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GENERAL STATEMENT

This report presents an outline of research and related activities carried out in our institute during the year 1988. Only new developments or changes will be summarized here.

Support of research activities

Total expenditures, not including those for facilities, for 1988 FY (April 1988–March 1989) amounted to 1 billion 166 million yen, a half of which was for personnel expenses. In addition, grants-in-aid amounting to 210 million yen were rendered to selected staff members by the Ministry of Education, Science and Culture (Monbusho). A Gene Library Laboratory was added to the DNA Research Center and the Radioisotope Center was newly established. Accordingly, new positions for two associate professors, a research staff member (*joshu*), a technician, and an administrative official were allotted. Thus the total number of positions available for regular staff is 101, of which 59 are for research professionals. Concerning facilities, a five-storied building (total floor area of 2389 centares) for the Radioisotope Center and a central machinery house were completed on December 5. The new building included two rooms for P3 level and three for P2 level recombinant DNA experiments. Now that molecular genetics has become the main focus of genetic research, the completion of a modern building for high technology using radioisotopes is extraordinarily important for the further development of the institute.

Opening of the Graduate University for Advanced Studies

As was announced in the last issue, October marked the opening of the Graduate University for Advanced Studies, which is based on six national inter-university research institutes (Institutes of Statistical Mathematics, High Energy Physics, Molecular Sciences, Genetics, Basic Biology, and Physiological Sciences). Our institute will function as the Department of Genetics, which, together with the Institute for Basic Biology and the Institute for Physiological Sciences, represent the Graduate School of "Life Sciences." We are preparing for the admission of about six students who will major in genetics next April.

Honors

Prof. M. Kimura, Head of the Department of Population Genetics, retired at the end of March. He is continuing his research as a Visiting Professor. Upon approval at a council meeting in June, Prof. M. Kimura and Prof. Kin-ichiro Miura of the University of Tokyo, who held professorship from 1969 to 1984 at the institute, were conferred the title of Professor Emeritus, according to the regulations of the institute.

I am happy to note that three of our colleagues were honored in the past year. Prof. Kimura was awarded the 4th International Prize for Biology, for his outstanding contributions to the modern development of both the theoretical aspects of population genetics with his study on diffusion models, and to evolutionary genetics with his neutral theory of molecular evolution. This prize was founded in order to encourage studies in biology, in commemoration of the Emperor's sixty year reign and his years of study in biology. Prof. K. Miura was awarded the Japan Academy Prize for his "Molecular genetic studies on double-stranded DNA viruses, especially the discovery of the cap structure of RNA." While he was in the institute, he discovered that one end of RNA, to which genetic information of eukaryotic cells and their viruses is transcribed, has a special structure called a "cap structure." Associate Prof. K. Tutikawa won an Encouragement Award from the Environmental Mutagen Society of Japan for "The mouse spot test: Development of the method with a new cross." This represents a system to detect *in vivo* somatic mutations induced by mutagens, which is currently being used by several industries for mutagen testing of new drugs and chemicals.

Personnel changes

The past year saw a number of personnel changes in the professional staff of the institute. While Drs. Ryuji Fukuda (Laboratory of Molecular Genetics), Tadashi Inoue (Laboratory of Mutagenesis), and Kenichi Aoki (Laboratory of Population Genetics) left for universities as professors or associate professors, the following ten appointments were made: Takeshi Seno, professor; Dai Ayusawa, associate professor; and Sumiko Kaneda (Laboratory of Mutagenesis), Hidenori Tachida (Laboratory of Population Genetics), Etsuko Moriyama (Laboratory of Evolutionary Genetics), Yoshio Tateno and Hiroyuki Hirano (Genetic Stock Research Center), research staff members; Nobuo Shimamoto, associate professor; and Kenichi Matsu-

moto (DNA Research Center), and Ikuo Nakamura (Experimental Farm), research staff members. Dr. Yoshio Sano and Dr. Takashi Gojobori were promoted to associate professors. Associate Prof. Yoshito Sadaie was transferred from the Laboratory of Mutagenesis to the newly established Radioisotope Center as its head.

International exchanges and symposia

The institute's staff were also very active in international personnel exchanges in the past year. Ten staff members attended the XVIth International Congress of Genetics held in Toronto, Canada, in August. Among them, M. Kimura, Professor Emeritus, was inivited to give a plenary lecture on his neutral theory of molecular evolution, which evoked a great deal of attention in local newspapers. Including that congress, members of our staff went abroad on 41 occasions for the purpose of presenting research results at various scientific meetings, exchanging information, and carrying out collaborative studies or field investigations. On the other hand, 58 scientists who visited our institute from abroad delivered lectures at the Biological Symposium or exchanged information and views on recent studies. Those who stayed longer than one month and carried out collaborative studies with our staff were: Miss Pascale Barbier, Université des Sciences et Techniques du Languedoc, Montpéllier, France; Dr. Dong Sang Suh, Genetic Engineering Center, Korean Advanced Institute of Technology, and Dr. Sam-Eun Kim, Sericultural Experiment Station, Rural Development Administration, Republic of Korea; Dr. Ling Hua Tang, Institute of Food Crops, Jiangsu Academy of Agricultural Sciences, Miss Xiao Mei Wu and Miss He Zhao, Lanzhou Institute of Biological Products, Ministry of Public Health, People's Republic of China; Prof. W. B. Provine, Cornell University, and Prof. D. M. Shankel, University of Kansas, U.S.A., and Prof. M. Rahat, Hebrew University, Israel.

Two international meetings were organized by our staff members. One was the Fourth International Symposium on "Population Biology of Genes and Molecules" held in conjunction with the Award of the International Prize for Biology Nov. 28-Dec. 2 in Tokyo. With support from Monbusho, ten scientists from abroad and ten Japanese were invited. Besides Prof. Kimura's award lecture, recent results of studies by other top scientists were presented to an audience of about one hundred. The other meeting was the 2nd International Conference on Mechanisms of Antimutagenesis

and Anticarcinogenesis held in Ohito Dec. 4–8, organized by Prof. Yukiaki Kuroda of the Laboratory of Phenogenetics and Prof. D. M. Shankel who was in Japan for cooperative studies with Prof. Kuroda. Interest in environmental mutagens and carcinogens has recently been shifting to identifying desmutagens, antimutagens and anticarcinogens. This is a new field initiated by the late Prof. Tsuneo Kada of the Laboratory of Mutagenesis (died Nov. 14, 1986). The conference was successful with 273 attendants, of which 99 were from abroad.

