned from a λ phage clone containing a chromosomal fragment around min 10, which we picked up from the aligned genomic library of Kohara et al. [Kohara, Y., Akiyama, K. & Isono, K. (1987) Cell 50, 495–508]. The cloned gene was identified as the *ispA* gene on the basis of recovery and amplification of FPP synthase activity in an *ispA* strain. A 1,542-nucleotide sequence of the cloned fragment was determined. This sequence specifies two open reading frames, ORF-1 and ORF-2, encoding proteins with the expected molecular weights of 8,951 and 32,158, respectively. A part of the deduced amino acid sequence of ORF-2 showed a similarity to the sequences of eucaryotic FPP synthases and the *crtE* product of a photosynthetic bacterium. The plasmid carrying ORF-2 downstream of the lac promoter complemented the defect of FPP synthase activity in the

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** Department of Biochemistry and Engineering, Faculty of Engineering, Tohoku University, Sendai.
ispA mutant. This implies that the product encoded by ORF-2 is the ispA product. Maxicell analyses indicated that a protein of molecular weight 36,000, approximately consistent with the molecular weight of the deduced ORF-2-encoded protein, is the gene product.

**Kinetic Study on Transcription by Immobilized Operons: Requirement for $\beta$, $\gamma$-pyrophosphate Bond of ATP in Initiation by Prokaryotic RNA Polymerases**

Hiroyuki TERADA* and Nobuo SHIMAMOTO

The requirements of the $\beta$, $\gamma$-pyrophosphate bond of ATP in transcription initiation by prokaryotic RNA polymerases has long been questioned. We devised a novel method for showing 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*, bacteriophage T7, T3, and SP6 RNA polymerases. Transcription was started by adding a set of substrates, and then interrupted rapidly diluting substrates to 0.1 $\mu$M. Elongation was resumed by adding the second set of substrates. Using either ATP or an ATP analogue as one of the four substrates, we clarified that the $\beta$, $\gamma$-pyrophosphate bond of ATP increased the yield of long transcripts in the early stage of elongation but did not affect it much in the late stage. Moreover, abortive initiation was activated with such an ATP analogue. The requirement for intact ATP in productive initiation was found in all the transcription systems examined. Thus ATP is likely to be used to keep the elongation complex in productive form in the early preiod of initiation.

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

Nobuo SHIMAMOTO

The first step of transcription is the search for and binding of RNA polymerase to a promoter, and this promoter search is the major determinant

* Faculty of Meieicire Kyushu University.
in selective expression of genes. The enzyme could directly bind to a promoter on a long DNA, or alternatively, first bind to DNA in a nonspecific manner and then form the promoter complex by one-dimensional diffusion along the DNA chain. Unfortunately, kinetic evidence for both of the conflicting models has been reported and further study should be based on more confident evidence. The immobilized operon provides a critically important method for determining which case is true. Linear DNA immobilized on a slide glass was prepared. Fluorescently labeled RNA polymerase was added to the linearly fixed DNA. The movement of a single molecule of RNA polymerase was followed through microscopy and recorded on a video-tape. The one-dimensional movement was detected and its contribution to the promoter binding is now being evaluated.

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

Nobuo SHIMAMOTO, Takeomi TSUKIHARA*, Khoichi TAKAHASHI*, and Katsuhiko KAMATA*

*E. coli* Single-Stranded DNA Binding Protein (SSB) is a 19kda protein and essential to replication and recombination. Under physiological conditions, it exists as a tetramer and binds to both single-stranded DNA and a group of mRNAs which have a homologous RNA element called SSB box. Both of the bindings are cooperative and the protein molecules form clusters on such nucleic acid molecules. These bindings suggest a coupling between replication and translation by exchanging SSB between replication forks and mRNAs.

As a first step for understanding the mechanism of these characteristic bindings, the protein was over-produced and purified to homogeneity. Crystals of the protein were formed with ammonium sulfate or ethanol and a larger crystal suitable for X-ray crystallography is being developed. The protein seems to have two structural domains although it is a small peptide.

* Faculty of Technology Tottori University.
III. MAMMALIAN GENETICS

Genetic and Geographical Differentiation of *Mus musculus* Subspecies in China

Kazuo MORIWAKI, Nobumoto MIYASHITA, Hitoshi SUZUKI*, Kimiyuki Tsuchiya**, Hiromichi YONEKAWA***, Cheng-huai WANG****, Xin-quao He***** and Mei-pei Jin******

Since 1984, genetic and geographical surveys of house mouse, *Mus musculus*, in China have been carried out. Molecular genetic data on the Chinese wild mice accumulated so far indicated that *Mus musculus musculus* subspecies inhabit areas north of the Chang Jiang River and *M. m. castaneus* to the south of it. To confirm this genetic and geographical distribution more precisely, a fifth survey on Chinese wild mice was carried out in December, 1990. RFLPs of their ribosomal and mitochondrial DNAs were analysed using wild mice collected from various areas in China, as summarized in Table 1.

As already indicated by our previous survey, the Chang Jiang River can be seen as a good geographical barrier between *musculus* and *castaneus* subspecies in the eastern half of China. In the western China, *castaneus* type mitochondrial DNAs seem to be predominant, suggesting a possibility that in ancient times, the *castaneus* subspecies had inhabited this area and later the area was invaded by the *musculus* subspecies, especially their males, from the eastern region. The intersubspecies hybrid whose nuclear genome is the *musculus* type and mitochondrial genome is from the *castaneus* subspecies, seem to be quite similar to the *molossinus* subspecies in Japan as already reported by us (Yonekawa et al., 1988).

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** Miyazaki Medical College, Miyazaki.
*** Tokyo Metropolitan Institute of Medical Science, Tokyo.
**** Lanzhou Institute of Biological Products, Lanzhou.
***** Shanghai Laboratory Animal Center, Shanghai.
Table 1. Geographical distribution of RFLPs in the ribosomal and mitochondrial DNAs as subspecies markers

<table>
<thead>
<tr>
<th>Place of collection</th>
<th>Subspecies specific rDNA with EcoRI</th>
<th>mtDNA with BamHI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Western China</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aksu (Xinjian Uygur prov.)</td>
<td>MUS</td>
<td>CAS</td>
</tr>
<tr>
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<td>MUS</td>
<td>CAS</td>
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<tr>
<td>Kashgar ( )</td>
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<tr>
<td>Urumqi ( )</td>
<td>MUS/CAS</td>
<td>CAS</td>
</tr>
<tr>
<td>Manasu ( )</td>
<td>MUS</td>
<td>MUS/CAS</td>
</tr>
<tr>
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<td>MUS</td>
<td>n. t.</td>
</tr>
<tr>
<td>Northern China</td>
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<td></td>
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<tr>
<td>Xinin (Qinhai prov.)</td>
<td>MUS/CAS</td>
<td>MUS</td>
</tr>
<tr>
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<td>MUS</td>
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<tr>
<td>Lanzhou ( )</td>
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<td>Hoikou (Hainan Is.)</td>
<td>CAS</td>
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<td>Sanya ( )</td>
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<td>n. t.</td>
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</table>

A High Incidence of Visible Mutation in Intra-MHC Recombinants

Toshikiko SHIROISHI, Tomoko SAGAI and Kazuo MORIWAKI

The B10.MOL-H-2 lines are congenic strains which carry the MHC region derived from Japanese wild mouse. We have produced a number of intra-MHC recombinants from crosses between one of such B10.MOL-H-2 lines, B10.MOL-SGR, and standard inbred strains, B10.A and B10. We found that these recombinants yielded multiple visible mutations. So far, six independent mutations have been detected in screening of approximately 2500 mice. Mating experiment indicated that three of them are recessive
single mutations and the remaining three are dominant single mutations. In mice, it is reported that the average rate of recessive and dominant visible mutation is $3.8 \times 10^{-6}$ and $4.4 \times 10^{-7}$ per locus per gamete, respectively. Thus, the rate of visible mutation observed in the intra-MHC recombinant is extremely high compared to the standard value. Further genetic analysis revealed no clear linkage of the mutated genes with the MHC on chromosome 17. Because parental strains, B10.MOL-SGR and standard inbred strains have not yielded mutations, the high incidence of the mutation is a unique genetic trait peculiar to intra-MHC recombinants. In order to reveal the molecular mechanism of this high incidence of the mutation, we are attempting to map the mutated genes by an analysis of linkage of mutation phenotypes with a number of multilocus DNA markers, such as endogenous murine leukemia proviruses.

Rescue of Lethal Adrenal-dysgenic Mice through Transgenesis

Hideo Gotoh, Tojihiko Shiroishi and Kazuo Moriwaki

The chromosome of an $H$-2 haplotype $aw18$ has a recessive lethal deletion encompassing genes for the steroid 21-hydroxylase and the complement component C4 in the class III region. Homozygotes for the $aw18$ haplotype die within two weeks after birth.

Following chemotherapy experiments in which crude afrenal homogenate was injected into such mice postnatally, an adult-aged $aw18$ homozygous female was obtained. The results of these preliminary experiments indicated that the cause of the lethality is adrenal dysfunction. The deletion of the steroid 21-hydroxylase gene is thought to be the most probable cause of this lethality.

To save the life of $aw18/aw18$ homozygote, the trait of a transgenic mouse was adopted. We prepared a 6.6 kb recombinant DNA fragment which is constituted from a MMTV-LTR sequence and the murine steroid 21-hydroxylase genomic gene. Using this DNA fragment, we produced six transgenic mice, and four of them were fertile.

The exogeneous transgene was introduced into the mutant mouse genome through mating. Firstly, a transgenic $+/+$ mouse was crossed with a $aw18/+$ heterozygote to obtain a transgenic $aw18/+$ mice. That mouse was then crossed with a $aw18/+$ heterozygote. A transgenic $aw18/aw18$ mouse should be produced with a second cross. Several crosses have been
performed for each transgenic lineage, and the genotype of offspring was determined at 14 days after birth by Southern analysis. Among the progeny of two independent transgenic lines, several live aw18 homozygotes carrying the transgene have been obtained. A pedigree tree illustrating the production of transgenic aw18/aw18 mice is shown in Fig. 1. All the transgenic aw18/aw18 mice surviving the first two weeks of postnatal life have shown normal development to date.

These results showed that the deficiency of steroid 21-hydroxylase is the cause of the lethality of aw18 homozygotes, and demonstrated the potential of gene therapy through transgenesis. This is the first demonstration of the genetic rescue of a lethal mutation in mammals. Further analyses are now being undertaken.
Genetic Control of Susceptibility to Urethane-Induced Lung Tumor in Mice

Nobumoto Miyashita and Kazuo Moriwaki

Many kinds of inbred, $H-2$ congenic, recombinant inbred (RI) strains, a newly-established strain derived from Japanese wild mice, M.MOLTEN2, and the crosses among these strains of mice were investigated for their susceptibility to the induction of lung tumor by urethane. In $H-2$ congenic strains with a C57BL/10 (B10) background, genetic polymorphisms between $K-E\beta$ and $E\beta-D$ contributed to differences in susceptibility to the development of the lung tumor. In the $H-2$-matched crosses between lung tumor sensitive A/WySnJ and the resistant B10.A/SgSnJ mice, the resistant B10.A phenotype behaved as a completely recessive. This indicated the presence of a single recessive major gene conferring resistance to the induction of lung tumor in the B10.A strain. This gene was identical to those already reported as 'pulmonary tumor resistance (ptr)' or 'pulmonary adenoma susceptibility-1 (Pas-1)'. In the crosses between the intermediately tumor-sensitive BALB/cByJ strain with the resistant C57BL/6ByJ strain and in CXB RI strains, more than 2 genes contributed to differences in susceptibility between these strains. Experiments on the crosses of the M.MOLTEN2 strain, which was derived from wild mice (Mus musculus molossinus), with A/J mice indicated that the allelic differences for at least 2 genes contributed to resistance to the induction of lung tumor. These genes were epistatic to A/J Pas alleles.

Amplified EF2 Related Sequence (MER) in Mouse Genome

Tsuyoshi Koide, Toshihiko Shiroishi, Masahiro Ishiura* and Kazuo Moriwaki

Elongation factor 2 (EF2) is an essential protein in the steps involved in the elongation of polypeptide chains during protein synthesis in eukaryotes. We have found that cells from standard laboratory mouse strains contain about 70 copies of amplified EF2-related sequences, called MERs, per haploid genome, in addition to a single copy of the EF2 gene. To characterize the structure of the MER and its flanking sequence, three independent clones were sequenced. The sequence of the MER is conserved well

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(more than 99%) in all three, except for the polymorphisms of number of repeats in (GAAA(A))n sequence located in the 3’ region of the MER. Furthermore, outer regions which surround the MERs are also highly conserved among these MERs. Therefore, it seems likely that one copy of the MER was integrated into a specific site in the mouse genome and then the number of copies of the MER, together with its large flanking sequence, increased about 70-fold during the evolution of the mouse. To determine when the MERs was amplified in the process of speciation in genus Mus, the degree of amplification of the MER was analyzed by Southern blot hybridization. In this study, we used genomic DNAs prepared from different subspecies of M. musculus, including wild mice collected in mainland China and Japan, and from different species of genus Mus. As a result, the copy number was found to be similar in different subspecies of musculus except for wild mice in Lanzhou, China, which showed a remarkably low copy number for the MER. In contrast, different species in genus Mus showed variations in degree of amplification of the MER. M. spicilegus yielded a more intensified band than M. musculus and M. spretoides, and the latter two showed almost the same number of copies. On the other hand, the existence of multicopies of MER was not detected in M. spreto, M. caroli, M. platythrix and M. leggada. These data indicated that the MER were amplified after differentiation of genus Mus and before subspecies differentiation of M. musculus.

A New Haplotype of the Mouse β-Globin Genes, Hbbw1, in Northwestern China


We have studied the genetic polymorphism of the hemoglobin beta chain (Hbb) of wild mice, Mus musculus, mostly in Asia. The wild population in the northwestern pars of China, Korea and the central part of Japan exhibited an almost monomorphic distribution of the Hbbp haplotype. The southern part of Asia shows lower frequency of Hbbp and a predominant distribution of Hbbd (Miyashita et al., 1985). In the process of the survey of Hbb polymorphism in China, we found another new haplotype

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of \( Hbb \), assigned \( Hbb^{w1} \). It is distributed in a wide area in northwestern China. Southern blot analysis of \( Hbb^{w1} \) and other \( Hbb \) haplotypes were carried out with \( Hbb^* \) DNA probes using nine restriction enzymes. Based on differences in restriction sites, sequence divergence was estimated. It was revealed that the distance between \( Hbb^{w1} \) and \( Hbb^* \) haplotype was 2.64\% diversity, but the distance between \( Hbb^{w1} \) and \( Hbb^p \) haplotype was only 0.011\% diversity. These results indicate that the \( Hbb^{w1} \) haplotype may be generated from the \( Hbb^p \) haplotype. \( Hbb-bl^{w1} \) and \( Hbb-b2^{w1} \) were cloned from genomic DNA and are in the process of being sequenced.

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

Tomoko Sagai, Norio Nakatsuji, Toshihiko Shiroishi and Kazuo Moriwaki

Embryonic stem (ES) cells are cell lines derived from the inner cell mass of a mouse blastocyst. When ES cells are microinjected into a host blastocyst, they can differentiate into any tissue, including germ cells and generate chimeric animals. Modified ES cells by homologous recombination in vitro can be used to introduce any mutations into the germ lines of chimeric mice.

The ES cell should provide a useful system for our various studies on B10.H-2 congenic strains. In particular, the study of the molecular mechanism of meiotic recombination in the \( H-2 \) region definitely requires this system. Thus far, ES cell lines have been produced from limited mouse strains, for example 129/Sv. At present, there are no ES lines derived from B10.H-2 congenic strains. Therefore we started to establish new ES cell lines from several different B10.H-2 congenic strains. Now we are cloning the first ES cell line from one of the congenic strains, B10.A (R209), which showed high recombination frequency in the \( H-2 \) region during meiosis. The cells of this line grow very rapidly on the feeder cells and the morphology is quite similar to a typical ES cell line, E14. A karyotype analysis showed that a majority of the cell population has normal diploidy and the clone was derived from a male blastocyst suitable for chimera formation.
Comparison of the DNA Sequence in the D-loop Region of Mouse Mitochondrial DNA. Four maternal Lineages in the Southern Population of Japanese "molossinus" Mice

Hiromichi Yonekawa, Sumiyo Watanabe, Nobumoto Miyashita and Kazuo Moriwaki

Based on mtDNA haplotype analysis with restriction enzymes, we previously proposed the hypothesis that Japanese wild mouse *Mus musculus molossinus*, is a hybrid from crosses between ancestral colonies, possibly very small, of *M. m. musculus* and *M. m. castaneus* (Yonekawa et al. (1988) *Mol. Biol. Evol.* 5, 63). To prove this hypothesis, it is necessary to analyze more precisely the population structure of Japanese "molossinus" mouse. To do so, however, there was a problem: Japanese wild mice showed no restriction polymorphism in the southern population with a *musculus* type mitochondrial DNA (mtDNA) and a very small degree of polymorphism in the northern population with *castaneus* type mtDNA. The problem was solved by introduction of a new technique, DNA sequence analysis combined with PCR technique.

The D-loop region of mouse mtDNA was amplified by PCR and then cloned in M13mp18 or M13mp19 RF plasmids. DNA sequencing was carried out at on least two recombinant M13 phage clones for every mouse mtDNA in order to eliminate misreading of the mtDNA sequence caused by misincorporation of nucleotides by Taq polymerase during PCR. Six sequencing primes were sued for the sequencing.