Service to scientific communities

The DNA Data Bank of Japan (DDBJ) continues to offer the following services through the efforts of the staff of the Laboratory of DNA Data Analysis: collection and input of data, sending data, on-line service by telephone, and issuing newsletters (Nos. 7 & 8). Training courses and demonstrations for beginners were carried out at general meetings of certain academic societies such as the Japan Cancer Association, and the Biochemistry and Molecular Biology Associations. The input of data applies to papers mainly published in Japanese academic journals. The 3rd edition issued in July, 1988, includes 230 entries and 345,850 bases, which corresponds to only one thirtieth of that of EMBL or GenBank; we have to make every effort to increase our data bases. In the past year an International Advisory Committee was set up in order to give advice regarding the procedure of the program. The committee recommended that DDBJ, EMBL and GenBank should collaborate in maintaining close contacts with each other. In order to assume a heavier responsibility in international collaboration, a drastic strengthening of the man-power and increase in the budget are indispensable. Prof. Hisao Uchida of Teikyo University, who is a Japanese representative to the Advisory Committee, has been providing advice to the operation of our DDBJ as a Visiting Professor since April.

Another project is to maintain and distribute the "*E. coli* Gene Library," which is a relational clone bank of the whole *E. coli* chromosome established by Drs. Yuji Kohara of Nagoya University and Katsumi Isono of Kobe University. At present it is taken care of by the staff of the Laboratory of Molecular Genetics. Since genome analysis of various organisms including human beings has become a most challenging project in biology, the physical mapping of the *E. coli* chromosome is very important as a first step. From its start in September, 1987 till May, 1988, 17,433 clones in total were dis-

tributed upon request for 37 domestic and 90 foreign projects.

The third project is to supply specific strains of mouse, *Drosophila*, *E. coli*, *B. subtilis*, etc. The total number supplied in 1987 amounted to 369 (42 sent abroad) and the total number of strains supplied was 4,388 (131 sent abroad). The fourth is to systematize, with the staff of the Laboratory of Genetic Resources, data-base information about location, characteristics, availability of distribution, etc., of a variety of experimental organisms preserved in universities and research institutes in Japan. "Silkworm strains in Japan, 1988" and "Rice Genetics Newsletter, No. 4" were also issued and distributed.

Service to the public

The institute held its yearly open house for the public on April 23. Some of the research activities of each laboratory were exhibited using panels, microscopes, microcomputers, and molecular models. Educational lectures, "Genetic individuality, natural environment, and the survival of human beings" by Prof. Takashi Imamura and "Evolution at the molecular level" by Associate Prof. Takashi Gojobori were given. The double cherry blossoms on the campus were at their best and some 3,000 visitors in the neighborhood enjoyed them. On November 12, public lectures were given at the National Science Museum in Tokyo; the titles were "Regulation of stem cell differentiation in Hydra" by Associate Prof. Toshitaka Fujisawa and "The origin and molecular evolution of AIDS viruses" by Dr. Gojobori. In spite of it being a Saturday afternoon about 120 eager people from universities and institutes listened to the lectures, which were followed by rather specialized questions and lively discussions.

Need for more facilities and man-power

Five years have passed since the institute was switched over to one for joint use by universities. We have accepted upon application, 38 collaborative programs, 11 workshops, 5 graduate students, 7 research fellows and 10 scholarships from industries, and 3 entrusted research projects from private corporations. Yet, there remain a number of problems to be solved in order to achieve the reality of reform. Among other things, construction of a new building for research to accommodate laboratories for joint use by visiting professors, and lodgings for visiting researchers with welfare facilities are of the highest priority. We are all eager to do our best to

accomplish the missions of our institute. We hope to have continued encouragement and support from all persons concerned.

At the completion of my term of directorship, I will retire on October 1st, 1989. I herewith express my heartfelt gratitude to all those who encouraged and helped me during my six years of service.

E. Matsunaga

STAFF

Director

MATSUNAGA, Ei, D. Med., D. Sc.

Members

1. *Department of Molecular Genetics*

ISHIHAMA, Akira, D. Sc., Head of the Department

Laboratory of Molecular Genetics

ISHIHAMA, Akira, D. Sc., Professor
FUJITA, Nobuyuki, D. Sc.
NAGATA, Kyosuke, D. Pharm.

Laboratory of Mutagenesis

SENO, Takeshi, D. Sc., Professor
AYUSAWA, Dai, D. Ag., Associate professor
TEZUKA, Hideo
KANEDA, Sumiko, D. Pharm.

Laboratory of Nucleic Acid Chemistry (Guest member)

YOSHIKAWA, Hiroshi, D. Sc., Professor
UCHIDA, Hisao, D. Sc., Professor
MIZUMOTO, Kiyohisa, D. Sc., Associate professor

2. *Department of Cell Genetics*

MORIWAKI, Kazuo, D. Sc., Head of the Department

Laboratory of Cytogenetics

MORIWAKI, Kazuo, D. Sc., Professor
IMAI, Hirotami T., D. Sc., Associate professor
SHIROISHI, Toshihiko, D. Sc.

Laboratory of Microbial Genetics

YASUDA, Seiichi, D. Sc., Associate professor
NISHIMURA, Yukinobu, D. Sc.
HARA, Hiroshi, D. Sc.

Laboratory of Cytoplasmic Genetics (Guest member)

YONEKAWA, Hiromichi, D. Sc., Associate professor

3. *Department of Ontogenetics*

KURODA, Yukiaki, D. Sc., Head of the Department

Laboratory of Developmental Genetics

SUGIYAMA, Tsutomu, Ph. D., Professor
NAWA, Saburo, D. Sc., Professor
FUJISAWA, Toshitaka, Ph. D., Associate professor
SHIMIZU, Hiroshi, D. Eng.

Laboratory of Phenogenetics

KURODA, Yukiaki, D. Sc., Professor
MURAKAMI, Akio, D. Ag., D. Sc., Associate professor
MINATO, Kiyoshi, M. Sc.
YAMADA, Masa-Aki, D. Ag.

Laboratory of Physiological Genetics (Guest member)

SHIMADA, Yutaka, D. Med., Professor
IDE, Hiroyuki, D. Sc., Associate professor

4. *Department of Population Genetics*

OHTA, Tomoko, Ph. D., D. Sc., Head of the Department

Laboratory of Population Genetics

OHTA, Tomoko, Ph. D., D. Sc., Professor
TAKAHATA, Naoyuki, D. Sc., Associate professor
TACHIDA, Hidenori, D. Sc.

Laboratory of Evolutionary Genetics

TUTIKAWA, Kiyosi, Associate Professor
GOJOBORI, Takashi, D. Sc., Associate professor
MORIYAMA, Etsuko

Laboratory of Theoretical Genetics (Guest member)

KIMURA, Motoo, Ph. D., D. Sc., Professor
MIYATA, Takashi D. Sc., Associate professor

5. *Department of Integrated Genetics*

IMAMURA, Takashi, D. Med., Head of the Department

Laboratory of Human Genetics

IMAMURA, Takashi, D. Med., Professor

FUJIYAMA, Asao, D. Sc., Associate professor
HORAI, Satoshi, D. Med.
NAKASHIMA, Hitoshi

Laboratory of Agricultural Genetics

MORISHIMA-OKINO, Hiroko, D. Ag., Professor
ENDO, Toru, D. Ag., Associate professor
SATO-HIRAOKA, Yoichiro, D. Ag.

Laboratory of Applied Genetics (Guest member)

WATANABE, Takeshi, D. Med., Professor
YONEZAWA, Katuei, D. Ag., Professor

6. *Research Facilities*

Genetic Stock Research Center

SUGIYAMA, Tsutomu, Ph. D., Head of the Center
MIYASHITA, Nobumoto, D. Med.
WATANABE, Takao K., D. Sc., Associate professor
UEDA, Hitoshi, D. Ag.
SANO, Yoshio, D. Ag., Associate professor
HIRANO, Hiroyuki, D. Ag.
NISHIMURA, Akiko, D. Ag.
IYAMA, Shin-ya, D. Ag., Associate professor
TATENO, Yoshio, Ph. D., D. Sc.

DNA Research Center

SENO, Takeshi, D. Sc., Head of the Center
SHIMAMOTO, Nobuo, D. Sc., Associate professor
IKEMURA, Toshimichi, D. Sc., Associate professor
MATSUMOTO, Kenichi, D. Ag.
HIROSE, Susumu, D. Sc., Associate professor
MIYAZAWA, Sanzo, D. Sc., Associate professor
HAYASHIDA, Hidenori

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Experimental Farm

IYAMA, Shin-ya, D. Ag., Head of the Farm
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ONIMARU, Kimiharu, Chief of the Section

8. ***Department of Administration***

HARA, Toshio, Head of the Department
UJIIE, Jun, Chief of the General Affairs Section
TANIGUCHI, Hiroshi, Chief of the Finance Section

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SENO, Takeshi; Professor, Laboratory of Mutagenesis

SUGIYAMA, Tsutomu; Professor, Laboratory of Developmental Genetics

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YAMAGUCHI, Hikoyuki; Manager, Professor of University of Tokyo

PROJECTS OF RESEARCH FOR 1988

1. DEPARTMENT OF MOLECULAR GENETICS

Laboratory of Molecular Genetics

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

Laboratory of Mutagenesis

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

Laboratory of Nucleic Acid Chemisry

Studies on regulatory mechanisms of chromosome replication (YOSHIKAWA)
Molecular mechanisms of RNA 5′-cap formation (MIZUMOTO)
Studies on molecular mechanisms of transcription and replication of Sendai virus (MIZUMOTO)

2. DEPARTMENT OF CELL GENETICS

Laboratory of Cytogenetics

Studies on species differentiation of mouse from cyto- and molecular genetic view points (MORIWAKI)
Immunogenetical studies on the mouse MHC (SHIROISHI and MORIWAKI)
Theoretical bases for chromosomal evolution in mammals and ants (IMAI)
Cyto- and molecular mechanism of meiotic recombination in mice (SHIRO-

ISHI, IMAI and MORIWAKI)

Laboratory of Microbial Genetics

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

Laboratory of Cytoplasmic Genetics

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

3. DEPARTMENT OF ONTOGENETICS

Laboratory of Developmental Genetics

Genetic analysis of developmental mechanisms in hydra (SUGIYAMA, FUJISAWA and SHIMIZU)
Studies on transformation and cell differentiation in higher organisms (NAWA and YAMADA)
Mitochondrial DNA organization in male-sterile cytoplasms of rice (NAWA and SANO)

Laboratory of Phenogenetics

Genetic studies on insect cells in tissue culture (KURODA and MINATO)
Developmental genetic studies on animal cells in tissue culture (KURODA)
Genetics of somatic mammalian cells in culture (KURODA)
Genetic studies on the reproduction in *Bombyx* (MURAKAMI)
Ecogenetic studies on the nerve system characters in *Bombyx* (MURAKAMI)
Studies on the male-specific killing activity of SRO in *Drosophila* (YAMADA)

Laboratory of Physiological Genetics

Electron microscopic studies on differentiation of animal cells (SHIMADA and KURODA)
Effect of morphogens on pattern formation in hydra (IDE and SUGIYAMA)

4. DEPARTMENT OF POPULATION GENETICS

Laboratory of Population Genetics

Theoretical studies of population genetics (OHTA, TAKAHATA and AOKI)
Studies on molecular evolution from the standpoint of population genetics (TAKAHATA and OHTA)
Theoretical studies on the evolution of multigene family (OHTA)
Theory of gene genealogy (TAKAHATA)
Theoretical studies on the evolution of altruism (AOKI)
Population genetical studies on gene-culture coevolution (AOKI)

Laboratory of Evolutionary Genetics

Studies on molecular evolution (GOJOBORI)
Radiation genetics in mice (TUTIKAWA)

Laboratory of Theoretical Genetics

Theoretical studies of population genetics and molecular evolution (KIMURA)
Computer studies on the molecular evolution (MIYATA)

5. DEPARTMENT OF INTEGRATED GENETICS

Laboratory of Human Genetics

Molecular genetics of growth and differentiation of human blood forming cells (IMAMURA, FUJIYAMA and NAKASHIMA)
Molecular genetics of human metabolic disorders (IMAMURA, FUJIYAMA and NAKASHIMA)
Studies on DNA polymorphisms in human populations (HORAI and MATSUNAGA)
Mitochondrial DNA restriction analysis of non-human primates (HORAI, MATSUNAGA, HAYASAKA, SHOTAKE and NOZAWA)

Laboratory of Agricultural Genetics

Evolutionary studies in wild and cultivated rice species (MORISHIMA and

SATO)
Genecological studies on pollination in rice plants (SATO and MORISHIMA)
Genetic studies on rice reserve proteins (ENDO)