We first sequenced 19 mtDNAs obtained from four *Mus musculus* subspecies and from one closely related species *Mus caroli*. Based on the sequence data, we constructed a phylogenic tree using the UPG method and the NJ method. Comparing the tree with that constructed using restriction enzyme site variation, we found that both trees are very similar to each other in topology. This suggests that substituted nucleotides in the mtDNA D-loop region, like restriction site variation, can be used as a diagnostic marker for subspecies identification.

Then we sequenced 11 mtDNAs obtained from mice belonging to the southern population. Although restriction analysis failed to detect any polymorphism in these mtDNA, sequence analysis showed that at least four different mtDNAs exist in the population. We designated them as major type (7 mtDNAs out of 11), Miyazaki type (2 out of 11), Ogasawara
type (1 out of 11) and Tsushima type (1 out of 11). In the previous issue (pp. 80–81, 1989), we described the existence of two highly variable segments in the D-loop region. The nucleotide substitutions detected among the four types were also found in the segments.

Sperm Head Abnormalities in Interspecific Hybrids between *Mus musculus* and *M. spretus*, and *M. spicilegus*

Michiko Niwa*, Akihiko Mita and Kazuo Moriwaki

*Mus spretus* originated in the Western Mediterranean region including Southern France, Iberia and Northern Africa. This mouse can interbreed under laboratory conditions with inbred strains of *M. musculus*. Offspring can be obtained in both directions of this interspecific cross. The F1 females are fertile and they can be backcrossed to males of either species. On the other hand, the F1 males are sterile. *M. spicilegus* originated in eastern Europe where there is sympatry with *M. musculus*. Hybrids between them may be obtained, but have not been studied in detail about their reproductive ability.

We observed the morphology of sperm from F1 hybrids between inbred strains and SEG (*M. spretus*), and ZBN (*M. spicilegus*) (Table 1). Almost all sperm from F1 males between inbred mouse and *M. spretus* was abnormal. The number of sperm was low and testis weight was significantly reduced (almost half of parents). We observed 15 male offspring bred

<table>
<thead>
<tr>
<th>strain or hybrid</th>
<th>No. of ♂♂ observed</th>
<th>abnormal rate of sperm (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6 (B6)</td>
<td>5</td>
<td>11.1</td>
</tr>
<tr>
<td>C3H/He (C3H)</td>
<td>3</td>
<td>21.4</td>
</tr>
<tr>
<td>M. MOL-MSM</td>
<td>7</td>
<td>2.1</td>
</tr>
<tr>
<td>SEG (<em>M. spretus</em>)</td>
<td>4</td>
<td>4.3</td>
</tr>
<tr>
<td>ZBN (<em>M. spicilegus</em>)</td>
<td>5</td>
<td>0.4</td>
</tr>
<tr>
<td>(B6×SEG) F1</td>
<td>3</td>
<td>95.4</td>
</tr>
<tr>
<td>(C3H×SEG) F1</td>
<td>6</td>
<td>98.9</td>
</tr>
<tr>
<td>(MSM×SEG) F1</td>
<td>3</td>
<td>91.4</td>
</tr>
<tr>
<td>(B6×ZBN) F1</td>
<td>4</td>
<td>100.0</td>
</tr>
</tbody>
</table>

* Saitama Cancer Center Research Institute, Saitama.
from a (B6 × SEG) F1 ♀ × B6 ♂ cross. Four males had normal values for sperm morphology and testis size, 4 had abnormal sperm and normal testis size, and 7 had abnormal sperm and small testis. It may be said that the sperm abnormality do not related tightly to the testis size, and that 4 males out of 15 are fertile. Guénet et al. (1990) reported that no more than two genes are related to hybrid sterility between laboratory mice and M. spretus and at least one is X-linked. Our results indicate the sperm abnormality of hybrids between the inbred strain and M. spretus may cause hybrid sterility in this case. F1 males between C57BL/6 (an inbred strain) and ZBN (M. spicilegus) also produced abnormal sperm, but the number of sperm and testis weight were similar to their parents. We cannot take offspring from the crosses between these F1 progeny and B6.

Further study on more backcross progeny from hybrids between laboratory mice and M. spretus, and M. spicilegus is necessary to learn the mechanisms for hybrid sterility.
IV. DEVELOPMENTAL AND SOMATIC CELL GENETICS

Head Regeneration and Budding in Hydra

Tsumi Sugiyama

Hydra tissue has four types of morphogenetic capacities involved in forming "head-type structures". They are the capacities (1) to form a secondary head, (2) to inhibit secondary head formation, (3) to form a bud, and (4) to inhibit bud formation. Evidence exists which indicates that a secondary head-forming/inhibiting mechanism is involved in determining head regeneration, whereas a bud-forming/inhibiting mechanism is involved in determining bud formation. At present, however, the relationship between the two mechanisms is not clear.

In order to gain an understanding of the relationship of the two mechanisms, lateral transplantation procedure (Browne, 1909) and ring transplantation procedure (Ando et al., 1989) were employed to examine and compare the relative levels of the four morphogenetic capacities in the tissue of Hydra magnipapillata (strain 105). The following observations were made. (1) Secondary head-forming capacity forms a gradient from the head to the foot along the body column of intact hydra, whereas bud-forming capacity forms a gradient in the opposite direction. (2) Both the secondary head-inhibiting and the bud-inhibiting capacities form gradients from the head to the foot in intact hydra. However, the secondary head-inhibiting capacity is unstable and falls significantly immediately after head removal in the regenerating tissue, whereas the bud-inhibiting capacity does not decrease under the same condition. (3) Repeated treatment of intact hydra with diacylglycerol produces a significant increase in the secondary head-forming capacity, but no changes in the bud-forming capacity.

These observations provide strong evidence for the view that, although head-type structures are formed by both head regeneration and budding, these two processes are regulated by two different mechanisms.
A cDNA Clone Specific for Stenotele Differentiation in *Hydra*

Toshitaka Fujiwara

Nematocytes are *hydra*’s stinger cells which are primarily used for capturing prey and for protection. There are 4 types of nematocytes (stenoteles, holotrichous isorhizas, atrichous isorhizas and desmonemes), all of which are differentiated from interstitial stem cells. The pathways of nematocyte differentiation from stem cells are well characterized at the cellular level. The main objective of this study was to analyze nematocyte differentiation at the molecular level.

An attempt was made to isolate genes which are specifically expressed during nematocyte differentiation. First, a cDNA library was produced from fractionated cells in the interstitial cell lineage. Using this library clones specific to interstitial cells were differentially screened. Individual clones were tested for specific expression through *in situ* hybridization. One of the clones called 5/5 was proven to be stenotele specific. *In situ* hybridization combined with BUdR labeling showed that the gene is expressed starting from about 66 h after the stenotele commitment and lasting for 12 h. This is the time when stenotele capsules become visible. The nucleotide sequence of the cDNA reveals that the gene codes a protein with 56 amino acids and that its function, however, is unknown since it is a new sequence which does not have homology with known genes.

Ovipositing Behavior in *Bombyx* Adults

a. Non-mated Females

Akio Murakami

Concerning the life-history of the silkworm (*Bombyx mori* L.), not a few number of characteristics including voltinism (number of generations per year), hatching and eclosion are subjected to considerable influences from the circadian rhythms of gene expression involved in them. For the hatching and eclosion of these moths, the circadian rhythms have been noted at the population level but not at the individual level. As reported herein, however, the ovipositing behavior of unmated females follows a distinct circadian rhythm. We conducted observations of the ovipositing process by making use of the females’ habit of secreting a cohesive substance together with their eggs: on a record paper moving 10 or 20 mm per unit
time was placed an acryl box of 3 cm each side which would not interfere with the movement of the paper; one female each was fixed in the box and the number of eggs laid per individual and per unit of time was determined.

In the case of mated females, since oviposition is completed four or five hours after sunset within a day of emergence, it is difficult to refer to it as periodic behavior in the strict sense. In contrast to this, freshly emerged (non-mated) females show almost no sign of ovipositing behavior on that day but start to lay eggs about two hours after sunset on the second day of eclosion; before or after sunrise of the following day, the oviposition is discontinued once and, as on the preceding day, reopened about two hours after sunset; eggs are laid down slowly and constantly until the dawn of the following day. A similar rhythm, though varied according to the number of eggs in the body of each female, continues to be observed for four or five days after eclosion. To sum this up, from these ovipositing behaviors of unmated females, the presence of a circadian rhythm was demonstrated at the individual level of this insect.

**Oviposition Behavior in Bombyx Adults**

b. Mated Females

Akio Murakami

Eclosion of adults occurs within a period of time from thirty minutes to two hours after sunrise regardless of the strain. Males tend to emerge significantly earlier than females. Twenty to thirty minutes after eclosion, moths of each sex initiate the courtship behavior followed by copulation; naturally, the male and female remain in this state for two or three hours and then part from each other spontaneously to dissolve the copulation. In the case of artificial dissolution of copulation, although copulating moths are separated usually any time between one and three o’clock in the afternoon, the mated females begin to deposit about two hours after sunset regardless of the time of the separation. During the subsequent four to five hours, the females keep on laying eggs actively and all the eggs in the body are laid down within this period of time. These observations, confirm that the ovipositing behavior of mated females also follows a circadian rhythm as in non-mated females.

As mentioned in the preceding report, in the case of non-mated females, the deposition of unfertilized eggs lasts [intermittently] for four to five
days. It is supposed that the difference in the oviposition rate observed between unmated- and mated females can be attributed to the presence/absence of effects from oviposition-stimulating factors (OSF) which are secreted accompanying copulation. The above finding, however, suggests the possibility that the secretion of OSF is regulated by a circadian rhythm.

When 20 to 35 mated females are kept together in a small styrol container (diameter: 10 cm; height: 5 cm), considerably more active ovipositing behaviors can be seen as early as 20 minutes after dissolution of copulation no matter what the time it is. The females continue to lay their eggs actively for about 1.5 hours and almost all the eggs in their body are laid down within two hours. These observations seem to indicate the involvement of an instinctive aspect in ovipositing behaviors: under such conditions as mentioned above, the neurosystem for oviposition of each female is stimulated by the competitive instinct to assure the survival of their respective descendants—probably owing to visual recognition of rivals or physical contact with them—so that a fairly high degree nervous activity come into action in a different manner from instructions by intrinsic circadian rhythm or the induction of oviposition accompanying copulation. That is, although the secretion of OSF occurs during a time period from sunset to sunrise essentially according to the biorhythm, the time of secretion can be easily modified through the nervous system by giving it powerful stimuli such as the competitive instinct. One can infer that hormone-like oviposition stimulating factors (OSF) are already accumulated in a sufficient amount in the body at the time of eclosion of females. It appears that the oviposition time is determined by a time measurement/regulation mechanism located in the central nervous system as is the case for voltinism and hatching/eclosion which are based on circadian rhythm and photoperiodism.

**Genetic Studies on Growth and Aging in the Silkworm (Bombyx Mori)**

**a. Growth Rate in Growth Period**

Akio Murakami

A consistent report on the duration time of the whole growth period, from embryo through larva to pupa, has not yet been published on the silkworm. For this insect, a relatively detailed observation on the larval period has been conducted, breeding records at the National Sericultural Experiment Station, M. A. F. F. are complete for a number of preserved
RESEARCH ACTIVITY IN 1990

silkworm stocks. We analyzed duration time in the larval stage making use of registered data in the record. Consequently, the distribution of larval periods is shown to occur in a clear binominal fashion with a mean value of 23.5 days under standard rearing conditions. There are a few extreme cases: 16 days at the minimum (in the case of the bimoulter strain) and 30 days at the maximum (in the case of the tetramoulter strain). However, the larval period is markedly not influenced by moultinism or the number of moulting times. The present findings and other observations clearly suggest that duration and/or growth rate in larval stages are under the control of polymeric genes. It is a widely known fact that rearing temperatures influence on the growth rate or growth period in the larval stage, but there are no serious effects with the standard culture temperatures of around 19–25°C on growth. A similar situation is also observed in the embryonic stage. In other words, the growth rate is essentially under the control of a certain genetic system, but not markedly influenced by environmental conditions. These findings suggest that a strong function related to homeostasis operates throughout the whole larval (and/or embryonic) stage. It is likely that in Bombyx the growth period and growth rate in the larval stage are subjected to relatively small variation.

With the courtesy of the Taiwan Sericultural Improvement Station (Taipei), we received the breeding records of the year 1986 in which there were registering 172 tetramoulter strains of Chinese, European, Japanese, and tropical races reared for three seasons (spring, summer and autumn) under natural climatic condition in Taipei and Taigo. Utilizing the observation data registered on the record, we analyzed a duration time of the whole growth period from the embryo stage to pupa. The result of the analyses indicated that the whole growth period in the temperate race is estimated to be about 50 days without any extreme fluctuation, while the 14 tropical tetramoulter strains are divided into two groups in response to the period of growth: one is about 43 days and the other is 35 days. Accordingly, growth rate during the whole growth period in the tropical race comes to be about 1.2 and 1.4 times faster than for the temperate ones. In addition, duration time in any stage comprising the growth period in a tropical race is intrinsically shorter than for the temperate race. Although the whole larval period fluctuates to some extent among the strains analyzed, the ratio of duration time in each developmental stage to the whole lifespan in the insect is almost the same. Consequently, it can be
said that the tropical race has a certain genetic factor promoting growth rate through the whole growth period. A preliminary attempt to detect such a presumptive genetic factor, \( pgr \), for example, has been carried out on a plan making use of several tropical races preserved in our country and in consequence a few candidates were obtained. The genetic characterization of the candidates is in progress. It is worthwhile to note that the following mutants, \( pre \) (or \( pgr \)), are perform their whole lifespan without any pathological symptoms. It is also clear that the function of these mutants responsible for the growth period is axiomatically different from the mutant, \( sdi \) (short duration of imaginal lifespan), which only expresses its function in the post growth period in adults. Accordingly, it became evident that the mechanism of aging in \( Bombyx \) is made up of at least two steps, the growth period concerned in growth rate and the post growth period related to senescence rate.

**Genetic Studies on Growth and Aging in the Silkworm (Bombyx Mori)**

**b. Growth Rate in the Pupal Stage**

**Akio Murakami**

Genetic analyses in the growth of silkworms, a lepidopteran insect, have been carried out to better understand mechanisms concerned in aging and/or senescence. There are drastic changes in morphological as well as physiological activity with the developmental stage from embryos to adults as it is a holometabolic insect. Growth periods can be distinguished from aging periods on the developmental process: the growth period is composed of the following three developmental stages, embryo, larva and pupa, and the aging period is composed on only the adult stage. There is relatively abundant biological information on the growth period, the embryo and larval stages, while there is scanty information on the pupal and adult stages. Such being the situation, the duration of the adult stage was investigated and results show the adult stage in females is about 1.5 times longer than that in males, regardless of strain. In addition, it was clearly shown that the duration of adult lifetime, 5 days to 8 days depending on strain and sex, is under the control of a particular genetix system and in addition, an autosomal recessive gene mutant \( sdi \) (short duration of imaginal lifespan), was detected.

Male adults emerged usually about one day earlier from pupae than
females regardless of strain, indicating that the pupal period in males is, as a rule, shorter to some extent than that in females of this insect. It should be noted that the growth rate of the male is several hours faster than the female. But female moths in an F₁ hybrid from a cross between one of the common bivoltine strain, J106, and a tropical multivoltine strain, Cambodge, emerged a few days earlier than males. A similar event was observed in the F₁ hybrid moths obtained from a cross between a Japanese bivoltine strain, J106, and a Chinese univoltine strain, Santosanmin. Of course, the adult lifespan for F₁ females, was about 1.5 times longer than for males. These findings clearly indicated that the genetic system involved in the pupal lifespan is different from that in the adult lifetime. From the results of genetic analyses on the phenomenon, it became clear that the genetic factor responsible for the early emergence of the female moths is a recessive gene \textit{pre} (precocious), which is located on the X-chromosome. It appears that the gene \textit{pre} operates mainly on the pupal stage, since the larval period is almost the same regardless of sex or genetic constitution. Known X-linked gene \textit{(Lm)} alleles, which are responsible for maturation in the silkworm, are sensitive to change in environmental conditions, while the \textit{pre} gene is indifferent to them. As stated already, however, the growth rate in male pupae of the silkworm is slightly but significantly faster than for females or the eclosion of female moths is delayed only for a day as compared with the males regardless of either strain or hybrid line. This difference seems to originate in the function of the Y-chromosome which determines femaleness.

In the silkworm, both embryonic and larval stages are sensitive to changes in environmental conditions, while both pupal and adult stages are insensitive to them. Thus, it is possible to say that pupal growth rate is principally under the control of a particular genetic system rather than the neuro-endocrine system which is under the influence of some environmental conditions. In brief, the gene \textit{pre} is not identical to \textit{Lm} gene-alleles.