Laboratory of Applied Genetics

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

6. RESEARCH FACILITIES

Genetic Stock Research Center

Genetic studies of speciation in rice (SANO and HIRANO)
Studies on plant gene expression (HIRANO and SANO)
Theoretical studies on breeding techniques (IYAMA)
Genetic studies of trees in natural forest (IYAMA)
Documentation of genetic stocks in Japan (IYAMA)
Studies on the management system of genetic stocks information (IYAMA)
Theoretical studies on molecular phylogeny (TATENO)
Evolutionary genetics of *Drosophila* (WATANABE)
Molecular genetics of insect development (UEDA)
Coordination of DNA synthesis and cell division in *E. coli* (NISHIMURA)
Mapping of a whole set of cell division genes in *Escherichia coli* K12 (NISHIMURA)
Genetic mechanisms for regulating tumor development in the laboratory and wild mice (MIYASHITA and MORIWAKI)

DNA Research Center

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

Control of gene expression in eukaryotes (HIROSE and UEDA)

Radioisotope Center

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

RESEARCH ACTIVITIES IN 1988

I. MOLECULAR GENETICS

Promoter Selectivity of *E. coli* RNA Polymerase: Cloning and Characterization of *E. coli* Promoters

Masayo KUBOTA, Yukiko YAMAZAKI, Nobuyuki FUJITA and Akira ISHIHAMA

The two parameters of promoter strength, affinity to RNA polymerase and rate of open complex formation, have been determined for a number of *E. coli* promoters from previously identified genes (reviewed in Ishihama, A. (1988) *Trends Genet.* 4: 282–286). To make a collection of promoters with yet unidentified unique properties, we have attempted, in parallel with the above approach, to randomly isolate *E. coli* DNA sequences promoting transcription of the *ampC* gene. Because a linear correlation exists between *ampC*-encoded β-lactamase production and the level of ampicillin resistance expressed, the *ampC* gene has advantages over certain other indicator genes, in particular for the direct selection of strong promoters or up-mutant promoters.

For this purpose, we used pJAC4 (Jaurin, B. and Cohen, S. N. (1985) *Gene* 39: 191–201) as a transcriptional fusion plasmid vector, into which 100–500 bp *Sau*3A fragments of total *E. coli* DNA were inserted. About 500 independent transformants were isolated, which were resistant to a low level (10 μg/ml) of ampicillin. Production of β-lactamase encoded by *ampC* could be determined accurately by measuring the maximum concentration of ampicillin that allowed colony formation. The values ranged from 10 μg/ml to over 5 mg/ml ampicillin.

The origins of these DNA fragments are being analyzed by hybridization with a gene library of the whole *E. coli* chromosome. Using the same promoter cloning system, the determination of *in vivo* strength is in progress for the promoters so far characterized using the *in vitro* mixed transcription system.

Promoter Selectivity of *E. coli* RNA Polymerase: Subunit ω Plays an Essential Role in Stringent Control

Kazuhiko IGARASHI, Nobuyuki FUJITA, Hiroshi OKAMOTO*
and Akira ISHIHAMA

RNA polymerase of *Escherichia coli* is composed of at least four different subunits, α, β, β' and one of various σ subunits. In addition, subunit ω, 10 KDa in size, is tightly associated with both the core and holoenzyme at a molar ratio of 0.5 to 2 per enzyme. The function of this subunit, however, remains unclear. Recently, the gene (*rpoZ*) coding for the ω subunit has been cloned and sequenced. On the basis of the DNA sequence, the *rpoZ* gene was found to be located next to *spoT*, the gene involved in ppGpp metabolism. This finding raised the possibility that the ω subunit might play a role in stringent control.

In order to examine whether the ω subunit influences ppGpp-sensitivity of RNA polymerase, we isolated both the ω subunit, and ω-free core enzyme from urea-treated RNA polymerase and reconstituted holoenzymes with or without the ω subunit. RNA polymerase lacking the ω subunit was found insensitive to ppGpp in an *in vitro* mixed transcription system (Igarashi, K. *et al.*, submitted for publication). Addition of the purified ω subunit restored ppGpp-sensitivity of ω-free RNA polymerase. These results indicate that the ω subunit is a regulatory subunit of RNA polymerase and that it is involved in the ppGpp-mediated alteration of promoter selectivity.

Previously, we reported that some *E. coli* mutants with mutations in the *rpoB* gene encoding β subunit exhibited a relaxed phenotype and that the mutant RNA polymerases were insensitive to inhibition by ppGpp (Glass, R. E. *et al.*, (1986) *Mol. Gen. Genet.* 203: 492–495). To link these two independent observations together, we propose that the target of ppGpp is subunit ω, which associates with RNA polymerase at the stringent control domain on the β subunit. However, the possibility that ppGpp interacts with the β subunit with this interaction being regulated by the ω subunit has not yet been excluded.

* Department of Biochemistry, Tohoku University School of Medicine, Sendai.

RNA Polymerase Sigma-related Protein in *Escherichia Coli*: Purification and Characterization of Proteins which Crossreact with Anti-sigma Peptide Antibodies

Rei UESHIMA, Nobuyuki FUJITA and Akira ISHIHAMA

Anti-sera against a synthetic tetradecameric peptide with the sequence DLIQEGNIGLMKAV, which is highly conserved among σ factors of bacterial RNA polymerases, specifically crossreact with several *E. coli* proteins in addition to the known σ factors (σ^{70} and σ^{32}) (Fujita, N. and Ishihama, A. (1987) *Mol. Gen. Genet.* 210: 5–9). In order to examine the nature of these sigma-related proteins, we purified four major proteins with apparent molecular weights of 34 KDa, 27 KDa (A component), 27 KDa (B component) and 23 KDa. N-Terminal amino acid sequence analysis revealed that three of these protein were as yet unidentified novel proteins and that one was ribosomal protein S2. By glycerol gradient centrifugation under both high- and low-salt conditions, however, these proteins did not stably associate with the RNA polymerase core enzyme.

The immunological crossreaction of σ^{70} and all these sigma related proteins with the anti-sigma peptide antibodies was interfered with the addition of the 14-meric synthetic peptide. Among three hexameric peptides with sequences identical to portions of the 14-meric sigma consensus sequence, GLMKAV could compete with all four sigma-related proteins, while none of these peptides could effectively compete with σ^{70}. These observations suggest that the anti-sigma peptide serum used in this study contained at least three different species of antibodies, each recognizing a specific epitope within the consensus sigma sequence. In addition, the antiserum might have contained an antibody which recognizes a highly ordered structure formed by the consensus sigma sequence within σ^{70}. In order to detect novel sigma factors, therefore, antibodies against such a conformational epitope should be used.

RNA Polymerase and Transcription Signals of *Micrococcus luteus*

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

The G+C content (74%) of *Micrococcus luteus* DNA is one of the highest

* Department of Biology, Faculty of Science, Nagoya University, Nagoya.

in bacteria. Through analysis of transcription initiation sites for the *spc* and *str* operons, we found that it nevertheless carries *E. coli*-type promoters (Nakayama, M. *et al.* (1989) *Mol. Gen. Genet.*, in press). In order to compare the promoter selectivity of RNA polymerase between *M. luteus* and *E. coli*, we purified RNA polymerase from both *M. luteus* and *E. coli*. Some *E. coli* promoters could be correctly transcribed by the *M. luteus* enzyme as well as by the *E. coli* enzyme, but others could not. On the basis of core enzyme-stimulating activity, we identified and purified one sigma factor from *M. luteus*. This *M. luteus* sigma factor, with an apparent molecular weight of 60 KDa on SDS-PAGE, could enhance transcription by *E. coli* core RNA polymerase. The sigma factor of *M. luteus* is therefore functionally exchangeable with that of *E. coli*.

In addition to this major sigma factor, we found a minor species, which was different from the major one in both promoter selectivity and molecular weight. Because both sigma factors were identified in cells under non-stressed conditions, they might be vegetative sigma factors.

Molecular Mechanism of Influenza Virus RNA Transcription. I. Analysis of Promoters for Viral RNA Polymerase

Akira ISHIHAMA, Jeffrey D. PARVIN*, Peter PALESE*, Mark KRYSTAL*,
Ayae HONDA**, Atsushi KATO***, Susumu UEDA***,
Akiko YOKOIYAMA, Yukiko YAMAZAKI,
Kunitoshi YAMANAKA and Kyosuke NAGATA

The RNA-dependent RNA polymerases of animal RNA viruses have been studied with regard to enzyme structure and reaction mechanisms. Little is known, however, about the elements on template RNA which promote transcription and replication by the RNA polymerases. Such promoter analysis is important for understanding how viral RNA polymerases recognize specific viral RNAs from among the many host-encoded RNAs found in infected cells.

Previously we demonstrated that the genomic RNA of influenza virus was in a panhandle structure with base-pairing of both termini (Honda, A *et al.* (1987) *J. Biochem* 102: 1241–1249). Furthermore, it was found that

* Department of Microbiology, Mount Sinai Medical School, New York.
** Bristol-Meyer's Research Institute, Tokyo.
*** Nippon Institute of Biological Science, Ohme, Tokyo.

influenza viral RNA polymerase is bound at the basepaired stem of this panhandle, leading to the suggestion that the panhandle structure is required for recognition by the viral RNA polymerase.

In order to test this possibility, we prepared a short model template of 53 nucleotides in length, by transcribing plasmid DNA with T7 RNA polymerase. The termini of this model RNA are identical to the 15 and 22 nucleotide sequences conserved at the 3′ and 5′ termini of RNA segment 8 from all influenza A viruses. In parallel, we prepared a mixture of RNA polymerase (PB1-PB2-PA complex) and NP by CsCl centrifugation of RNP cores (Honda, A. *et al.* (1987) *J. Biochem.* 102: 1241–1249). The RNA-free RNA polymerase fraction specifically recognized the model RNA template and, in the presence of ApG primer, transcribed it into template-sized complementary RNA (Parvin, J. D. *et al.*, submitted for publication). Additional template constructions containing only the 3′ half of this model template (and therefore the 3′ end of genomic RNA) were shown to be as efficiently transcribed as the original template. These results indicate that the promoter for primer-directed transcription lay solely within the 15 nucleotide 3′ terminus of viral RNA. The role of individual nucleotides within the putative promoter region was also analyzed by site-specific mutagenesis.

Molecular Mechanism of Influenza Virus RNA Transcription. II. Matrix Protein Epitopes Involved in Transcription Inhibition

Raleigh W. HANKINS*, Kyosuke NAGATA, Doris J. BUCHER**
and Akira ISHIHAMA

Recent studies have revealed that influenza virus matrix (M) protein inhibits vRNA transcription by binding to NP protein in the basic viral transcribing unit (RNP cores) consisting of vRNA, PB2, PB1, PA and NP. The inhibition mechanism is, however, not understood yet.

Experiments using monoclonal antibodies against the M protein enabled us to identify the epitope(s) on the M protein involved in this transcription inhibition. When a battery of 15 monoclonal anti-M antibodies was

* Present address: Laboratory of Microbiology, Health Sciences Research Institute, Yokohama.

** Department of Microbiology and Immunology, New York Medical College, New York, USA.

analyzed on western blot, five distinct banding patterns were observed. A representative antibody of each group was analyzed as to the effect of the M protein on transcription inhibition. From these data, the critical sites on M for transcription inhibition were deduced. It appears now that the regions on M in the vicinity of amino acid residues #70 and #140 are physically close together in the final 3-dimensional conformation of the M protein, and that the residues in between form a loop and are thus removed from the functional site.

Molecular Mechanism of Influenza Virus RNA Transcription. III. Binding Mode of Influenza Virus Nucleoprotein to RNA

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

Ribonucleoprotein (RNP) cores (RNA polymerase-RNA-NP complexes) were isolated from detergenet-treated influenza virus A/PR/8/34 by glycerol gradient centrifugation (Honda, A. *et al.* (1987) *J. Biochem.* 101: 1241–1249). RNP cores were dissociated into P-RNA (RNA polymerase-RNA) complexes and nucleoprotein (NP) by CsCl centrifugation (Honda, A. *et al.* (1988) *J. Biochem.* 104: 1021–1026). P-RNA complexes were capable of synthesizing only small-sized RNA. By adding NP to P-RNA complexes, RNP complexes were reconstituted, which supported the synthesis of template-sized RNA as did native RNP cores. Furthermore, RNP cores, but not P-RNA complexes, were able to produce infections viruses, when transfected into MDCK cells. These results suggest that NP is required for efficient elongation of the RNA chain.

To elucidate the binding mode of NP to RNA, we optimized the conditions for binding of NP to RNA by using filter binding assay and gel shift assay. NP binds to any RNA longer than 15 nucleotides; NP-RNA complexes formed at 30°C are more resistant to high concentrations of NaCl than those formed at 0°C; divalent cations are not required for the binding; treatment of NP with N-ethylmaleimide gives no effect on its RNA binding, whereas treatment with alkaline phosphatase enhances its RNA binding. The mode of RNA binding was analyzed by the RNase "reverse-printing" method using RNase V_1. Reconstituted NP-RNA complexes as well as native RNP cores carry RNase V_1-sensitive sites for every 15 nucleotides.

* Department of Genetics, School of Medicine, Osaka University, Osaka.

The biological activity of these reconstituted complexes is being tested. In parallel, we are developing a bacterial expression system for large scale production of NP.