**Genetic Studies on Growth and Aging in the Silkworm (Bombyx Mori)**

\textbf{c. Embryonic Diapause as a Prolongation of Lifespan}

\textit{Akio Murakami}

Genetic studies on growth in the silkworm, \textit{Bombyx mori}, have mainly focused so far on two main life history characteristics, voltinism (the number
of generation times per year) and moultinism (the number of ecdysial times in the larval stage). The former has the function of adaptation for the maintenance of survival of individual life. Looking at voltinism from another angle, this characteristic is also acts upon the determination of the diapause period or when the insect enters into the diapause state. In *Bombyx*, the diapause—a period of inactivity and suspension of growth—takes place in the embryo stage. The event is accompanied by greatly decreased metabolic activity. The diapause state in the insect lasts a considerably long time ranging from a few months up to about one year partly depending on genetic background. The diapausing eggs in all strains hatch the following spring. It is important to note that the diapause characteristic fluctuates according to several environmental conditions, including photoperiodism experienced in the parental generation (or rearing season). In fact, the diapause event is under the control of a genetic system connected with the central nervous system (brain) through which the management of various environmental information is performed. In other words, the diapause period is determined by some genetic and environmental factors, which determine the time for entering the dormant state.

In the univoltine race, for example, the whole growth period is about 50 days under standard rearing conditions, exclusive of the embryonic diapause term. Even if the adult lifetime which ranges from about two to ten days in the race is added to the duration time of the growth period. The whole lifespan lasts no more than 60 days, while the diapause period in this race is at least 10 months. The situation is complicated in the tropical multivoltine race: the diapause period is subjected to a wide fluctuation from zero to about 10 months depending on what time the embryo enters into the dormant state. Also, the determination of diapause periods is partly depends on the rearing place or latitude of the parental generation. In the multivoltine race, an early stage diapause event (estivation and/or hibernation) is observable even late in June and is a late stage event in December under the climatic conditions in Japan. In addition, there are cases of the insect being completely free of the diapause state. In general, the tropical multivoltine race has a short growth period, *ca.*40 days, exclusive of the duration of the diapause state, but the duration of the adult stage does not deviated from the standard lifetime in the insect. It should be also emphasized that awakened embryos after the dormant state proceed...
in their development without any abnormal symptoms and have normal lifespan and life functions. Certain genetic factors leading to a low metabolic state will produce a long-lived silkworm strain.

As referred to an early part of the communication, moultinism is also related to the growth events in *Bombyx*. This life history characteristic is under the control of some major genes as well as under the influence of environmental conditions. In *Bombyx*, there are known to be various types of moultinisms, bimoulters, pentamoulters and tetramoulters, among which the tetramoulters are the most common type followed by the trimoulters. It is a well-known fact that in this insect, a change of the number of moulting times per larval stage is no more than once regardless of strain: for example, from tetramoulters to trimoulters, and *vice versa*. Prior to moult, about two days are required to make the new epidermis and cuticle for each ecdysis, so larval growth is not apparently observed during the preparation for ecdysis. It is also a well-known fact that marked metabolic activity occurs in preparation for ecdysis. From a series of analyses on the relationship between moultinism and the duration time in a given larval instar (or between the two moult), it was revealed that a reduction of moulting times compensates for an increase in duration time in the instar between the two moult events. The increase and decrease of moult times per larval stage does not have a great influence on the duration time in the larval stage. Accordingly, the ecdysial event is no more than a secondary factor in prolonging the entire lifespan of the silkworm.

**A New Approach to Major Genes which Control Lifespan in Insects**


Longevity is one of the most fascinating and challenging research subjects in life science, but had been left out of genetic studies at the molecular level because of the general opinion that the biological event is under the control of polygenic factors. However, a pioneering work by Yonemura *et al.* (1986) has clearly shown that a certain major gene controls lifespan in
purebred strains of *Drosophila melanogaster*. A similar finding was also reported in *D. melanogaster* by Luckinbill et al. (1987). Recent experiments by Yonemura *et al.* (1989, 1990) revealed the mode of major gene inheritances in relationship to the lifespan in adult *Drosophila*; they identified genes concerned in longevity, which are designated as a *Jm* (Jumyo gene). The *Jm* alleles also control developmental speed in larvae, indicating that the alleles express their first function in the late larval stage to the early pupal one. In other words, the longevity potential of the alleles is differentiated during this period (Yonemura *et al.*, 1991).

In *Bombyx*, Murakami (1887) detected certain major genes concerned in the lifespan: a recessive gene, *sdi*, which controls the short duration in the imaginal lifetime and a sex-linked recessive gene *pre* (precocious), which accelerates growth rate in pupal stages (Murakami, in this Report). He also found a strain preserving a genetic factor, which influences a high growth rate throughout the whole growth period, ranging from the embryonic to pupal stages. There is generally observed a typical binominal distribution pattern on duration time in larval as well as pupal stages in 179 *Bombyx* strains analyzed so far, which may show that the lifespan character is still influenced by a number of polymeric genes. However, it seems to be impossible to completely deny a possibility that the effect of some major genes on the character in question decides the mode of lifespan distribution originated in the polymeric gene system.

So far we have analyzed (Yonemura *et al.*, in preparation), a remarkable change in the protein profile of both the short- and long-lived strains of *D. melanogaster* during the first few days after emergence. Only one protein was shown to be different between the strains. The protein quickly disappeared after emergence in the short-lived strain, while it remained for two days or more in the long-lived strain. The protein in question was extracted from young pupae of the long-life strain by phosphate buffered saline and purified by column chromatography procedures. The chemical characteristics of the protein were as follows: M. W. 76,600 (by SDS-PAGE), isoelectric point pH 6.5, molecular extinction coefficient $A(1\%\text{c}) = 18.3$, and a glycoprotein containing 3% hexoses (Okano *et al.*, in preparation). This protein was termed Ju-myo protein (JP).

When *D. melanogaster* adults were fed with JP mixed in food every day after day 5 of emergence, a significant life-prolongation effect was observed on both short- and long-lived strains, even with the extreme dilution of
0.0005 ug/ml (Yonemura et al., unpublished data), while all the other proteins, devoid of JP, had no effect at all. Details of this series of experiments will be published elsewhere.

Thus, we obtained a clue to the molecular approach for longevity making use of JP and/or a pure line with known Jm-alleles in D. melanogaster. Furthermore, an antibody against JP has now been produced, which will facilitate cloning of the Jm genes. Use of the antibody will make possible a search for the presence of the Jm gene or JP in animals other than D. melanogaster, such as Bombyx, mice, etc. This line approach will make a strong contribution to elucidating genetic mechanisms of the determination of lifespan and aging.

**Permeability of Eggs and Abnormal Development in Drosophila**

Kiyoshi Minato

In general, *Drosophila* eggs are impermeable to aqueous solutions even after dechorionation. However, "premature eggs" forced to be laid in rapid succession (Yoon & Fox, '65) or eggs laid spontaneously but still being at an early stage of development (Limboug & Zalokar, '73) are known to be, when dechorionated, permeable to aqueous solutions. Previously, we also found that eggs laid freshly by females and collected successively for short time intervals in *Drosophila melanogaster* failed to develop normally and did not hatch when dechorionated and incubated in aqueous media such as water and 0.9%NaCl, but they developed normally and hatched successfully when incubated in non-aqueous media such as paraffin-oil. The findings suggested that the above abnormal development of eggs occurred as a result of permeability to aqueous solutions of the eggs in "premature" or early stage of embryonic development.

The time dependencies of the phenomenon and the characteristics of the developmental anomalies induced were investigated in detail. Consequently, eggs which were incubated after the cysytial blastoderm stage (2 h at 25°C) developed normally and hatched successfully, but eggs before the cysytial blastoderm stage developed abnormally and never hatched. Thus, there was a critical difference in the reaction of eggs incubated in aqueous media before and after the blastoderm stage, and therefore a layer of cells formed on the surface of the egg in this stage seemed to be responsible for the impermeability of eggs or the establishment of a waxy coating.
on the vitelline membrane.

As to the differences in incubation media, when eggs were incubated in water, the eggs showed little sign of development and became gradually opaque, and after several hours swelled, and didn’t hatch. Incubated in 0.9% NaCl, eggs developed to a later stage and in most cases formed a structure like the cellular blastoderm only in the posterior-half of the embryo. They developed to the embryo stage with the amorphous and opaque structure anteriorly and a herniated bag-like structure posteriorly. These embryos became somewhat shrunken and hence detached from the vitelline membrane at both anterior and posterior ends, and did not hatch.

**Conditional Radiation Sensitivity in the Wasted Mouse Mutant**

Hideo Tezuka, Dai Ayusawa and Takeshi Seno

A quantitative study on the alteration in development of wasted marrow CFU-S and CFU-E revealed that (a) at weaning 21-22 days of age, the number of CFU-S per femur (bone marrow) and per spleen had already changed, while that of CFU-E per femur did not change, (b) at 26-27 days of age, the numbers of all these stem cells and progenitor cells decreased markedly.

A qualitative study on the developmental alteration of CFU-E revealed that (a) at the weaning age, the responsiveness of wasted marrow CFU-E to erythropoietin (epo) did not change, as shown by the colony forming ability of the cell in culture under different epo concentration, (b) at 26-27 days of age, however, CFU-E from wasted marrow required 8-10 fold concentrations of epo to form the same number of colonies as the control did. The number of wasted marrow CFU-E colonies formed significantly decreased, compared with the control, under the same epo concentrations at any level. This indicates that the responsiveness of wasted marrow CFU-E to epo at 26-27 days of age is lower than that at 21-22 days of age.

The time course in changes of CFU-E responsiveness to epo coincided with the appearance of radiation sensitivity in the wasted mouse, indicating that the change in CFU-E responsiveness to epo may be the cause of the radiation sensitivity of the cell.

To investigate this hypothesis, the radiation sensitivity of bone marrow CFU-E from wasted and control mice was determined under different epo concentrations. Results showed that radiation sensitivity of both CFU-Es from wasted and control mice was dependent on epo concen-
In addition, CFU-E from wasted marrow and from control, formed a similar number of colonies under different epo concentrations, when unirradiated (10-fold concentration of epo for wasted CFU-E). Under these culture conditions, these two cells showed similar radiation sensitivity. This suggests that the radiation sensitivity of marrow CFU-E may be determined by how epo interacts on the cell and consequently how the cell is stimulated to proliferate and differentiate.

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

Yoshie OHOYA*, 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 a first step in checking 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. A new purification procedure was developed by using centrifugal evaporation as the first concentrating method. The specific activity was increased 1,000-fold in this step. The yield and the specific activity of the purified growth factor was improved. A cDNA library was constructed from mRNA prepared from the primary-cultured cell to clone the cDNA of the growth factor.

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In Vitro Amplification of whole cDNA from a Single Embryo of the Nematode *C. elegans* and Its Application to Differential Screening

Yuji Kohara

The nematode *C. elegans* is one of the best systems for studying the network of gene expression regulation during development at the level of individual cells. Aiming to isolate genes which are expressed at specific stages or in specific cells, a new method to make a series of cDNA probes from single embryos at various stages was developed. The method consists of (1) disruption of a staged-single embryo under a dissecting microscope and extraction of total RNA, (2) first strand cDNA synthesis using a specific primer of oligo (dT)$_{12}$ linked with a 20 mer tag sequence at the 5'-side and [α$^{32}$P] dCTP at high specific activity instead of cold dCTP, (3) shortening of the cDNA to about 1 Kb on average by disintegration of incorporated $^{32}$P (4) dG tailing (20-30 mer) by Terminal transferase, (5) amplification of the cDNA by PCR using the tag sequence used in the first strand synthesis and oligo (dC)$_{12}$ linked with another tag sequence as primers.

Through this method, cDNA were amplified from individual embryos every 2 hr after the first cleavage. To assess uniform amplification, aliquots of amplified cDNA were dot-blotted onto strips of nylon membrane and probed with various genes whose expression patterns are known: *myo-2* (pharyngeal muscle specific myosin heavy chain), *vit-2* (vitellogenin, one of the most abundant mRNA but not present in embryo and *unc-30* (essential gene for differentiation of two motor neurons, which was kindly provided by Dr. R. Hoskins, MRC-LMB). The *myo-2* probe gave signals at the dots from 9.5, 11.5 and 13.5 hr embryos, *vit-2* gave no signal and *unc-30* gave signals at the dots from 7.5, 9.5 and 11.5 hr embryos. These results coincide with the expression patterns of these genes perfectly.

The set of amplified cDNA were $^{32}$P-labeled and probed a embryonic cDNA library made by J. Ahlinger (Univ. of Wisconsin). Although there were not any big differences in the patterns of positive signals between far different stages (probably due to the artificial rearrangement of the cDNA insert during library construction), by comparing the signals in autoradiograms very carefully, several clones seemed to be differentially expressed. To examine these, the dot-blot strips were hybridized with $^{32}$P-labeled
cDNA inserts from individual clones. For example, clone 4–3 gave a very strong signal at the dot from the 1.5 hr embryo, a weak signal for the 3.5 hr embryo and virtually no signal at 0 hr and other stages. Clone 4–1 gave a strong signal for the 7.5 hr embryo. A control clone 1–1 which did not show differential expression gave strong signals at almost the same level through out all the stages. These hybridization patterns were essentially reproducible in another experiment using amplified cDNA from another series of embryos. The cDNA in clone 4–1 and 4–3 were mapped near zyg-11 (on chromosome II) and mec-1 (on chromosome V), respectively, by probing so-called “polytene filters”, membrane filters blotted with a set of ordered YAC (Yeast Artificial Chromosome) clones which covers virtually the whole genome. Characterization of these cDNA is in progress.
In order to clarify the nature of "near neutrality" in molecular evolution and polymorphism, extensive simulation studies were performed. Selection coefficients of new mutations are assumed to be small so that both random genetic drift and selection contribute to determining the behavior of mutants. The model also incorporates normally distributed spatial fluctuation of selection coefficients. If the system starts from "average neutrality", it will move to a better adapted state, and most new mutations will become "slightly deleterious". Monte Carlo simulations have indicated that such adaptation is attained, but that the rate of such "progress" is very low for weak selection. In general, the larger the population size, the more effective the selection becomes. Also, as selection becomes weaker, the behavior of the mutants approaches that of completely neutral genes. Thus, the weaker the selection, the smaller is the effect of population size on mutant dynamics. An increase in heterozygosity with population size is very pronounced for subdivided populations. The significance of these results is discussed in relation to various observed facts on molecular evolution and polymorphism, such as generation-time dependency and overdispersion of the molecular clock, or contrasting patterns of DNA and protein polymorphism among some closely related species. For details, see Genetics 126, 219–229.

How Gene Families Evolve

Tomoko Ohta

Theories and facts of gene family evolution are reviewed. Concerted evolution is commonly observed for gene families which originated a long time ago, however there are many different types of multigene families, from uniform to diverse. The rate of homogenization caused by unequal crossing-over, gene conversion, etc. has been evolutionarily adjusted for
each gene family. When new functions are needed by organisms, gene families may evolve into superfamilies, in which no further concerted evolution takes place, and each member of the family may acquire an indispensable function. The homeobox-containing gene family is a most exciting example of such a superfamily. For details see *Theor. Pop. Biol.* 37, 213–219.

**Evolution of the Multigene Family: A Case of Dynamically Evolving Genes in the Major Histocompatibility Complex**

Tomoko Ohta

It is now known that many multigene families exist in eukaryote genomes. Multigene families that are known to be evolving under continued occurrences of unequal crossing-over and gene conversion provide a different picture of evolution from that of conventional population genetics. As an example of an evolving multigene family, evolution and variation at major histocompatibility complex loci were reviewed. The exceptionally high polymorphisms at the class I and class II loci of the major histocompatibility complex (MHC) have been of great interest for many years. In addition, recent studies indicate that amino acid substitution at antigen recognition sites (ARS) is more rapid than synonymous substitution, contrary to the general pattern of nucleotide substitution in evolution. However, such acceleration seems to be limited to a certain period after gene duplication. In order to explain such an unusual pattern, a population genetic model of diversifying selection was constructed. To make the model fit the data, diversifying selection needs to work on enhancing diversity not only between alleles at the same locus, but also between genes at different loci belonging to the gene family. Gene conversion among genes is also incorporated by choosing parameter values that are thought to be realistic. Simulation studies reveal that very weak selection at individual amino acid sites can explain the unusual pattern of evolution and polymorphism at MHC loci under this model. The applicability of the present model is discussed by surveying other examples, such as evolution of protease inhibitors and immunoglobulin variable regions which show a similar pattern of acceleration of amino acid substitutions. For details, see “*Evolution of Life,*” eds. S. Osawa and T. Honjo, Springer-Verlag, Tokyo, pp. 145–159.
The Major Histocompatibility Complex and the Quest for Origins
Jan Klein and Naoyuki Takahata

Two lines of pursuit in biological research hold the promise of particularly great satisfaction if drawn to a successful conclusion. One has the potential of helping the unfortunate among us who are stricken by some physical or mental impairment. The other touches upon the timeless question Où venons-nous? Whence do we come? Unlike Paul Gauguin, those of us engaged in finding answers to this question do not paint broad canvases that appeal to our senses and feelings or evoke a vague awareness of an answer. Our work can rather be compared to a mosaic to which each of us contributes but a single piece and on which the answer emerges in sharply delineated contours as the effort continues over generations. Some of us are lucky enough to be able to place a piece in a prominent position, others contribute dispensable or less conspicuous chips, while others still are among the unfortunate ones who see the pieces they laboriously placed in the mosaic removed again and replaced by better fitting fragments. But we are all working together on this venture in a collective effort to create a composition which will affect others. As for ourselves, we would not trade the opportunity to place a small piece into the mosaic Où venons-nous? for anything in the world. In this article, we discuss one genetic system where evidence for trans-species transfer of polymorphism is unassailable, with the hope that once we learn where we come from, we may begin to fathom the enigma of our existence. This system is the major histocompatibility complex. See Immunological Reviews 13, 5–25, 1990 for details.