Transcription and Replication of Influenza Virus RNA: An *in vitro* System Using Isolation Nuclei from Virus-Infected Cells

Kyosuke NAGATA, Hiroshi SAKAGAMI* and Akira ISHIHAMA

We developed an *in vitro* system for transcription and replication of influenza virus RNA using isolated nuclei from influenza virus-infected HeLa cells (Takeuchi K. *et al.* (1987) *J. Biochem.* 101: 837–845). Fractionation and reconstitution of the system, indicated that a regulatory factor(s) that can be dissociated from vRNA-RNA polymerase complexes upon exposure to high ionic strength is involved in the switch from mRNA to cRNA synthesis (Nagata, K., *et al.* (1989) *J. Biochem.* 106: 205–208). Such a factor has been suggested as being either encoded by the virus genome or induced by virus infection. To gain further insight into influenza virus replication, attempts were made to discover specific inhibitors of viral replication.

It has been shown that pine cone extract (PCE) contains not only a potent antitumor substance(s) (Sakagami, H. *et al.* (1987) *Anticancer Res.* 7: 1153–1160), but also a substance(s) inhibitory to virus replication (Harada, H. *et al.* (1988) *Anticancer Res.* 8: 581–588). We examined the effect of PCE fractions on influenza virus replication. Acidic substances of PCE markedly suppressed the growth of the influenza virus in MDCK cells. Significant inhibition of both viral protein synthesis in infected cells and RNA-dependent RNA polymerase activity in virions was observed with these acidic fractions. These results suggest that the PCE contains anti-influenza virus substance(s). Using an *in vitro* system for transcription and replication, we are attempting to accurately define the molecular mechanism for the action of PCE.

* First Department of Biochemistry, School of Medicine, Showa University, Tokyo.

Translational Regulation of Influenza Virus mRNAs

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

In influenza virus-infected cells, ten kinds of viral proteins are synthesized and their synthesis is regulated with respect to total amount and timing. Although recent studies revealed that transcription is the key step in the control of gene expression in the influenza virus, we found that the translation efficiency of influenza viral mRNAs from at least nonstructural (NS) and neuraminidase (NA) genes is subject to temporal control following viral infection (Yamanaka, K. *et al.* (1988) *Virus Genes* 2: 19–30).

In order to test whether this control is operative for translation of other influenza viral mRNAs, cDNAs for each of the genome RNA segments of influenza virus A/PR/8/34 were cloned and various portions of these cDNA clones, containing the ATG initiation codon for translation, were inserted into the 5′-leader sequence of the chloramphenicol acetyltransferase (CAT) gene in a pSV2cat vector. Cells were first transfected with one of the recombinant plasmids and then super-infected with influenza virus. When plasmids carrying the 5′ proximal regions of the genes, which are expressed early after virus infection, were transfected, maximal CAT activity was obtained at an early stage of infection. In contrast, plasmids containing cDNA segments for the late genes, directed the highest CAT activity at the late stage of infection. These results suggest that translational regulation takes place during differential control of gene expression among eight RNA segments of the influenza viral genome. To identify a signal(s) and a cognate trans-acting factor(s) responsible for such translational regulation, *in vitro* translation experiments using extracts from infected cells are currently ongoing.

Mechanism of Anti-Influenza Virus by Mouse Mx Gene

Akiko YOKOIYAMA, Kyosuke NAGATA, Yoichiro IWAKURA**
and Akira ISHIHAMA

The inbred mouse strain A2G has been shown to be resistant to influenza virus infection. This resistance is determined by a single dominant allele

* Department of Genetics, School of Medicine, Osaka University, Osaka.
** Institute of Medical Science, University of Tokyo, Tokyo.

designated Mx^+. Several attempts have been made to determine the mechanism(s) for induction of the anti-influenza viral state.

First, an attempt is being made to establish mouse cells producing the Mx protein. For efficient expression of the Mx gene, we inserted human metalothionein IIA promoter upstream of Mx cDNA. The recombinant plasmid was transfected into mouse C127 cells (Mx^-) with a pSV2neo by the calcium-phosphate method and Neo-resistant colonies were selected by G-418. Production of the Mx protein in transfected cells is being checked by Northern and Western blottings. Second, we started the production of transgenic mice carrying the Mx^+ gene. In parallel with these *in vivo* experiments, we are purifying the Mx protein from *E. coli* cells carrying an Mx plasmid, for analysis of its effect on *in vitro* transcription and replication of the influenza virus.

Regulation of Transcription by Nuclear Factor I, a Protein Essential for Adenovirus DNA Replication

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

Nuclear Factor I (NFI) has been isolated from uninfected HeLa cell nuclei as an essential factor for the initiation of adenovirus DNA replication, and shown to be a sequence-specific DNA-binding protein (Matsumoto, K. *et al.* (1989) *J. Biochem.* 105: 927–932). NFI binding sites (FIB sites) possibly include the CCAAT-box which often plays a role as a regulatory element of transcription. Cloning of NFI cDNA is important for understanding the physiological function of NFI.

For this purpose, we have devised a novel strategy using a prokaryotic transcriptions system. An oligonucleotide containing the FIB site was inserted into the promoter region of the *E. coli metZ* gene so as to overlap the transcription initiation site. Since the promoter −10 and −35 regions upstream from the initiation site remained intact in this construct, designated Zi12, we expected that the inserted FIB site might function as an operator. In the *in vitro* transcription system using purified *E. coli* RNA polymerase, purified NFI inhibited transcription from the Zi12 promoter by interfering with the formation of the open complex. When a reporter gene is under control of such an *E. coli* promoter-NFI operator sequence, it appears

* Faculty of Pharmaceutical Sciences, University of Tokyo, Tokyo.

possible to clone NFI cDNA by monitoring the repression of the reporter gene. This strategy is also applicable for the cloning of other sequence-specific DNA-binding proteins. Although NFI has been reported to be a transcriptional activator, it may also function as a transcriptional repressor in eukaryotes.

Soluble 5′-Nucleotidases from Rabbit Heart

Yukiko YAMAZAKI, Y. L. TRUONG*, J. M. LOWENSTEIN*
and Akira ISHIHAMA

Two molecular species of soluble 5′-nucleotidases, termed N-I and N-II, from rabbit heart, were separated using phosphocellulose chromatography and characterized with regard to reaction properties and protein structure. N-I has a preference for AMP over IMP as a substrate, while N-II prefers IMP over AMP. Both enzymes require Mg^{2+} as a divalent cation, but differ in optimum Mg^{2+} concentrations. High concentrations of NaCl inhibit N-I but activate N-II.

N-I has been purified to apparent homogeneity by phosphocellulose, DEAE-Sepharose, AMP-agarose chromatography and PROTEIN-PAK 300 HPLC. Enzyme activity cosedimented with a 94,000-dalton polypeptide in glycerol gradient. By polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate, N-I migrated as fast as a protein with the molecular weight of 42,000, indicating that N-I is a dimer of 42,000 dalton subunit. Among the 5′-nucleotides tested, N-I showed the following order of substrate preference: AMP>CMP>UMP>IMP=GMP. However, N-I displayed a sigmoidal saturation kinetics ($h=1.76$, $s_{0.5}=6.8$ mM) in the absence of ADP and a hyperbolic profile ($h=1.0$, $s_{0.5}=3.4$ mM) in the presence of ADP. Neither ATP nor 2,3-diphosphoglycerate activate purified N-I.

These properties suggest that N-I is a novel species of soluble 5′-nucleotidase and is responsible for the release of adenosine from AMP under conditions of hypoxia or increased work load.

* Graduate Department of Biochemistry, Brandeis University, Waltham, MA 02254, USA.

Structure and Complete Nucleotide Sequence of the Human Thymidylate Synthase Gene

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

Using DNA mediated transformation of mouse cell mutant strains deficient in thymidylate synthase (TS), biologically active human genomic DNA fragments for the enzyme were cloned and their nucleotide sequences were determined. Cloning, structure and functional analysis of human TS cDNA have been previously reported (Ayusawa *et al.*, 1984, *J. Biol. Chem.* **259**, 14361; Takeishi *et al.*, 1985, *Nucl. Acids Res.* **13**, 2035; Kaneda *et al.*, 1987, *Nucl. Acids Res.* **15**, 1259). The human TS gene consists of seven exons separated by six introns. The organization of exons and the size of each introns were consistent with those for mouse gene except for intron 3 (6.3 kb) which is larger than its mouse gene counterpart (Deng, Li, Jenh and Johnson, 1986, *J. Biol. Chem.* **261**, 16000). Within introns, a total of 7 *Alu*-type sequences are distributed. However, no *Alu*-type sequences are found in intron 3. Intron 3 of the human TS gene has an interesting feature in that it contains a long L1 repetitive sequence. The amino acid sequence deduced from the open reading frame of the L1 sequence was found to be highly homologous (90%) to that encoded by the known human teratocarcinoma L1 RNA species (Skowronski, Fanning and Singer, 1988, *Mol. Cell. Biol.* **8**, 1385) and contained several blocks of the sequences homologous to RNA-dependent DNA polymerases of various origins. Intron 3 also contains a Hind III repeat sequence and 44 times tandem repeats of the 6 bp element of GGT(or A)GAT. The 5′ flanking region of the human TS gene lacks the canonical 5′ transcriptional regulatory sequence elements "TATA", "CAAT" and "GC box" in the vicinity of the presumed cap sites. In the 5′-untranslated region of cDNA, a consensus GC box sequence (GGGCGG) for the binding site of the transcription factor Sp1, was found in reverse sequence. The gene is rich in GpC sequences that fit the general properties of the growth-associated mammalian genes. The CpG sequence distribution is skewed and concentrated in the

* INRS-Sante, Universite du Quebec, Pointe-Claire, Canada.
** School of Food and Nutritional Sciences, University of Shizuoka, Shizuoka, Shizuoka-ken.
*** Saitama Cancer Center Res. Inst., Saitama-ken.

5′-flanking region, exon 1 and intron 1. Comparison of the human and mouse sequences upstream from the ATG initiator codon revealed many significant blocks of sequence homology, although the triple tandem repeat of a 28-bp sequence and its inverted sequence found in the human sequence were not present in the mouse counterpart.

Functional Analysis of the Human Thymidylate Synthase Gene with the Use of a Minigene

Sumiko KANEDA, Dai AYUSAWA and Takeshi SENO

We reported the total sequence of the human thymidylate synthase (TS) gene of about 23 kb that consists of 7 exons and 6 introns flanked by 5′ and 3′ sequences. In order to locate the regulatory sequences within the gene, for its cell cycle-dependent expression, we analyzed the effect of introns on the expression of the TS minigene when stably introduced into TS-negative mutant cells from mouse FM3A and rat 3Y1 cell lines. The minigene that contained only intron 1 transformed the TS-negative cells at a frequency of about 2×10^{-4} which was comparable to that obtained with TS cDNA ligated to the expressible plasmid vector. The intronless minigene and the minigene that contained only intron 2, both transformed the cells at frequencies 2 orders of magnitude lower than the above value. We also showed that at least 300 bp 5′ upstream of the ATG initiation codon are required for expression of the minigene with intron 1. We further examined the intron 1 sequence of 1.6 kb in terms of enhancer activity, by introducing a part of or whole sequence of intron 1 into recombinant plasmid pA_{10} Cat_2. Results showed that the 5′ proximal half of intron 1 is enough for full enhancer activity. It is noted that within this 800 bp region, 3 consensus sequences (GGGCGG) for the binding site of transcription factor Sp1 were located while no other GGGCGG sequence was found in the TS gene. Further study is necessary, however, to show whether or not the function of intron 1 in the minigene is associated with the cell cycle-dependent expression of the gene.

Characterization of DNA Double Strand Scission in Mouse Cells Induced by Thymidylate Deprivation

Dai AYUSAWA, Hideo TEZUKA and Takeshi SENO

We previously isolated mouse cell mutants deficient in thymidylate synthase from the FM3A line. When deprived of thymidine, the mutant cells, which are auxotrophic for thymidine, die rapidly. The phenomenon, so called thymineless death, was originally studied in *Escherichia coli.* A positive correlation between thymidylate deprivation (often called thymidylate stress for eukaryotic cell systems) and the induction of DNA double stand breaks has been demonstrated in the mutant cells by analyzing DNA size, using alkaline sucrose density gradient centrifugation and neutral filter elution. It has been also demonstrated that cell death and DNA breaks occur in S-phase cells. Thus, they are tightly associated with DNA replication (Ayusawa *et al.*, 1983, *J. Biol. Chem.* **258**, 12448). We have also suggested that an inducible endonuclease is involved in DNA double strand scission (Ayusawa, Arai, Wataya and Seno, 1988, *Mutat. Res.* **200**, 221). By using pulsed-field gel electrophoresis we measured the size of chromosomal DNA fragments produced by thymidylate stress. The results showed that the double strand scission has some specificity in nature: (a) DNA fragments of a rather uniform size of 50–150 kb, were produced in thymidylate-deprived cells, thus migrating as a discrete band across the gel electrophoresis that was apart from the remaining intact chromosomal DNA, and (b) DNA fragments accumulated as a function of time of thymidylate stress, but the size of the fragments did not change. These results indicate that the sites where DNA double strand breaks occur are not random, but predetermined. Since the size of the DNA fragments coincides with the size of the mammalian replicon unit and the breaks are tightly associated with ongoing DNA replication, we hypothesize that thymidylate stress induces DNA double strand breaks at the origin or terminator region of each of the clustered replicons, but not at the ongoing replication fork regions. Characterization of the DNA fragments in terms of nucleotide sequence in both termini, and nature of the inducible endonuclease involved in the scission, are under investigation.