Allelic Genealogy and MHC Polymorphisms
Naoyuki Takahata

Allelic genealogy, the genealogical relationships among different alleles, exhibits very different patterns depending on the underlying classic (neutral, deleterious, advantageous and overdominant) selection regimes and is used to elucidate the evolutionary mechanisms responsible for the extraordinary polymorphisms observed at the MHC (Major Histocompatibility Complex) loci. The polymorphisms can be most easily accounted for by overdominant selection. Two models of frequency dependent selection often invoked in relation to MHC polymorphisms actually cannot explain either
the long separation time of different alleles or a large number of segregating alleles under realistic circumstances, and hence they are incompatible with MHC polymorphisms. If we apply a strict minority advantage model, however, it is possible to generate exactly the same pattern of allelic genealogy as that of overdominant alleles, but in practice it is not easy to justify such a model on biological grounds. See Takahata, N. and Crow, J. F. (ed.) Population Biology of Genes and Molecules (Proceedings of the 4th Symposium of the International Prize), pp. 267–286, Baifukan, Tokyo 1990 for details.

**Population Biology of Genes and Molecules**

Proceedings of the 4th Symposium of the International Prize for Biology
Edited by Naoyuki Takahata and James F. Crow

The International Prize for Biology was established in April 1985 in celebration of the sixtieth year of the reign of the Emperor of Japan. It commemorates his long devotion to biological research. The first two Prizes were awarded in systematics and taxonomy, fields in which His Majesty was personally interested. In the following year the field was developmental biology. The current Prize is in population biology and was awarded to Professor Motoo Kimura on November 28, 1988. The serious condition of His Majesty prevented the Emperor from receiving Professor Kimura following the Prize ceremony. The award was granted in the presence of the then Crown Prince, now Emperor of Japan.

For the past thirty years Motoo Kimura has made many important contributions to mathematical, population, and evolutionary biology.

One of his most important contributions has been the development of diffusion models and applying these to significant problems in genetics and evolution. Starting as a graduate student he published a series of papers treating the transient distribution of gene frequencies in finite populations. These papers have provided a basis for studying the evolutionary change of natural populations and are now regarded as classics in population genetics. He has since studied additional problems including the probability of fixation of mutant genes, the dynamics of linked loci, the infinite allele and infinite site models, and the genetic consequences of geographical structure. With the advent of molecular biology, he pioneered a new field in which principles of both population and molecular genetics are synthesized and the resulting theory serves as a basis for investigating the mechanism
of evolution at the molecular level.

In 1968 Kimura proposed the neutral theory of molecular evolution, suggesting that evolutionary changes of proteins occur mostly by random fixation of neutral or nearly neutral mutations rather than positive Darwinian selection. Furthermore, the theory implies that a large fraction of the protein polymorphism in natural populations represents a transient phase of molecular evolution, because those genotypic mutants that are destined to spread into a species take a long time until fixation and on their way take the phenotypic form of protein polymorphism. The neutral theory has had an enormous impact not only in population genetics, but in evolutionary biology in general. It has stimulated research throughout the world. During the past twenty years the evolutionary study of DNA and proteins has generated a large amount of new data to support Kimura's hypothesis, and the theory is now widely regarded as substantially correct. It has become the guiding principle, the starting point for analyzing and interpreting molecular data. His 1983 book, *The Neutral Theory of Molecular Evolution*, is already regarded as a milestone in evolutionary biology.

Kimura's work in unifying the theory of population genetics and the data of molecular evolution will undoubtedly remain as one of the most important achievements of twentieth century biology.

This Symposium, held in Tokyo the day after the Prize was awarded, emphasized population genetics, quantitative genetics, and molecular evolution, subjects of great interest to Kimura and to which he has made such important contributions.

Although the International Prize is awarded by Japan, the recipient is chosen on a world-wide basis by an international committee. It is especially pleasing that this year's recipient is from Japan. See Takahata, N. and Crow, J. F. (ed.) *Population Biology of Genes and Molecules* (Proceedings of the 4th Symposium of the International Prize for Biology), Baifukan, Tokyo 1990 for details.

**Allelic Genealogy under Overdominant and Frequency-Dependent Selection and Polymorphism of Major Histocompatibility Complex Loci**

Naoyuki Takahata and Masatoshi Nei

To explain the long-term persistence of polymorphic alleles (trans-specific
polymorphism) at the major histocompatibility complex (MHC) loci in rodents and primates, a computer simulation study was conducted on the coalescence time of different alleles sampled under various forms of selection. At the same time, average heterozygosity, the number of alleles in a sample, and the rate of codon substitution were examined to explain the mechanism of maintenance of polymorphism at the MHC loci. The results obtained as follows. (1) The coalescence time for neutral alleles is too short to explain the trans-specific polymorphism at the MHC loci. (2) Under overdominant selection, the coalescence time can be tens of millions of years, depending on the parameter values used. The average heterozygosity and the number of alleles observed are also high enough to explain MHC polymorphism. (3) The pathogen adaptation model proposed by Snell is incapable of explaining MHC polymorphism, since the coalescence time for this model is too short and the expected heterozygosity and the expected number of alleles are too small. (4) From the mathematical point of view, the minority advantage model of frequency-dependent selection is capable of explaining a high degree of polymorphism and trans-specific polymorphism. (5) The molecular mimicry hypothesis also gives a sufficiently long coalescence time when the mutation rate is low in the host but very high in the parasite. However, the expected heterozygosity and the expected number of alleles tend to be too small. (6) Consideration of the molecular mechanism of the function of MHC molecules and other biological observations suggest that the most important factor for the maintenance of MHC polymorphism is overdominant selection. However, further studies are necessary to compare the overdominance and frequency-dependent selection hypotheses. See Genetics 124, 967–978, 1990 for details.

A Simple Genealogical Structure of Strongly Balanced Allelic Lines and Trans-Species Evolution of Polymorphism

Naoyuki TAKAHATA

Different alleles undergoing strong symmetric balancing selection show a simple genealogical structure (allelic genealogy), similar to a gene genealogy described by the coalescence process for a sample of neutral genes randomly drawn from a panmictic population at equilibrium. The only difference between the two genealogies lies in different time scales. An approximate scaling factor of the allelic genealogy relative to that of the neutral gene
genealogy is \( \sqrt{S/(2M)}[(\ln[S/(16\pi M^3)])^{-3/2}] \), where \( M=Nu \) and \( S=2Ns \) (\( N \); effective population size, \( u \); mutation rate to selected alleles per locus per generation, \( s \); selection coefficient). The larger the value of \( \sqrt{S/M} \) (\( \geq 100 \)), the larger the scaling factor. These findings, supported by simulation results, allow us to apply all theoretical results of the coalescence process directly to the allelic genealogy. Combined with trans-species evolution of the MHC (major histocompatibility complex) polymorphism, for which balancing selection is believed to be responsible, the allelic genealogy predicts that the number of breeding individuals in the human population could not be as small as 50~100 at any time of its evolutionary history. The analysis appears to contradict the founder principle as being important in recent mammalian evolution. See *Proc. Natl. Acad. Sci. USA* **87**, 2419–2423, 1990 for details.

**Genealogy of Neutral Genes in Two Partially Isolated Populations**

Naoyuki Takahata and Montgomery Slatkin

Gene genealogy in two partially isolated populations which diverged at a given time \( t \) in the past and have since been exchanging individuals at a constant rate \( m \) is studied based upon an analytic method for large \( t \) and a simulation method for any \( t \). Particular attention paid to the conditions under which neutral genes sampled from populations are mono-, para-, and polyphyletic in terms of coalescence (divergence) times of genes. It is shown that the probability of monophyly is high if \( M=2Nm<0.5 \) and \( T=t/(2N)>1 \) where \( N \) is the size of ancestral and descendant haploid populations, in which case most gene genealogies are likely to be concordant with population relatedness. This probability decreases as the sample size of genes increases. On the other hand the case where the probability of monophyly is low implies either a situation where \( M>1 \) and any \( T \) or where \( M<1 \) and \( T<1 \), but the clear distinction between these conditions appears very difficult to make. These results were also examined for a gene genealogy reconstructed from nucleotide differences. It is then shown that results based upon coalescence times remain valid if the number of nucleotide differences between a pair of genes is not much smaller than 10. To observe such large nucleotide differences in small populations and therefore infer a reliable gene genealogy, we must examine a fairly long stretch of DNA sequences. See *Theor. Pop. Biol.* **38**, 331–350, 1990 for details.
Incomplete Maternal Transmission of Mitochondrial DNA in Drosophila

Rumi Kondo, Yoko Satta, Etsuko T. Matsuura, Hiromi Ishiwa, Naoyuki Takahata and Sadao I. Chigusa

The possibility of incomplete maternal transmission of mitochondrial DNA in Drosophila, previously suggested by the presence of heteroplasmy, was examined by intra- and interspecific backcrosses of D. simulans and its closest relative, D. mauritiana. mtDNAs of offspring in these crosses were characterized by Southern hybridization with two α32P-labeled probes that are specific to paternal mtDNAs. This method was sufficient for detecting as little as as 0.03% paternal mtDNA in a sample. Among 331 lines that had been backcrossed for ten generations, four lines from the interspecific cross D. simulans (female) × D. mauritiana (male) showed clear evidence for paternal leakage of mtDNA. In three of these, the maternal type was completely replaced while the fourth was heteroplasmic. Since the total number of fertilization carried out in this experiment was $331 \times 10 = 3310$, the proportion of paternal mtDNA per fertilization was estimated as about 0.1%. The mechanisms and evolutionary significance for paternal leakage are discussed in light of this finding. See Genetics 126, 657-663, 1990 for details.

Evolution of Drosophila Mitochondrial DNA and the History of the Melanogaster Subgroup

Yoko Satta and Naoyuki Takahata

The nucleotide sequences of a common region of 15 mitochondrial DNAs (mtDNAs) sampled from the Drosophila melanogaster subgroup were determined. The region is 2527 bp long, encoding most of the NADH dehydrogenase subunit 2 (ND2) and cytochrome oxidase subunit 1 (COI) genes punctuated by three tRNAs. The comparative study revealed (i) the extremely low saturation level of transitional differences, (ii) recombination or variable substitution rates even within species, (iii) long persistence times of distinct types of mtDNA in D. simulans and D. mauritiana, and (iv) an apparent lack of within-type variations in island species. Also found was the high correlation among transitional rate, saturation level, and GC content (or codon usage). It appears that D. simulans and D. mauritiana have maintained highly structured populations for more than
one million years. Such structures are consistent with the origination of *D. sechellia* from *D. simulans*. Yet, geographic isolation is so weak as to show no evidence for further speciation. Moreover, one type of mtDNA shared by *D. simulans* and *D. mauritiana* suggests either their recent divergence or ongoing introgression. See *Proc. Natl. Acad. Sci. USA* 87, 9558–9562, 1990 for details.

**A Study on a Nearly Neutral Mutation Model in Finite Populations**

Hidenori TACHIDA

As a nearly neutral mutation model, the house-of-cards model was studied in finite populations using computer simulations. The distribution of the mutant effect was assumed to be normal. Behavior was mainly determined by the product of population size, $N$, and standard deviation, $\sigma$, of the distribution of the mutant effect. If $4Na$ is larger than one, a few advantageous mutants are quickly fixed in early generations. Then, most mutation become deleterious and a very slow increase of the average selection coefficient follows. It takes a long time for the population to reach an equilibrium state. Substitutions of alleles occur very infrequently in the later stage. If $4Na$ is the order of one or less, the behavior is qualitatively similar to that of the strict neutral case. A gradual increase in the average selection coefficient occurs and in generations several times the inverse of the mutation rate, the population almost reaches an equilibrium state. Both advantageous and neutral (including slightly deleterious) mutations are fixed. Except in the early stage, an increase in standard deviation of the distribution of the mutant effect decreases the average heterozygosity. The substitution rate is reduced as $4Na$ is increased.

Three tests of neutrality, one using the relationship between the average and the variance of heterozygosity, another using the relationship between average heterozygosity and the average number of substitutions and Watterson’s homozygosity test were applied to the results of the present model. It was found that deviation from the neutral expectation becomes apparent only when $4Na$ is more than two. Also a simple approximation for the model was developed which works well when the mutation rate is very small. For details, see *Genetics* 128, 183–192.
Fixation Probability in Spatially Changing Environments

Hidenori TACHIDA and Masaru IIZUKA*

The fixation probability of a mutant in a subdivided population with spatially varying environments was investigated using a finite island model. This probability is different from that in a panmictic population if selection is intermediate to strong and migration is weak. An approximation was developed to compute the fixation probability when migration among subpopulations is very weak. By numerically solving the two-dimensional partial differential equation for the fixation probability in the two subpopulation case, the approximation was shown to give fairly accurate values. With this approximation, we show in the case of two subpopulations that the fixation probability in subdivided populations is generally greater than that in panmictic populations. The increase is most pronounced when the mutant is selected for in one subpopulation and is selected against in the other subpopulation. Also it was shown that when there are two types of environments, further subdivision of subpopulations does not cause much change in the fixation probability unless the product of the selection coefficient and the local population size is less than one.

Relationship between DNA Polymorphism and Fixation Time

Fumio TAJIMA

When there is no recombination among nucleotide sites in DNA sequences, DNA polymorphism and fixation of mutants at nucleotide sites are mutually related. Using the method of gene genealogy, the relationship between DNA polymorphism and the fixation of mutant nucleotide was quantitatively investigated under the assumption that mutants are selectively neutral, that there is no recombination among nucleotide sites, and that the population is a random mating population with $N$ diploid individuals. The results obtained indicate that the expected number of nucleotide differences between two DNA sequences randomly sampled from the population is $42\%$ less when a mutant at a particular nucleotide site reaches fixation than at a random time, and that heterozygosity is also expected to be less when fixation takes place than at a random time. However, the

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amount of reduction depends on the value of $4N\nu$ in this case, where $\nu$ is the mutation rate per DNA sequence per generation. The formula for obtaining the expected number of nucleotide differences between the two DNA sequences for a given fixation time was also derived, and indicates that, even when it takes a large number of generations for a mutant to reach fixation, this number is 33% less than at a random time. The computer simulation conducted suggests that the expected number of nucleotide differences between the two DNA sequences at the time when a advantageous mutant becomes fixed is essentially the same as that of a neutral mutant if the fixation time is the same. The effect of recombination on the amount of DNA polymorphism was also investigated using computer simulation. For details, see *Genetics* 125, 447-454.

Relationship between Migration and DNA Polymorphism in a Local Population

Fumio Tajima

The expected amount of DNA polymorphism, measured in terms of the number of nucleotide differences between the two DNA sequences randomly sampled from subpopulations, was studied using the stepping-stone model and the finite island model, under the assumption that the migration rate is not the same among different subpopulations. The results obtained indicate that the expected amount of DNA polymorphism in the subpopulation with a lower migration rate is smaller than that with a higher migration rate. This suggests that marginal populations tend to have lower levels of DNA polymorphism than central populations, if the migration rate in the marginal populations is lower than that of the central populations. For details, see *Genetics* 126, 231-234.

A Simple Graphic Method for Reconstructing Phylogenetic Trees from Molecular Data

Fumio Tajima

A simple graphic method was proposed for reconstructing phylogenetic trees from molecular data. This method is similar to the unweighted pair-group method with arithmetic mean (UPGMA), but the process of
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computation of average distances and reconstruction of new matrices, required in the latter method, was eliminated from this new method, so that one can reconstruct a phylogenetic tree without a computer unless the number of operational taxonomic units (OTUs) is very large. Furthermore, this method allows a phylogenetic tree to have multifurcating branches whenever there is ambiguity with bifurcation. For details, see *Mol. Biol. Evol.* 7, 578–588.

**Some Consideration on Diversifying Selection**

Fumio TAJIMA and Terumi MUKAI

Diversifying selection due to genotype-environment interaction can increase genetic variation in natural populations. It is known, however, that the conditions for stable genetic polymorphism or marginal overdominance are quite restricted in this selection model. In this study a simple model of diversifying selection was examined, and the following results were obtained: (1) Even when the conditions for marginal overdominance are not satisfied, if diversifying selection is operating, the frequency of mutants can be higher than that in the case of simple mutation-selection balance. (2) This selection model causes a large amount of genetic load (environment load), even when the conditions for marginal overdominance are not satisfied, namely even when the equilibrium frequency of mutant is very low. From these results it can be concluded that the number of loci on which this type of diversifying selection is operating is very small, if any. For details, see *Jpn. J. Genet.* 65, 193–200.

**An Estimate for the Total Rate of Mutations in Humans**

Motoo KIMURA

Applying the neutral theory of molecular evolution, I intend to show that the total mutation rate per genome per generation in man must be very much higher than earlier estimates. To show this, I use the estimated value of the evolutionary rate of the mouse globin pseudogene, which is approximately \( k_1 = 5 \times 10^{-9} \) per nucleotide site per year (Li *et al.* (1981) *Nature* 292, 237–239). If the neutral theory is valid, this represents the total mutation rate per site per year in the mouse. This is because, according to the neutral theory, the rate of mutant substitution at the molecular level
is equal to the neutral mutation rate, and also, all the mutations of the pseudogene ("dead gene") are selectively neutral. I will also utilize the observation (Li and Tanimura (1987) *Nature* 226, 93–96) that the rate of synonymous substitutions in rodents is about 7 times higher than that in higher primates.

The human genome consists of about three billion \((3 \times 10^9)\) nucleotide sites, so if we assume that the average human generation span is 20 years, we can obtain the total mutation rate per genome per generation as follows.

\[
V_T = (3 \times 10^9) \times (5 \times 10^{-6}) \times 20 \div 7 ,
\]

or approximately \(V_T = 43\), meaning that the total number of new mutations per generation due to base substitutions amounts to 43 per gamete, and twice as many per zygote. This is a very high value compared with the traditional estimates of the genomic mutation rate (i.e., much smaller than unity). According to the genetic load theory, the mutational load becomes intolerably high unless the great majority (say, 99% or more) of these mutations are selectively neutral (i.e., nondeleterious).

**Recessive Hereditary Deafness, Assortative Mating, and Persistence of a Sign Language**

Kenichi Aoki and Marcus W. Feldman

We modelled the cultural transmission of a sign language when there is one-locus genetic variation for deafness and hearing. Our premises are that the deaf are more motivated to learn sign language than the hearing, and that a vertically transmitted sign language, unlike recessive hereditary deafness, cannot "jump a generation". Conditions were obtained for persistence (i.e., protection from loss) of signers. These conditions are more easily satisfied the greater is the fraction of the hearing who also learn sign language and as the frequency of the recessive gene for deafness increases. Persistence is also facilitated by assortative mating for deafness, but not by assortment for signing. With vertical transmission only, it is necessary that one signer parent be able to transmit sign language with greater than one-half the efficiency of two. Under the assumption that the hearing do not learn sign language, the following additional results were obtained. Persistence is more likely with dominant as opposed to recessive inheritance. When recessive hereditary and acquired deafness
co-occur, increasing the frequency of the latter has an opposite effect, depending on the degree of assortment. Opportunities for the deaf to learn sign language outside the family do not seem to affect the conditions for persistence. For details, see *Theor. Popul. Biol.* 39, 358–372.

**Some Theoretical Aspects of the Origin of Cultural Transmission**

Kenichi Aoki

Cultural transmission can be roughly defined as the transfer of information between individuals by social learning. Despite its manifest importance in the determination of various aspects of human behavior, the question of its origin(s) has not received due attention. Previous theoretical studies, notably by Cavalli-Sforza and Feldman, have indicated that there may be serious obstacles to the evolution of a genetically determined capacity for cultural transmission. This study reviews some new theoretical results that extend their work and notes the conditions that may favor the occurrence of cultural transmission. Basically, our claim is that an increase in frequency of communicators was linked to the spread, by vertical transmission, of a specific adaptive trait. Hence, if cultural processes are constrained by evolutionary history, we expect that a propensity should exist for children to acquire useful innovations from their parents. An attempt was made to interpret the observations of cultural transmission in birds, nonhuman primates, and a hunter-gatherer group in terms of this and other predictions. The theoretical results presented here may also be useful in suggesting further empirical studies that can improve our understanding of the origin(s) of cultural transmission. For details, see *Evolution of Life* (S. Osawa and T. Honjo, eds.), pp. 439–449, Springer-Verlag, Tokyo, 1991.
VI. EVOLUTIONARY GENETICS

Evolutionary Origin of Human and Simian Immunodeficiency Viruses

Takashi Gojobori, Etsuko N. Moriyama, Kazuo Ikeo and Yasuo Ina

Controversy continues to exist on which viruses the human immunodeficiency viruses (HIVs) originated from. To settle this controversy, we analyzed nucleotide sequences of simian immunodeficiency viruses (SIVs) and HIVs from the view point of molecular evolution. In particular, we compared the nucleotide sequences of the entire genomes, gene region by gene region, between a given pair of viruses. The viral strains used in this study include HIVs and four types of SIVs; SIVs isolated from mandrills (SIV_{mnd}), African green monkeys (SIV_{agm}), sooty mangabeys (SIV_{sm}), and rhesus macaques (SIV_{mac}). The phylogenetic trees obtained showed that the HIV-1 and HIV-2 groups, SIV_{agm}, and SIV_{mnd} all diverged from one ancestor at almost the same time, although SIV_{sm} and SIV_{mac} definitely belong to the HIV-2 group. Moreover, the evolutionary position of SIV_{agm} and SIV_{mnd} on the phylogenetic tree depended on the gene examined. From these results, we concluded that the present HIVs may have emerged as different variants of SIVs in Old World monkeys, possibly from recombination between different strains of viruses related to SIVs. For details, see Proc. Natl. Acad. Sci. USA. 87, 4108–4111, 1990 and Sixth International Conference on AIDS 2, 124.

Statistical Methods for Estimating Sequence Divergence

Takashi Gojobori, Etsuko N. Moriyama and Motoo Kimura

Methods for estimating the number of nucleotide base substitutions are crucial for studies of molecular evolution. Knowledge of the number of base substitutions is particularly important for computing the evolutionary rate and constructing phylogenetic trees at the DNA level. From the perspective of population genetics, nucleotide and amino acid substitutions can both be treated as stochastic processes. Methods for estimating the number of DNA base substitutions, however, are different
from those for estimating the number of amino acid substitutions. Because only four kinds of nucleotide bases exist (usually denoted A, T, C, and G), multiple and superimposed nucleotide substitutions at the same site may occur undetected especially when sequence divergence is great. For example, when two comparable DNA sequences have different bases, say A and G, at the corresponding (i.e., homologous) site, a change of $A \rightarrow T \rightarrow G$ may have occurred for one sequence and no change for the other at that site. The observed number of nucleotide differences between the two DNA sequences is thus frequently different from the total number of nucleotide substitutions that have actually occurred during their divergence. Statistical methods for estimating the number of nucleotide substitutions are therefore required for comparative studies of DNA sequences. We described various methods for estimating the number of nucleotide substitutions. In particular, we discussed the advantages and disadvantages of these methods. For details, see *Methods in Enzymology* (Ed. R. F. Doolittle) 183, 531–550.

**Molecular Clock of Viral Evolution, and the Neutral Theory**

Takashi Gojobori, Etsuko N. Moriyama and Motoo Kimura

Evolution of viral genes is characterized by an enormously high speed compared with that for nuclear genes of eukaryotic organisms. The evolutionary rates and patterns of base substitutions were examined for retroviral oncogenes, human immunodeficiency viruses (HIV), hepatitis B viruses (HBV), and influenza A viruses. Our results show that the evolutionary process of these viral genes can readily be explained by the neutral theory of molecular evolution. In particular, the neutral theory is supported by our observation that synonymous substitutions always greatly predominate over nonsynonymous substitutions, even though the substitution rate varies consideranly among the viruses. Furthermore, the exact correspondence between the high rates of evolutionary base substitutions and the rates of production of mutants in RNA viruses fits very nicely with predictions of the theory. The linear relationship between substitution numbers and time was examined to evaluate the clocklike property of viral evolution. The clock appears to be quite accurate in the influenza A viruses in man. For details, see *Proc. Natl. Acad. Sci. USA.* 87, 10015–10018.
Patterns of Amino Acid Substitutions in the Envelope Protein of Human Immunodeficiency Viruses and Its Application to Vaccine Development

Takashi Gojobori and Etsuko N. Moriyama

A major obstacle to the development of a HIV vaccine is the variability of the env gene region in HIVs. Although the env region is variable, certain sub-regions may have less variation than others. To identify these sub-regions, we first computed the frequency for each amino acid, site by site, in the amino acid sequences of the envelope protein for various isolates of HIV-ls. We then identified four conserved sub-regions within the gp120 region of the envelope protein, and these were also found to be relatively hydrophilic. Moreover, we estimated the direction of amino acid substitutions, using the phylogenetic tree for the envelope protein of HIVs. We then made a 20 × 20 matrix for amino acid substitutions which can be used to predict the future direction of amino acid substitutions in HIVs. In fact, it is possible to synthesize peptides with various combinations of the predicted amino acids, taking into account the possible directions of amino acid substitutions in the conserved sub-region. For detail, see Population Biology of Genetics and Molecules (eds. N. Takahata and J. F. Crow), Baifu-Kan, Tokyo, pp. 323–340, 1990.

Rates and Patterns of Nucleotide Substitutions for Nuclear Genes of Drosophila Species

Etsuko N. Moriyama and Takashi Gojobori

It is known that the nuclear genes of the Drosophila species evolve at rates two to four times higher than those of mammals. These rates are also known to vary with the genes (10.8–20.2 × 10⁻⁹/site/year) (see Jpn. J. Genet. 52, 139–147, 1987 and Genetics 122, s41, 1989). To examine the relationships between the evolutionary rate and the mutation mechanism in Drosophila, we estimated the patterns of nucleotide substitutions for nuclear genes in Drosophila. We then compared the patterns of Drosophila with those for rodents, artiodactyls and primates. In Drosophila, most nuclear genes had almost the same patterns of nucleotide substitutions at the third codon position, although at the second codon position the pattern varied with genes examined. Furthermore, the relative frequency of trans-
version substitutions was nearly equal to that of transition substitutions in \textit{Drosophila}. For other organisms, on the other hand, transitions occurred more frequently than transversions. Therefore, the mutation mechanism in the \textit{Drosophila} genome may be different from that in the mammalian genome. Nevertheless, we cannot exclude the effect of the shorter generation time of \textit{Drosophila} on the evolutionary rate. For details, see \textit{Jpn. J. Genet.} \textbf{55}, 529–531, 1990.

**Molecular Evolution of Human T-Cell Leukemia Virus**

\textbf{Yasuo Ina} and Takashi Gojobori

Phylogenetic trees for the human T-cell leukemia virus type I (HTLV-I) and its related viruses were constructed using of nucleotide sequences of the long terminal repeat (LTR) and the \textit{tax} gene. The trees showed that the viruses diverged from a common ancestor and that they are classified into two groups whose hosts are either primates or bovines. However, the topology of the trees for the viruses differed from that for the hosts. This suggests that HTLV-I and HTLV-I-related viruses evolved independently of host-species divergence. It also implies that interspecies transmission between human and monkeys occurred in the past. The nucleotide diversity of the \textit{tax} genes of HTLV-I was estimated to be 0.025. This value is more than 10 times larger than that of human globin genes, but it is about 20 times smaller than that of human influenza A viruses. Thus, the genetic variability of the HTLV-I genes seems to be higher than that of nuclear genes but much lower than the genes of typical RNA viruses. Furthermore, we examined functional constraints on the overlapping region of the \textit{rex} and \textit{tax} genes of HTLV-I. The results obtained show that for the overlapping region, the \textit{tax} gene has much stronger constraints against amino acid changes than the \textit{rex} gene. For details, see \textit{J. Mol. Evol.} \textbf{31}, 493–499.
Host-independent Evolution of the Hepadnavirus Family

Yasuo Ina, Masashi Mizokami*, Etsuro Orito*, Nobutoshi Kameshima*, Masahiko Yamamoto*, Etsuko N. Moriyama and Takashi Gojobori

Nucleotide sequences of 25 hepatitis B viruses (HBVs), 6 woodchuck hepatitis viruses (WHVs), 1 ground squirrel hepatitis virus (GSHV), 2 duck hepatitis B viruses (DHBVs) and 1 heron hepatitis B virus (HHBV) have been determined to date. To examine whether the evolution of the hepadnavirus family is host-dependent, we constructed phylogenetic trees by use of the nucleotide sequences. Although the phylogenetic trees for the viruses had the same topology of that for their host-species, the trees for the viruses showed that the divergence times of the viruses are much more recent than those of their host-species. These results strongly support our previous study (Proc. Natl. Acad. Sci. USA. 86, 7059-7062), in which we used 18 sequences of the hepadnavirus family. Thus, the host-independent evolution of the hepadnavirus family was confirmed.

Furthermore, we estimated the pattern of nucleotide substitutions in order to know characteristics of the mutation mechanism. The rate of nucleotide substitution between C and T was the highest for HBV. This feature is very different from those of retroviral genes (the rate of nucleotide substitution between G and A is the highest) but similar to those of eukaryotic genes, although it is believed that HBV replicates itself by reverse transcriptase. Based on the results, we proposed a model of mutation mechanism of HBV. The model suggests that pyrimidine analogues could be more effective anti-HBV drugs than purine analogues. Ueda et al., have recently reported that oxetanocin A (OXT-A), analogue of aranine, inhibited HBV replication more effectively than 3'-azido-3'-deoxythymidine (AZT), analogue of thymidine. For details, see the Abstracts of the Fourth International Congress of Systematic and Evolutionary Biology (ICSEB IV) 154.

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Molecular Evolution of Kunitz-type Protease Inhibitor
Kazuho IKEO, Kei TAKAHASHI and Takashi GOJOBORI

The Kunitz-type protease inhibitor is known as a serine protease inhibitor. It is also known that this inhibitor is mainly found in blood or saliva as a kind of soluble protein. Recently, an insertion of the Kunitz-type sequence was found in the protein sequence of the amyloid beta precursor. It is known that this protein accumulates in the neuritic plaques and cerebrovascular deposits in the patients with Alzheimer's disease. Collagen type VI in chicken also has an insertion of Kunitz-type sequence. To elucidate the evolutionary origin of these insertion sequences, we constructed a phylogenetic tree using all the available sequences of the Kunitz-type inhibitors. This tree showed that the ancestral gene of the Kunitz-type inhibitor appeared about 500 million years ago. Thereafter, this gene duplicated itself many times and some of these duplications were inserted into other protein-coding genes. During this process, the insertion sequence in the amyloid precursor diverged from its ancestral gene about 350 million years ago, and was inserted into the amyloid beta gene after duplication. Although the function of these insertion sequences is unknown, we have shown that these insertion sequences have an evolutionarily close relationship with each other.

Patterns of Nucleotide Substitution in Class I Major Histocompatibility Complex Genes
Tadashi IMANISHI and Takashi GOJOBORI

The major histocompatibility complex (MHC) genes are highly polymorphic in humans so that the value for heterozygosity exceeds 0.9. There has been controversy over the proposed causes of such extremely high polymorphism in MHC genes. Recently, it was proposed that MHC polymorphism is probably generated by overdominant selection. If this is the case, selective forces may have significantly affected the patterns of nucleotide substitution in MHC genes. To examine this possibility, we examined the patterns of nucleotide substitution in MHC genes.

To estimate the substitution patterns, we used the following method. First, we reconstructed the phylogenetic trees of human class I MHC genes. Second, we estimated the nucleotide sequences of the ancestral genes by
using the maximum parsimony method, and determined the direction of nucleotide changes from a particular nucleotide (A, T, C, or G) to another. Finally, we calculated 12 parameters of relative substitution frequencies, which comprised the substitution patterns.

The substitution pattern at the antigen recognition sites (ARS) in functional MHC genes was remarkably different from that at the remaining coding region (Non-ARS). In particular, the proportion of transitions \( (P_t) \) among all substitutions was extremely low at the third codon positions of ARS. In the HLA-A genes, \( P_t \) at the third codon positions was only 6% in ARS, whereas it was 69% in Non-ARS. In HLA-B, the corresponding values were 30% in ARS and 80% in Non-ARS, respectively. On the other hand, \( P_t \) in a class I pseudogene (HLA-AR) was 57% showing a strong similarity to \( P_t \) in other pseudogenes.

Since pseudogenes are selectively neutral, the pattern in pseudogenes is almost identical with the pattern for spontaneous substitution mutations. The pattern in functional genes usually deviates from the pattern in pseudogenes because they are subject to natural selection or functional constraints. In protein coding genes, transitions at the third codon positions rarely cause amino acid replacements, while about half of all transversions do so. Accordingly, \( P_t \) at the third codon positions decreases if the rate of amino acid replacements is enhanced by natural selection, but increases if amino acids are conserved by functional constraint. Our observations imply that the ARS region is subject to natural selection favoring amino acid replacements while the Non-ARS region is subject to functional constraints.

**Establishment of a Phylogenetic Survey System for AIDS-related Lentiviruses and Demonstration of a New HIV-2 Subgroup**

Tomoyuki Miura, Masashi Fukasawa, Etsuko N. Moriyama, Takashi Gojobori, Koh-ichi Ishikawa, Hajime Tsujimoto and Masanori Hayami

We designed a universal primer (UNIPOL) for DNA amplification of AIDS-related viruses. The phylogenetic tree constructed from the presumed sequences amplified with UNIPOL was representative of the tree calculated from whole pol gene sequences so far reported. UNIPOL was able to amplify the sequences of all four major groups of primate lentiviruses and also that of a distinct virus from a Ghanaian patient with an AIDS-related
complex, designated GH-2. This strain rarely hybridizes with known HIV/simian immunodeficiency virus (SIV) DNA probes. Sequence analysis of the only amplified fragment revealed quickly that GH-2 was quite similar to the recently reported HIV-2_{ALT} (D205) and that these two viruses form a new subgroup distinct from known HIV-2 and SIV_{mac}/SIV_{sm} in the large HIV-2 group. This system will be useful for further phylogenetic study of various primate lentiviruses. For details, see *AIDS* 4, 1257–1261, 1990.

*Maternal hybrid rescue (mhr) Gene which Rescues Embryonic Lethal Hybrids from Drosophila simulans Females Crossed with D. melanogaster Males*

Kyoichi SAWAMURA and Takao K. WATANABE

Hybrid females from the crosses of *D. simulans* ♀ × *D. melanogaster* ♂ are embryonic lethal, while hybrid males from the reciprocal crosses are larval lethal. We found new *D. simulans* strains which rescued the former embryonic lethality. The rescue gene(s) was named *maternal hybrid rescue (mhr)*, since it had a maternal effect. It was recessive and located on the second chromosome. Embryonic lethality was caused by the incompatibility between the cytoplasm of *D. simulans* (C,) and the X chromosome of *D. melanogaster* (X_m), whereas the larval lethality was caused by the non-existence of the X chromosome of *D. simulans* (X_s). Therefore, hybrid males (C_sX_mY_s) from the crosses of *D. simulans* X/X/Y ♀ × *D. melanogaster* ♂ should be doubly lethal, for both the embryonic and larval stages. The embryonic lethality was rescued by *mhr*, and the larval lethality was rescued independently by the *Hybrid male rescue (Hmr)* gene of *D. melanogaster*.

*Zygotic hybrid rescue (Zhr) Gene which Rescues Embryonic Lethal Hybrids from Drosophila melanogaster Males Crossed with Sibling Species*

Kyoichi SAWAMURA and Takao K. WATANABE

Interspecific hybrid females from the crosses of *D. simulans* ♀ × *D. melanogaster* ♂ are generally embryonic lethal. They were zygotically rescued by two paternal strains. We analyzed them genetically, and named their rescue genes *Zygotic hybrid rescue (Zhr)* and *Zygotic hybrid rescue-
temperature sensitive (Zhr<sup>ts</sup>). They were located on the X chromosome of <i>D. melanogaster</i>, proximal to 1-62.6 and to 1-56.7, respectively. Zhr also rescued the embryonic lethalities of hybrid females from the crosses of <i>D. mauritiana</i> ♀ × <i>D. melanogaster</i> ♂ and of <i>D. sechellia</i> ♀ × <i>D. melanogaster</i> ♂, but Zhr<sup>ts</sup> did not. However, if some chromosomal parts of <i>D. mauritiana</i> or <i>D. sechellia</i> were replaced by the <i>D. simulans</i> chromosome homozygously, they were also rescued by Zhr<sup>ts</sup>. Finally, we demonstrated the independence of three hybrid lethalities in crosses between <i>D. melanogaster</i> and <i>D. simulans</i>: (1) embryonic lethality caused by incompatibility between the cytoplasm of <i>D. simulans</i> and the X chromosome of <i>D. melanogaster</i>; (2) larval lethality caused by the non-existence of the X chromosome of <i>D. simulans</i>; and (3) temperature sensitive pupal lethality caused by incompatibility between the X chromosomes of these two species.
Characterization of Genetic Polymorphisms in Tandem Repeated Sequence Blocks

Takashi Imamura

Tandemly repetitive DNA sequences are thought to have the potential to express the locus-specific organization of their folding structure in the nucleus. Because of the inherent possibilities of rapid evolutionary sequence alterations, including a tolerance for mutation events, repetitive sequence units are predestined to develop and balance locus-specific repetitive higher order structures. This potential may create a specific chromatin folding structure whenever there is a selection force at the position of this repetitive DNA sequence in the genome. Human tandemly repetitive sequences are of interest for two reasons. First they define the boundaries or the signposts of the physical and genetic maps of human chromosomes and thus particularly important in the physical and genetic mapping of the human genome that is now being attempted. Second, some of them are essential for human chromosome function. In this study, I attempted to explore the principal organization of human telomere-associated (or proterminal) DNA at 48 distinct telomeres and that of an apparently new repeat sequence family which was found in PCR-amplified genomic DNA. The preliminary results suggest that these DNAs and organized as a string of distinct repeated sequence elements. The chromosomal distribution of these sequences could be highly polymorphic. These data thus identify a new source of human genetic variation, emphasizing the evolutionary conservation of the organization of tandemly repeated sequence family.

Human telomeres are similar to those of simple organisms. It has been shown that the telomere repeat of (TTGGGG)n from *Tetrahymena thermophila* will cross-hybridize with human telomeres, and that the cloned *Arabidopsis* telomere repeat (TTAGGG)n also recognizes human telomeres. Human telomeres have repeating units of (TTAGGG), equivalent to those of the trypanosome telomere, which suggest that in humans, as in yeast, DNA sequences that are significant for telomere function are telomeric simple repeated sequences. I used a series of synthetic oligonu-
cleotides and restriction enzymes to clone directly this sequence from human chromosomal DNA, mainly by using a ploymerase chain reaction. The present and previous reports suggest that human telomeres are not uniform throughout their length, comprising several multiple, different, but related tandem motifs. In somatic tissue cells, the major, most distal, component consists of 5–10 kb of repeats that are very similar to the sequence (TTAGGG)n. A (TTAGGG)4 probe can now be used to identify cloned telomeric DNA fragments and the flanking sequences derived from both ends of each human chromosome. Study is now being directed towards cloning and sequencing of the subtelomeric or so-called proterminal single copy fragments adjacent to these repeating units, which should prove to be invaluable genetic markers for completing the linkage map of the human genome and for characterizing chromosome rearrangements involving telomeres, which may have been stabilized during evolution.

In the course of this study an unrelated new family of repetitive DNA was identified. A tight genomic linkage was observed through the use of non-radioactive in situ hybridization assay between this repetitive DNA and acrocentric chromosomes 13, 14, 15, 21 and 22; the centric region of chromosome 1 also had this repeated sequence block, identified by the strong fluorescent signal on metaphase chromosome preparations. These results suggest that the long range sequence organization is not related to any satellite or alphoid DNA sequence. From the sequence analysis of a series of these repeat units, the polymorphic sequence divergence will be estimated in due course.

Analysis of Genetic Polymorphisms in the Human Alu Sequence Family and the Flanking Regions for Genetic Mapping of Chromosomes 18 and X
Hitoshi Nakashima, Masako Sakai, Tomoko Hasegawa and Takashi Imamura

As a step towards constructing the complete genetic linkage map of chromosome 18, we made a gene library from a mouse hybrid cell line with an extra human chromosome 18, using the pWE15 cosmid cloning vector. We attempted to place these clones at distinct bands of chromosome 18 using in situ hybridization techniques and high sensitivity microscopic fluorescent imaging apparatus. Genetic polymorphisms in regions that
are identified with these clones were analysed with one of the clone located either on the short or on the long arm of chromosome 18. For this purpose we isolated several fragments from each cosmid clone that contains the Alu family sequence and the flanking DNA including the poly (A) tail sequence. The results suggest that the Alu family sequence and the flanking region are polymorphic and could be site-specifically identified. We are continuing this line of work, aiming at the construction of a high resolution genetic linkage map for chromosome 18 with a series of at least 100 well spaced polymorphic markers.

One of the clones was found to be hybridized specifically with DNA at the centromere of chromosome X. Using this clone as a probe, both the genetic polymorphism in the centric DNA and the parental origin of chromosome X could be identified through in situ hybridization.

**Beta-Thalassemia Resulting from Compound Heterozygosity for Globin Gene Mutation in a Japanese Family: Evidence for Multiple Origin of the Thalassemia gene in Asian Populations**

Hitoshi NAKASHIMA, Yutaka CHIFU, Eisuke YOKOTA and Takashi IMAMURA

Thalassemias are a heterogeneous group of inherited disorders of hemoglobin synthesis, all being characterized by the absence or reduced output of one or more of the globin chains of hemoglobin. This leads to an imbalanced globin chain synthesis. One of the most remarkable aspects of thalassemia is how it has been possible to relate the diverse clinical manifestations to primary genetic defects in the α- or β-globin genes. Thalassemias are the most common single gene disorder in the world population. Genetic studies at the DNA level disclosed more than 40 different molecular varieties of thalassemia. Thus, it is common for individuals to receive genes for more than one type of thalassemia.

Although thalassemias are not frequent in Japanese, we encountered a patient with relatively severe disease resulting from heterozygosity for two types of β-thalassemia. One mutation is a C to T substitution at IVS-2 position 654 and the second is a G to A substitution at IVS-2 position 1. These two different mutations in the β-globin allele are associated with the restriction fragment length polymorphism (RFLPs) haplotype IX, defined
in Mediterranean population, and X not yet defined, respectively. The IVS-2 654 mutation is commonly found in the Chinese population, associated with haplotype I. The IVS-2 1 mutation was found in a Mediterranean family, associated with haplotype IX, but as yet there is no known case of this mutation in Asiatic populations. Thus, studies of mutation at the DNA level and the linked polymorphisms in and around the β-globin gene suggest that these mutations have arisen independently in different populations.

Before it was possible to understand the remarkable heterogeneity of β-thalassemia by analysing human DNA directly, a great deal was written about the distribution of β-thalassemia mutations, as was the case for the sickle cell gene, under the assumption that population movements could be discerned from the distribution of genetic markers of this type. More recently, however, it has been possible to define precise molecular lesions and β-globin gene haplotypes by examining the pattern of restriction fragment length polymorphisms (RFLPs) in and around the β-globin gene carrying a thalassemia mutation. The particular arrangement of RFLPs is referred to as the β-globin gene haplotype. These results suggest that the mutation may have had multiple origins.

Molecular Phylogeny and Evolution of Human Mitochondrial DNA

Satoshi Horai

Using restriction fragment length polymorphism of mitochondrial DNA (mtDNA), my colleagues and I first showed that the Japanese population could be separated into two distinct groups: A group with smaller frequency (group I) first diverged from the other group with larger frequency (group II). By a more exhaustive survey, the existence of the two groups was confirmed in two other Japanese populations, though the frequencies of the groups are different among populations. A phylogenetic analysis among three major racial groups indicated that the group I Japanese and the majority of Negroids first diverged from the rest of Japanese (group II) and Caucasoids. Later, we extended our analysis to various ethnic groups of humans to study their evolutionary relationships by the sequence analysis of the major noncoding region of mtDNA. Based on the sequence comparison of over a hundred of individuals, remarkable features of nucleotide
substitutions and insertion/deletion events have been revealed. The nucleotide diversity among the sequences is estimated as 1.45%, which is three to six-fold higher than the corresponding value estimated from restriction enzyme analysis of the whole mtDNA genome. More recently we applied polymerase chain reaction (PCR) to molecular evolutionary studies on not only contemporary but archaeological samples. We have succeeded to amplify mtDNA extracted from an ancient Japanese bone, whose age is estimated at about 6000 years B.P., and determined the nucleotide sequence of part of the major noncoding region. Sequence comparison shows that the ancient individual has a close phylogenetic affiliation to Southeast Asians.

It is obvious that the Mongoloids can be separated into two subpopulations in the present sequence analysis. This observation confirmed our earlier study which clearly demonstrated the existence of two distinct groups in the Japanese by restriction enzyme analysis. Most of those who belong to the group I cluster in the Japanese have the deletion of 9-bp in region V (Cann and Wilson 1983). This deletion is observed not only in East Asians, Indonesians, Taiwan Chinese but also in Polynesians with a very high frequency. The same deletion has never found in Caucasoids and Negroids. Thus, the existence of two major groups is one of characteristics found commonly among Mongoloid populations. Nowadays Mongoloid descendants live in North and South America, Oceania, South East Asia, East Asia and Siberia, adapting themselves to various environment on the earth. An extended study of mtDNA polymorphism and its genealogy by using both contemporary and archaeological samples will be required to confirm the origins and dispersal of the two subpopulations of Mongoloids.


**Mitochondrial DNA Evolution in Primates: Transition Rate Has Been Extremely Low in the Lemur**

Masami Hasegawa*, Hirohisa Kishino*, Kenji Hayasaka and Satoshi Horai

Based on mitochondrial DNA (mtDNA) sequence data from a wide range of primate species, branching order in the evolution of primates was
inferred by the maximum likelihood method of Felsenstein without assuming rate constancy among lineages. Bootstrap probabilities for being the maximum likelihood tree topology among alternatives were estimated without performing a maximum likelihood estimation for each resampled data set. Variation in the evolutionary rate among lineages was examined for the maximum likelihood tree by a method developed by Kishino and Hasegawa. From these analyses it appears that the transition rate of mtDNA evolution in the lemur has been extremely low, only about 1/10 that in other primate lines, whereas the transversion rate does not differ significantly from that of other primates. Furthermore, the transition rate in catarrhines, except the gibbon, is higher than those in the tarsier and in platyrrhines, and the transition rate in the gibbon is lower than those in other catarrhines. Branching dates in primate evolution were estimated by a molecular clock analysis of mtDNA, taking into account the rate of variation among different lines, and the results were compared with those estimated from nuclear DNA. Under the most likely model, where the evolutionary rate of mtDNA has been uniform within a great apes/human clade, human/chimpanzee clustering is preferred to the alternative branching orders among human, chimpanzee, and gorilla. For details, see J. Mol. Evol. 31, 113–121 (1990).

A Common Mitochondrial DNA Mutation in the tRNA<sub>Lys</sub> of Patients with Myoclonus Epilepsy Associated with Ragged-Red Fibers

Makoto Yoneda*, Yoshinori Tanno*, Satoshi Horai, Takayuki Ozawa**, Tadashi Miyatake* and Shoji Tsuji*

Nucleotide sequence analyses of muscle mitochondrial DNA (mtDNA) from a patient with myoclonus epilepsy associated with ragged-red fibers (MERRF) revealed 33 single base substitutions, including 23 in coding regions for mitochondrial polypeptides and 10 in non-coding regions, as compared with the normal human mtDNA sequence. Three substitutions, in COI, ND4, and Cytb, would result in amino acid substitutions, which are conserved among species. Of three patients with MERRF, all had an

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identical A to G base substitution only at nucleotide position 8344 in the tRNA$_{\text{Lys}}$ region. The substitution was not found in 15 controls. Various degrees of the combined enzymic defects in the oxidative phosphorylation system of mitochondria were found in the MERRF patients. The defects could be explained by altered function or processing of the mutant tRNA$_{\text{Lys}}$. This mutation in the tRNA$_{\text{Lys}}$ is the most probable cause of MERRF. For details, see *Biochemistry International*, 21(5), 789-796 (1990).

A Mutation in the tRNA$_{\text{Leu(UUR)}}$ Gene Associated with the MELAS Subgroup of Mitochondrial Encephalomyopathies

Yu-ichi Goto*, Ikuya Nonaka* and Satoshi Horai

Mitochondrial encephalomyopathies are usually divided into three distinct clinical subgroups: (1) mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes (MELAS); (2) myoclonus epilepsy associated with ragged-red fibers (MERRF); and (3) chronic progressive external ophthalmoplegia (CPEO) including Kearns-Sayre syndrome. Large deletions of human mitochondrial DNA and a transition mutation at the mitochondrial transfer RNA$_{\text{Lys}}$ gene give rise to CPEO including Kearns-Sayre syndrome and MERRF, respectively. Here, we report an A-to-G transition mutation at nucleotide pair 3,243 in the dihydrouridine loop of mitochondrial tRNA$_{\text{Leu(UUR)}}$ that is specific to patients with MELAS. Because this mutation creates an *Apa I* restriction site, we could perform a simple molecular diagnostic test for the disease. The mutation was present in 26 out of 31 independent MELAS patients and 1 out of 29 CPEO patients, but absent in the 5 MERRF and 50 controls tested. Southern blot analysis confirmed that the mutant DNA always coexists with the wild-type DNA (heteloplasmy). For details, see *Nature* 348, 651-653 (1990).

Chronic Progressive External Ophthalmoplegia: a Correlative Study of Mitochondrial DNA Deletions and Their Phenotypic Expression in Muscle Biopsies

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Deleted mitochondrial DNA (mtDNA) has been shown to coexist with

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normal mtDNA (heteroplasy) in muscles from chronic progressive external ophthalmoplegia, including Kerans-Sayre syndrome. In this study, we correlated heteroplasmic mtDNA abnormality with clinical, biochemical and histological findings with the following results: (a) large deletions ranging from 1.8 to 8.8 kb in 22 muscle specimens from 28 patients who had ophthalmoplegia clinically and focal cytochrome c oxidase (CCO) deficiency by histochemistry, (2) no difference in clinical and biochemical findings between patients with and without mtDNA deletions, (3) no relationship between the size, site or populations of deleted mtDNA and respiratory chain enzyme activities in muscles, (4) positive correlation between the number of CCO-deficient fibers and the populations of deleted mtDNA, and (5) higher incidence of CCO-negative fibers in patients with deleted mtDNZ than in those with no deletion of mtDNA. These results suggest that deleted mtDNA is, at least in part, responsible for focal CCO deficiency as a phenotypic expression and that the investigation on pathogenetic mechanism of focal CCO deficiency may provide a clue to understanding the underlying pathophysiology in this disorder. For details, see *Journal of Neurological Science*, **100**, 63-69 (1990).

Renal Tubular Involvement Mimicking Bartter Syndrome in a Patient with Kearns-Sayre Syndrome


A 10-year-old boy had short stature, external ophthalmoplegia, atypical retinal pigmentary degeneration, and sensorineural hearing loss (Kearns-Sayre syndrome). In addition to ragged-red fibers observed on modified Gomori trichrome staining, there were scattered fibers exhibiting no cytochrome c oxidase activity, indicating a focal deficiency. Cytochrome c oxidase and other respiratory chain enzyme activities were normal biochemically. The patient also had renal tubular dysfunction, including isosthenuria, decreased urine-concentrating ability, and excessive excretion of potassium and magnesium. In addition, he had hyperreninemia and hyper-
aldosteronism but no hypertension. The renal dysfunction was thought to have resulted from a primary defect in the thick ascending limb of the loop of Henle, mimicking Bartter syndrome. In contrast to previously described cases of cytochrome c oxidase deficiency with de Toni-Fanconi Debre syndrome, the patient had less intensive muscle abnormalities. A renal biopsy specimen showed ultrastructural changes in mitochondria that were similar to those seen in biopsy specimens of muscle. A large-scale deletion (8.8 kilobases) in mitochondrial DNA was found in biopsy specimens of muscle and kidney. For details, see *The Journal of Pediatrics*, 116(6), 904–910 (1990).

Mitochondrial Myopathies: Morphological Approach to Molecular Abnormalities


Cytochrome c oxidase (CCO) activity was shown by biochemical and histochemical examination to be decreased in the skeletal muscles of twelve patients with mitochondrial myopathies, especially in 8 chronic progressive external ophthalmoplegia (CPEO) cases, which included 2 Kearns-Sayre syndrome and 6 ocular myopathy patients. In 4 MELAS patients, NADH cytochrome c reductase activity was decreased. Immunocytochemical examination, using anti-CCO, anti-complex I and III rabbit sera revealed that CCO was stained more weakly in the muscle fibers of one of the CPEO patients than in those of the control. Immuno-electron microscopic examination of CCO, complex I and III, using a method of gold labeling, was also performed. Extensive labeling by gold particles, representing the localization of respiratory enzymes, could be seen in close vicinity to the cristae and inner mitochondrial membrane of normal shaped mitochondria. The concentration of gold particles was markedly decreased in one of the CPEO patients. To detect the localization of mitochondrial DNA or mRNA, in situ hybridization was performed on human biopsied muscles using a 35S labeled mitochondrial DNA probe. The wide distribution of autoradiographic grains for mRNA over the sarcoplasm of all muscle fibers

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was correlated with the distribution of immno-stained mitochondria. Southern blotting revealed large deletions of mitochondrial DNA in six of the patients with CPEO. In one of these patients, in situ hybridization showed a marked decrease in density of autoradiographic grains in muscle fibers. For details, see *Bioenergetics*, Edited by C. H. Kim and T. Ozawa, pp. 429–439 (1990).

**In Situ Hybridization of Muscle Mitochondrial mRNA in Mitochondrial Myopathies**

Shinji Nakamura*, Takeshi Satō*, Hiroko Hirawake*, Ryo Kobayashi* and Satoshi Horai

To determine whether a mitochondrial mRNA deficiency exists in mitochondrial myopathies, muscle biopsies from a patient with chronic progressive external ophthalmoplegia (CPEO) and a patient with mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes (MELAS) were studied using *in situ* hybridization. Histochemistry and immunohistochemistry were performed along with hybridization. Hybridization reactions were widely distributed over the sarcoplasm of all muscle fibers in the patient with MELAS. In the patient with CPEO, 80% of the fibers showed a marked decrease in density of autoradiographic grains. This marked decrease corresponded to the histochemical and immunohistochemical findings of a very weak staining of cytochrome c oxidase (CCO). The isotope-labeled mtDNA probe used in *in situ* hybridization in this study complements a part of subunit I of CCO and a part of subunit II of complex I in the mitochondrial gene. Our results suggest a defect in the mRNA in this CPEO patient. For details, see *Acta Neuropathol.* **81**, 1–6 (1990).

**A Tenascin-like Gene Found in the Human Major Histocompatibility Complex Class III Region**

Ken-ichi Matsumoto, Miyuki Arai, Nanayo Ishihara, Asako Ando**, Hidetoshi Inoko** and Toshimichi Ikemura

Walking and sequencing a genome portion centromeric to CYP21B in the human MHC class III disclosed a clustered occurrence of fibronectin type-III repeats in a ca. 50 kb DNA segment. The repeats are known to

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consist of ca. 90 amino acid residues and are present in a wide range of protein species. A homology search of Protein Databases showed our repeats to have the highest homology with repeats of tenascin, an extracellular matrix protein. Furthermore, we found that the OSG cDNA sequence, a portion of which was mapped on the opposite strand of CYP21B, had six type-III repeats followed by a fibrinogen domain and these and our repeats showed the same polarity. Upstream of the type-III repeats (at least 14 repeats in total), we found 18.5 EGF-like repeats and 4 heptad repeats. The organization of these repeats and of the fibrinogen domain at the C-terminal part was closely related with that of tenascin. Thus we concluded that there exists a tenascin-like gene in the HLA locus (Genomics in press).

Evident Diversity of Codon Usage Patterns of Human Genes with Respect to Chromosome Banding Patterns and Chromosome Numbers; Relationship between Nucleotide Sequence Data and Cytogenetic Data

Toshimichi Ikemura and Ken-nosuke Wada*

The sequences of the human genome compiled in DNA databases are now about 10 megabase pairs (Mb), and thus the size of the sequences is several times the average size of chromosome bands at high resolution. By surveying this sequence data, it may be possible to clarify the global characteristics of the human genome, through correlation of gene sequence data (kb-level) with cytogenetic data (Mb-level). By extensively searching the GenBank database, we calculated codon usages in about 2000 human sequences. The highest G+C percentage at the third codon position was 97%, and that of about 250 sequences was 80% or more. The lowest G+C% was 27%, and that in about 150 sequences was 40% or less. A major portion of the GC-rich genes was found to be on special subsets of R-bands (T-bands and/or terminal R-bands). AT-rich genes, however, were mainly on G-bands or non-T-type internal R-bands. Average G+C% at the third position for individual chromosomes significantly differed among chromosomes, and was related to T-band density, quinacrine dullness, and mitotic chiasmata density in the respective chromosomes (see Ikemura, T., Wada, K., and Aota, S., Genomics 8, 207–216 (1990); Ikemura, T., and Wada, K., Nucl. Acids Res. 19, 4333–4339 (1991)).

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VIII. APPLIED GENETICS

Spatial Pattern of Genetic Variation within Wild Rice Populations

Hiroko Morishima and Pascale Barbier*

The spatial pattern of genetic variation within natural populations is an important determinant of evolutionary and ecological genetic processes in plants. It is also a key factor in considering strategies for sampling natural populations. Fine scale spatial distribution of genetic variation within natural populations of Asian common wild rice (*Oryza rufipogon*) was investigated using isozyme markers.

Two wild rice populations examined were a perennial-type population growing in a roadside ditch (NE88) and a weedy type population growing at the fringe of a rice field (CP20, perennial-annual intermediate type), both being from the Central Plain of Thailand. Seeds were sampled on an individual basis with the locations of respective plants being recorded on a map. In the perennial population, sampling was done in a long line-transect along the ditch. In the weedy population, sampling was done along five smaller line transects. Progenies were grown in a plant-to-row experiment at Mishima, and allozyme variations at 7 polymorphic loci were examined using plumules and leaves at the tillering stage. The genotype of each mother plant (individual in natural population) was estimated by allelic segregation in progeny. In the perennial population, 39 different multilocus genotypes were identified among 60 plants. In the weedy population, 42 different multilocus genotypes were found among 54 plants. To obtain quantitative measures representing the degree of similarity in multilocus genotypes among individuals within populations, allozyme data were analyzed in each population by a multivariate technique called “Quantification-III” or pattern analysis. Inspection of the geographical distribution of the score for each plant on the map revealed the contrasting spatial patterns for the perennial and weedy populations. In the perennial population, distribution of the scores was structured forming “genetic neighbors”, in other words similar genotypes were closely distributed. It was also found that a number of “genets” existed although the plants prop-

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agate mainly by vegetative means in this population. In the weedy population which propagates both by seeds and vegetative means, however, distribution of genotypes seemed to be rather random.

Spatial distribution patterns of neutral genes such as isozymes may be a function of reproductive biology of the plants and the degree of habitat disturbance. Outcrossing rates of these two populations, which were estimated by the same set of data, proved to be about 50–55% for both populations. Habitat is relatively stable in the perennial population, but strongly disturbed in the weedy population. Under such circumstances, identities of the neighbors are easily preserved and genetic variation tends to be geographically structured in the former population. In the latter population, gene flow efficiently functions through a high rate of seed propagation blurring genetic neighbors. Further, strong habitat disturbance might aid in the decay of structured genetic variation.

A Nuclear Gene Inducing Fertility Restoration in Cytoplasmic Male Sterile Rice

Yoshio Sano

Cytoplasmic male sterility (CMS) is characterized by a failure of affected plants to produce functional pollen. The trait is maternally inherited, and most evidence associates it with changes in the mitochondrial genome. Although maternally inherited traits are stably transmitted to progenies in most cases, Laughnan and his coworkers found that spontaneous reversions to male fertility including cytoplasmic and nuclear changes occur not infrequently in S type male-sterile cytoplasm of maize. The nuclear genotype governs the frequency of reversions as well as the relative frequency of cytoplasmic and nuclear reversions. Recent molecular studies revealed that cytoplasmic reversions are also highly associated with reorganization of mitochondrial genomes. However, the genetic mechanism inducing spontaneous reversions remains to be studied. The present study gives evidence on the presence of a single nuclear gene which induces nuclear reversion in CMS rice.

Rice cytoplasms which exhibit CMS are classified into different types on the basis of the effects of different nuclear genes which can restore fertility (Rf genes). Normal cytoplasm allows fertile pollen development in all nuclear backgrounds. The (cms-bo) cytoplasm of Chinsurah boro II
exhibits male sterility when combined with the nucleus of Taichung 65 (T65A) as reported by Shinjo (1975). Fertility restoration of (cms-bo) cytoplasm is gametophytically controlled by Rf1. Although male sterility is stably expressed in the genetic background of T65A, Taichung 65 (T65B) preserved in our laboratory showed about 8% seed fertility when pollinated to male-sterile plants (cms-bo)rf1,rf1. Unexpectedly, a strange phenomenon was detected in later generations of the hybrid, namely seed fertility gradually increased with repeated selfings and finally almost fully fertile plants were obtained in the F6 generation. In this experiment, seed fertility was examined by strictly bagging to eliminate cross pollination. The occurrence of fertile segregants might be simply explained by the assumption that T65B carries recessive restorers and their accumulation gives rise to fertile plants in later generation of the hybrid. The assumption was tested by establishing plants with (cms-bo) cytoplasm and a T65B nucleus by successive backcrossings. The assumption was ruled out since resultant plants still showed about 6% seed fertility. Accordingly, the fertile plants observed in F6 might have resulted from mutational events such as found in the S-type of maize cytoplasm.

The cytoplasm of the fertile revertant (FR37-4) was examined in relation to cytoplasmic reversion. FR37-4 was repeatedly backcrossed with T65A possessing rf1,rf1. Results indicated that no cytoplasmic change was detected in FR-4 since the cytoplasm was completely male sterile in the T65A genetic background. Therefore, mutational events seem to have occurred in the nuclear genes. A gradual increase of fertility strongly suggested that multiple genes are involved in the fertile revertant. FR37-4×T65B F2 gave more fertile plants than (cms-bo)rf1,rf1×T65B F2 supporting the assumption that FR37-4 carries mutant nuclear genes. Conventional genic analysis for them, however, seems to be impossible because of instability.

The segregation manners observed in later generations of (cms-bo)rf1,rf1×T65B suggested that genes controlling partial fertility in the hybrid are associated with the induction of fertility reversion since F2 plants showing different degrees of fertility similarly exhibited a gradual increase in fertility with selfings. In other words, partially fertile plants seemed to produce fertility restoration with repeated selfings. Therefore, the genetic control for partial fertility was examined. When (cms-bo)rf1,rf1 was pollinated with the pollens of T65A×T65B F1, completely sterile and partially fertile plants segregated into a 1:1 ratio, suggesting that partial fertility is con-
trolled by a single dominant gene. From the assumption, when T65A × T65B F₂ individuals were crossed to (cms-bo) rf₁rf₁, three different types of F₂ individuals which produce only sterile plants, only partially fertile ones or both would arise. Results supported the above assumption showing the expected ratio of 1:1:2. In addition, an allelism test revealed that the gene seemed to be independent of Rf₁. The gene detected was designated Ifr₁(t). At present, whether Ifr₁(t) gives rise to mutational events only in the presence of (cms-bo) cytoplasm is under investigation.

**Developmental Genetics of Phenotypic Plasticity in Rice**

Mitsgu Eiguchi and Yoshio Sano

Plants utilize environmental signals for altering the timing of gene expression so as to respond to changing environments. A drastic change in morphology due to changing environments is known as phenotypic plasticity which is particularly important in plants because of their sessile life style. An example of plastic response is deepwater or floating rice, whose internodal elongation starts with increasing water depth as a means for escaping submergence, resulting in various changes in morphology. However, no or little internodal elongation occurs in non-deepwater rice as well as in air-grown deepwater rice. A number of recent experiments indicated that deepwater tolerance is related to enhanced internodal elongation resulting from the action of the plant hormones ethylene and gibberellin. Although the inheritance of deepwater tolerance seems to be complex, genetic and developmental regulation gives an excellent opportunity for understanding the mechanisms of phenotypic plasticity in plants. In the present study we successfully detected a major gene which is responsible for deepwater tolerance in rice. We report here that responding to flood, the gene for deepwater tolerance induces elongation of basal internodes whose intercalary meristem produces no elongation without the gene.

The materials used were a perennial type of *Oryza rufipogon* (W120 from India) and a near isogenic line of Taichung 65 with wx (T65wx). Generally, perennial types of *O. rufipogon* shows tolerance for deepwater since they prefer deepwater and stable habitats while Taichung 65 is a non-deepwater rice cultivar intolerant of flood. In order to introduce an alien factor(s) responsible for deepwater tolerance, the T65wx × W120 F₁ was successively backcrossed with T65wx as the recurrent parent. In each
generation, F2 plants were grown in a deepwater tank and subjected to flood at 6 weeks of age. The water level was raised 10 cm every other day to a maximum depth of 100 cm. Under those conditions, only 1/5 to 1/4 of F2 plants survived and came to flowering and these were used for further backcrossings. Segregation data in BC3F1, BC3F2 and BC3F3 consistently supported the assumption that deepwater tolerance is controlled by a single recessive gene which was designated dw3.

To examine gene expression of dw3 during the course of development, plants homozygous for dw3 were subjected to flood at different stages. When subjected to flood after 6 weeks of age the homozygotes survived with elongation of internodes. However, they produced no internodal elongation and died when subjected to flood before 4 weeks of age, suggesting that internodal elongation capability in response to flood is age specific. The elongation of internodes begins around the time of floral initiation in the recurrent parent T65wx. The homozygote for dw3 exhibited internodal elongation before the time of floral initiation only when subjected to flood. Thus, the dw3 gene changes the patterns of internodal elongation rather than causing an increase in the rate of internodal growth depending on environmental conditions.

Population Biology of Resistance to Bacterial Leaf Blight Disease in Rice

Chika Hamamatsu and Hiroko Morishima

Leaf blight is a serious bacterial disease in rice caused by Xanthomonas campestris pv. oryzae (Xco). Racial differentiation in virulence is known among Xco isolates. In rice, on the other hand, about 20 resistance genes which show differential responses to infection by different Xco races were identified. Our objective is to draw a coevolutionary scenario of rice and Xco by investigating how the genetic structure of host populations affects that of pathogen populations, and vice versa. Our target system is primitive or non-agricultural communities. Understanding plant-pathogen interactions in non-agricultural ecosystems, which seem to be well balanced without causing a serious damage to plants, may help us in considering strategies for controlling disease epidemics in agro-ecosystems.

Using wild and landrace (native cultivars) populations of rice collected in Thailand, Bangladesh and China, we surveyed between- and within-
populational variability in resistance to four Japanese Xco races. The following results were obtained.

1) Wild rice plants are generally more resistant to Xco than cultivars, although response patterns differ in respect to host genotypes and pathogen races.

2) Response to Xco is highly variable among individuals within the same population in wild rice as well as in landrace rice. The level of diversity in resistance is not correlated to diversity in isozymes nor to that in quantitative traits.

3) Intrapopulational diversities in resistance revealed in the present study seem to be partly due to segregation of major genes (race specific response, discontinuous variation) and partly due to polygenes (race non-specific response, continuous variation).

4) In the samples collected in Bangladesh, populations of cultivars adapted to deepwater conditions frequently contain individuals which are more resistant than those adapted to shallow-water conditions and coexisting wild rice populations. This suggests that deepwater conditions favoured epidemics of Xco and that under such environment disease-mediated selection works on host populations.

The mechanism for preserving genetic variability in resistance of hosts (and most probably in virulence of pathogens too) should be elucidated. The fitness cost of resistance in plants as well as of virulence in pathogens might be the core issue to be discussed in this argument. We are planning to examine variability in natural populations of pathogens. Further, several F2 populations are under study in order to examine whether or not presumed major genes segregating in our materials are identical to already known genes.

**Size-Shape Variation of the Silica Body in Wild and Cultivated Rices**

Hiroshi FUJIWARA*, Yo-Ichiro SATO and Hiroko MORISHIMA

Silica (SiO2) absorbed by plants and accumulated on the surface of cell walls is called “plant opal”, forming replicas of plant cells. It remains in the soil for a long time after the plants are deposited and decayed. Since a plant-derived silica body has a species-specific morphology, analysis of

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plant opal excavated from the soil enables us to assess old vegetation formed on the soil in the past.

Hoping to shed light on the evolutionary process of rice species and the history of rice cultivation, we are studying plant opal obtained from the soil in excavation sites in Japan and China. In order to identify the type of rice which carried the excavated plant opal, we have to have information on inter- and intra-specific variation of plant opal morphology in wild (*Oryza rufipogon*) and cultivated (*O. sativa*) rice species.

In the present study, 53 strains of *O. rufipogon* collected in various Asian countries were surveyed regarding size and shape of plant opal, and data were analyzed together with those previously obtained for *O. sativa*. With the purified silica bodies, three size measurements (length, width and thickness) and two ratios derived from the above measurements were obtained for each strain. As no single characteristic could discriminate clearly wild from cultivated types, the preceding five characteristics were collectively analyzed using multivariate techniques. On the two-dimensional plane defined by the first and second multivariate axes mathematically extracted, wild strains shared a distribution range with two thirds of the cultivated strains. The remaining Indica and Japonica cultivars were distributed outside this wild-cultivar complex range showing their respective distribution ranges.

The present study indicated that from plant opal data alone it is not possible to discriminate wild from cultivated types, but samples which are distributed outside of the wild-cultivar complex range when examined in a multivariate plane could be judged as a cultivated type and furthermore as either Indica or Japonica type. Variation among wild rice strains did not show clear distinctions between two major ecotypes, perennial and annual types, but a trend in geographical variation was detected. Morphological variation observed in the present study might be largely due to genetic factors because samples were taken from plants grown under uniform conditions. Environmental factors as well as intrapopulational variability should be examined. According to our preliminary results, shape components, rather than size components, seem to be more reliable in the morphological analysis of plant opal obtained from the soil.
New DNA Fingerprinting Procedure: Amplified Fragment Length Polymorphism of Hazy Associations (ALPHA)

IKUO NAKAMURA

A new DNA fingerprinting procedure based on PCR technique (Mullis et al., 1986) has been developed in our laboratory. The procedure, named ALPHA (Amplified fragment Length Polymorphism of Hazy Associations), is designed to detect the size polymorphism of DNA fragments amplified by a standard PCR reaction using a single primer with a lower annealing temperature which causes a hazy association (allowing for incomplete homology) between primer and template. Thus, the ALPHA procedure could amplify and analyze unknown fragments in the genome without any sequence information. In this regard, the ALPHA procedure is different from RFLP (Botstein et al., 1980) and other DNA fingerprint methods such as VNTR (Jeffrey et al., 1985).

In a model experiment, four different oligonucleotides (#1; 5'-GATGTCTTCTTCAAGACCTACTCAA-3', #2; 5'-TGTACAGTTGTCCA-GAGGATGC-3', #3; 5'-CAGATCTGCTGGGACTCTTT-3', #4; 5'-CATGATATTATGCTGGAGC-3') used as primer in PCR reactions were arbitrarily taken from the DNA sequence of the rice phytochrome gene reported by Kay et al. (1989). PCR reactions were carried out in a 25 μl reaction mix, which contained 2.5 μl of 10x PCR buffer (100 mM Tris-HCl, 15 mM MgCl₂, 800 mM KCl, 0.5% BSA, 1% Na-cholate, 1% Triton X-100), 200 μM of four dNTPs, 4 μM of primer, 5 ng of rice total DNA as a template, and 1 unit of Thermus thermophilus DNA polymerase. The reactions were carried out in a thermal programmer for 42 cycles under these conditions: 1 min. denaturation at 92°C; 1 min. annealing at 37°C; and 2 min. polymerization at 72°C. Aliquots of four μl were analyzed by 0.8% agarose gel electrophoresis.

No amplified product was found when the #1 or #2 primers were used for the PCR reaction. On the other hand, some fragments were amplified when the #3 or #4 primers were used. If single 20-mer nucleotides were used as the primer for amplification, the association possibility of the primer with the completely complementary sequence of template DNA would be $1 \times 10^{-12}$. This value corresponds to a genome size as large as $2 \times 10^{13}$ bp., which exceeds a rice genome of $5 \times 10^8$ bp. by long. Some primers have been known to associate with template DNA even with a sequence homology
of 80%. Supposing it were true, the primer should theoretically associate with the template at an average distance of 500 bp. In this case, no fragment could be amplified by the PCR reaction. But some amplified fragments were actually found, depending on the primer sequence, in our experiment. This means that nucleotide sequences are not completely random in the rice genome.

The stringency of associations between the primer and template could be controlled by the changing annealing temperature. When the annealing temperature was raised, the number of amplified fragments decreased and the size of fragments tended to be reduced. These changes of amplification pattern in the ALPHA procedure might reflect the secondary structure of template DNA. This is advantage of the ALPHA procedure because different levels of polymorphism could be detected using the same primer by changing the annealing temperatures. Two important factors might be responsible for the amplification events of ALPHA procedure: 1) few bases at the 3' end of the primer sequence need to be perfectly matched with the template in order to start DNA polymerization with a thermostable DNA polymerase. 2) the remaining part of the primer has the key for determining the thermostability of the primer-template complex. How hazy associations occur in the PCR reaction and what primer sequence is suitable for the ALPHA procedure remain to be investigated.

The ALPHA procedure is not only a useful DNA fingerprint technique for detection of polymorphisms between closely related strains in various species, but also a convenient technique for cloning of DNA markers useful for RFLP analysis.

DNA Markers Specific to Subspecies of *Oryza sativa*
Detected by the ALPHA Procedure

Ikuo Nakamura, Katsuhiko Kanda* and Yo-ichiro Sato

Asian cultivated rice (*Oryza sativa*) is classified into two subspecies, *indica* and *japonica* according to characteristics found in morphological and physiological analyses (Oka. *Ind. J. Ganet. Plant Breed* 18, 79–89, 1958). Recent studies carried out by many researchers indicated that this classification holds true at the molecular level. (isozymes: Graszmann. *TAG*, 74,

* Gakken Plant Technology Institute.
We were able to identify new DNA markers specific to *indica* ecospecies using the ALPHA procedure (see Nakamura in this report). Typical *japonica* (Taichung 65) and *indica* (Ac 419) cultivars were analyzed by the ALPHA procedure where a single primer (5'-CATGATTATTGGCGTGGAGC-3') and 5 ng of genomic DNA from each cultivar were included in the reaction mixture, and reactions of 42 cycles were carried out with temperatures of 94°C for denaturation, 37°C for annealing and 72°C for extension.

Three DNA markers (α₁-J₁, α₁-J₂ and α₁-J₃) were identified as the sizes of ca. 750, 720 and 670 bp fragments, respectively. As these markers were found only when template DNA was extracted from typical *indica* cultivars, they might be useful for distinguishing the two subspecies. We made a survey of variations in these markers among native rice cultivars using the ALPHA procedure. Among 60 tested cultivars, 20 had all three markers, 30 had none of them and 10 had one or two of them. They were classified as *indica*, *japonica* and "recombinant" types, respectively (Table 1).

<table>
<thead>
<tr>
<th></th>
<th>indica</th>
<th>japonica</th>
<th>recombinant</th>
<th>sum</th>
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<td><strong>Sato's</strong></td>
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<td>indica</td>
<td>20</td>
<td>0</td>
<td>6</td>
<td>26</td>
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<tr>
<td>japonica</td>
<td>0</td>
<td>30</td>
<td>4</td>
<td>34</td>
</tr>
<tr>
<td>sum</td>
<td>20</td>
<td>30</td>
<td>10</td>
<td>60</td>
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Results showed a good correspondence to classifications done according to Sato's discriminant function (Sato *et al.*, *In New Front. Breed. Res.* pp. 185–191, 1986). Furthermore, this procedure is a useful tool for analyzing the evolution of the genome structure in the *indica-japonica* complex, because it can clearly identify recombinant types. These markers were also useful for analysis of ancient rice seeds excavated in archaeological sites (*Ann Rep.* 40).
IX. DATABASE

Construction of Nucleotide Sequence Data Base
Yoshihiro Ugawa, Hidenori Hayashida, Sanzo Miyazawa,
Yoshio Tateno, and Takashi Gojobori

As one of various activities at the DNA Data Bank of Japan, we continued construction of the database of nucleotide sequences and distribution of these data to the academic community in Japan. In particular, we started a reorganization of DDBJ and a program for improvement of data bank services. At the same time, we spent considerable time preparing the DDBJ database Release 8. This release will contain more than 870 entries that correspond to more than 1,570,000 nucleotide bases. We also developed several UNIX programs which were necessary for construction of the database. Moreover, we started improving the management of our computer network system by reorganizing the relevant software.

We collaborated on construction of the internationally unified database with GenBank in the United States and the EMBL Data Library in EC. International collaboration is essential for not only contributing to the academic community in the world but also maintaining the present activities of DDBJ. We will definitely continue collaborative works with GenBank and EMBL.

Codon Usage Tabulated from the GenBank Genetic Sequence Data
Ken-nosuke Wada*, Yoshiko Wada**, Hirofumi Doi***, Fumie Ishibashi,
Takashi Gojobori and Toshimichi Ikemura

Now the codon usages in 15137 genes can be analyzed using the nucleotide sequence data obtained from the GenBank Genetic Sequence Data Bank (Release 65.0, Sep., 1990). This codon database was registered as CUTG by LiMB Database (Listing of Molecular Biology databases, constructed by

* From Chukyo University, School of Computer and Cognitive Sciences, Nagoya.
** Niigata University, School of Medicine, Niigata.
*** From International Institute for Advanced Study of Social Information Science, Fujitsu Limited.
Los Alamos National Laboratory). Because of the growing size of the database, it had become impossible to print the data. Distribution of the database with the electric version of the Sequence Supplement of Nucleic Acids Research using a CD ROM, is planned possibly beginning in 1991. This year was a transition year, and thus we have sent upon request, a magnetic tape or a hard copy listing the codon usages in 15137 genes. For details, see Kennoske Wada, Yoshiko Wada, Hirofumi Doi, Fumie Ishibashi, Takashi Gojobori and Toshimichi Ikemura (1991) *Nucl. Acids Res.* **19** (Supplement), 1981–1986.


GOJOBO, T. and MORIYAMA, E. N.: Molecular phylogeny of AIDS viruses and its


MATSUMOTO, K., ARAI, M., ISHIHARA, N., ANDO, A., INOKO, H. and IKEMURA, T.: Cluster
of fibronectin type-III repeats found in the human major histocompatibility complex class III region shows the highest homology with the repeats in an extracellular matrix protein, tenascin. Genomics (in press).


MUTO, A., ANDACHI, Y., YUZAWA, H., YAMAO, F. and OSAWA, S.: The organization...


PUBLICATIONS


SHIMIZU, N., TAKEUCHI, Y., NARUSE, T., INAGAKI, M., MORIYAMA, E. N., GOJOBORI, T. and HOSHINO, H.: Six strains of human immunodeficiency virus type-I isolated in


YONEDA M., TANNO Y., HORAI S., OZAWA, T., MIYATAKE, T. and TSUI, S.: A common mitochondrial DNA mutation in the t-RNA^{Lys} of patients with myoclonus epilepsy


**Yoshioka, Y., Fujita, Y. and Ohtsubo, E.:** Nucleotide sequence of the promoter-distal region of the *tra* operon including *traI* (DNA helicase I) and *traD* genes of plasmid R100. J. Mol. Biol. 214: 39–53, 1990.
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304th meeting Jan. 21 The development and use of a human genetic linkage map to locate genes responsible for inherited disorders (Tim P. Keith)

305th — Jan. 27 DNA methylation: How important in plant gene control? (Jarunya Ngernprarsitsiri)

306th — Mar. 2 How RNA splicing enables the expression of a maize gene with a transposable element insertion in an exon (O. E. Nelson Jr.)

307th — Mar. 12 Molecular genetics of Alzheimer's disease (Josephine Nalbantoglu)

308th — Mar. 20 Engineering virus resistance in transgenic plants (Gregg Clark)

309th — Mar. 22 Mini collagens a major component of hydra nematocyte capsules (Charles N. David)

310th — Mar. 28 Regulation of heat shock genes in Drosophila (Carl Wn)

311st — Mar. 30 The neutral mutation theory of molecular evolution and the disunity of evolutionary biology (William B. Provine)

312nd — Apr. 6 Transcriptional termination in *Escherichia coli*: Effects of translation and of sequence hypersymmetry on rho-independent termination (Richard S. Hayward)

313rd — Apr. 23 Intercellular signalling molecules regulate gene expression in myxococcus development (A. D. Kaiser)

314th — Apr. 27 The heat shock response in Hydra: Characterization of species unable to synthesize a major heat shock protein in response to stress (Thomas C. G. Bosch)
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331st — Oct. 29 Repetitive sequences organization and functions in the genome of Caenorhabditis elegans (Adriana La Volpe)
332nd — Nov. 8 Biological correlates of oncogene expression and function in chemically induced transformation (Mukkattu R. Das)
333rd — Nov. 26 Two problems in gonoalogy: Oldest and common ancestor alleles (Geoff Watterson)
334th — Dec. 5 The genome of Caenorhabditis elegans (John Sulston)
335th — Dec. 5 Genetic recombination: Chi and the Rec BCD pathway (Franklin W. Stahl)
336th — Dec. 17 The taxonomic status of guinea pig based on protein data (Dan Graur)

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365th meeting Feb. 16 Long-distance restriction mapping of human chromosome 21 with NotI linking clones (Misao Ohki)
336th — Mar. 9 Amino acid sequence homology between prostaglandin D synthetase and gramicidin S synthetase in relation to their physiological function (Hiroyuki Toh)
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368th — Apr. 19 Expression and functions of stress proteins—tumorigenesis · differentiation · heat shock—(Kazuhiro Nagata)
369th — Sept. 6 ACTINOBASE: An image database of actinomycetes (Yoshihiro Ugawa)
370th — Sept. 7 Biochemistry of the SV40 DNA replication (Toshiki Tsurimoto)
371st — Nov. 9 Morphogenesis and expression of tissuespecific genes (Sadao Yasugi)
372nd — Nov. 19 Roles of cell-cell interaction in determination
of developmental fate: Cell interaction in sexual reproduction of slime mold (Hideko Urusibara)

373rd — Nov. 15 Mouse embryogenesis and X chromosome (Nobuo Takagi)

374th — Dec. 11 Mechanism of length determination in bacteriophage lambda tails (Isao Katsura)

375th — Dec. 19 Regulation of the phosphate regulon in *Escherichia coli* (Kozo Makino)
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Pascale Barbier; Université des Sciences et Techniques du Languedoc, Montpellier, France

September 1, 1888–January 7, 1889
Guan Cheng Sun; Sericultural Research Institute, Chinese Academy of Agricultural Sciences, China

January 7, 1889–September 25, 1990
Yong Hong Wang; Lanzhou Institute of Biological Product, Ministry of Public Health, China

April 10, 1989–March 31, 1990
Fengshan Wang; Tianjin Institute of Industrial Hygiene and Occupational Diseases, China

January 10, 1990
Guangyu Zhou; Shanghai Institute of Biology and Chemistry, Academia Sinica, China

February 3
Yutaka Eguchi; National Institutes of Health, U. S. A.

February 21
Tim P. Keith; Collaborative Research Inc., U. S. A.

February 27
Jarunya Ngernprasirtsiri, Nagoya University

March 1–May 31
Richard S. Hayward; University of Edinburgh, U. K.

March 2
O. E. Nelson, Jr.; University of Wisconsin, U. S. A.

March 9–28
Charles N. David; University of Munich, W. Germany

March 12
Josephine Nalbantoglu; Institut national de la recherche Scientifique Quebec, Canada

March 20
Gregg Clark; Washington University, U. S. A.

March 28
Carl Wn; National Cancer Institute, NIH, U. S. A.

March 30
William B. Provine; Cornell University, U. S. A.

April 5–6
Masatoshi Nei; University of Texas, U. S. A.

April 23
A. D. Kaiser; Stanford University, U. S. A.

April 23–May 14
Thomas C. G. Bosch; University of Munich, W. Germany

April 17
Max E. Gottesman; Columbia University, U. S. A.

May 11
Verne M. Chapman; Roswell Park Cancer Institute, U. S. A.

May 30
Jean-Louis Guenét; Institut Pasteur, France
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<td>Mount Sinai School of Medicine, U.S.A.</td>
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<td>Song Mong Lee</td>
<td>Sericultural Experiment Station, Rural Development Administration, Korea</td>
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<td>Institut Jacques Monod, France</td>
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<td>Indian Statistical Institute, India</td>
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<td>University of Tubingen, E. Germany</td>
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<td>Cleveland Clinic Foundation Research Institute, U.S.A.</td>
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<td>Maurice S. Fox</td>
<td>Massachusetts Institute of Technology, U.S.A.</td>
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<td>Richard R. Burgess</td>
<td>University of Wisconsin, U.S.A.</td>
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<td>University of Colorado at Boulder, U.S.A.</td>
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<td>October 1</td>
<td>Kiyoshi Mizuuchi</td>
<td>National Institute of Diabetes and Digestive and Kidney Diseases, NIH, U.S.A.</td>
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<td>October 8-10</td>
<td>Masatoshi Nei</td>
<td>Pennsylvania State University, U.S.A.</td>
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<td>Hans R. Bode</td>
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<td>Adriana La Volpe</td>
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<td>Mukkattu R. Das</td>
<td>Center for Cellular and Molecular Biology, India</td>
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November 8  Marcus W. Feldman; Stanford University, U. S. A.
November 11–12  S. N. Ethier; Utah University, U. S. A.
November 12  Woong Jik Lee; Professor Emeritus, Seoul National University, Korea
November 21–29  Geoff Watterson; Monash University, Australia
December 3  Peter G. Condliffe; Scientist Emeritus, National Institutes of Health, U. S. A.
December 5  John Sulston; MRC Laboratory of Molecular Biology, U. K.
December 5  Franklin W. Stahl; University of Oregon, U. S. A.
December 17  Dan Grauer; Tel Aviv University, Israel
December 17  Shen Zhang; Da Sheng Wang; Zi jian Wang; Nai-Kai Zhu; Committee of Environment, Academia Sinica, China
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