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

Nobuo SHIMAMOTO

E. coli single-stranded DNA binding protein specifically binds to some group of mRNA in a cooperative manner. The importance of this binding has been suggested as follows. First, the binding takes place at physiological concentrations. Second, a significant amount of the binding protein was found complexed with RNA in cell lysate. Third, the complexed form of mRNA was an inactive template in a cell-free translation system *in vitro*. Fourth, the copy number of the protein in a wild type cell was within the range where two types of complexes are formed, one with DNA and the other with RNA. From these lines of evidence the following picture emerges. When cells grow in a favorable environment, replication forks is increased, catching more binding protein. Thus more mRNA is used in translation. The state of replication can be transmitted to a translation system in this way.

To check this model, I identified three RNA segment responsible for the specific and cooperative binding on *ssb* mRNA with homologous 12 base sequences, named SSB box, located outside of the coding regions. Genes *alkA* and *secY* mRNA have such sequences. The *in vitro* translation of these genes was inhibited by the binding protein but that of *recA* and *nusA*, with no such sequences, was not. Thus the binding protein may be a pleiotropic translation factor for presumable SSB translational regulons. An excess amount of the protein may be toxic by inhibiting expression of the whole regulon. An overproduction of the binding protein by mRNA without the SSB box should supply the excess amount of the protein. Results showed such an expression was lethal.

Kinetic Study on Transcription by Immobilized Operons: Release of *E. coli* RNA Polymerase σ Subunit Requires β-γ Phosphodiester Bond of Nucleoside Triphosphates

Nobuo SHIMAMOTO and Miki FUJIOKA

Transcription of genes is mediated by a protein complex which includes the DNA-dependent RNA polymerase. One of the largest targets for

research is clarifying the composition of subunits of the transcription complex. To investigate the relationship between the structure of the complex and the RNA product, one has to stop elongation and also to freeze the composition of subunits. We have devised a novel method for analyzing the structure of the transcription complex and product RNA. This method enables us to stop elongation without changing the ionic atmosphere.

A DNA fragment containing an operator and a promoter was fixed to agarose or acrylamide beads at its ends. The immobilized DNA was a template as good as the conventional free DNA for *E. coli* RNA polymerase. The immobilization of λP_R operon caused little change in the distribution of synthesized RNA species.

We applied this procedure to sigma subunit release in the early step of initiation of transcription by *E. coli* RNA polymerase. Our previous results can be interpreted in two ways. The release itself may need magnesium ion, or alternatively, nucleoside triphosphates might be needed for the release. This problem can be solved if RNA synthesis is rapidly quenched in the presence of magnesium ion by diluting with an excess amount of the reaction buffer. The latter interpretation was the case because σ release was quenched by such a dilution but not by dilution with ATP. The use of ATP analogues revealed an obligatory breakdown of $\beta-\gamma$ phosphodiester bond for σ release. Thus nucleoside triphosphates play two roles in transcription, as substrates for elongation and for σ release.

Global Variation in G+C Content along Vertebrate Genome DNA, and Chromosome Band Structures

Toshimichi IKEMURA, Shin-ichi AOTA*, Fumie ISHIBASHI
and Ken-ichi MATSUMOTO

In higher eukaryotes such as vertebrates, the codon-choice patterns of different genes in a single organism often differ significantly. Some genes are extremely G+C-rich at the codon 3rd position (e.g., 95% G+C), whereas some others are rather A+T-rich (e.g., 30% G+C). The G+C content of the 3rd letter was found to be correlated to the G+C content of a large genome portion surrounding the gene; exons of genes with a high G+C% at the 3rd position (e.g., >80% G+C) are usually surrounded by G+C-

* Present address, Department of Health & Human Services, National Institute of Health, U.S.A.

rich introns and G+C-rich flanking sequences (e.g., 60% G+C), and those with a low G+C% at the position (e.g., <50% G+C) are surrounded by A+T-rich introns and flanking sequences (e.g., 40% G+C). We arranged human DNA sequences in the GenBank according to the order of the Human Genetic Map (HGM9) and analyzed global variation in G+C content along the DNA sequences thus ordered. The analysis showed that the human genome is a mosaic of regions with clear differences in G+C content, and that the diversity at the codon 3rd position is related to the mosaic structures. A correlation between the G+C% levels for human DNA sequences and the chromosome band structures was observed. For details, see Ikemura, T. and Aota, S. (1988) *J. Mol. Biol.*, **203**, 1–13.

Purification of RNA Molecules with High Resolution by Two-dimensional Gel Techniques

Toshimichi IKEMURA

Most tRNA molecules of organisms such as *E. coli*, *Salmonella typhimurium*, *Serratia marcescens*, *Bacillus subtilis*, *Mycoplasma capricolum*, *Saccharomyces cerevisiae*, *Drosophila melanogaster*, mouse and chicken, could be purified by a two-dimensional gel system which we have developed. The method is based on the combination of acrylamide- and urea-concentration shifts: The first dimension is on a 14% acrylamide gel and the second dimension on a 22% acrylamide gel containing 40% urea. Large RNAs such as mRNAs also could be fractionated by other systems of two-dimensional gel electrophoresis, although the resolving power was far from satisfactory when compared with that for small RNAs. The first dimension is on a composite gel of polyacrylamide (2.8–3.0%) and agarose (1%), and the second dimension on a acrylamide gel (5.5–10%) containing 40% urea. P^{32}-labeled *S. cerevisiae* poly(A)$^+$ RNAs, whose sizes are from 1600 to 3500 nucleotides, can be separated with high resolution by this system. For details, see Ikemura, T. *Methods in Enzymology* (Academic Press), Vol. **180**, "RNA Processing" pp. 14–25, Purification of RNA molecules by gel techniques'

Computer Analyses for Searching for Protein Coding Regions in Human Genome DNA Sequences

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

Non-random usage of codons has been used by several researchers to search for protein coding regions in genome DNA sequences for organisms such as *E. coli*, *Drosophila* and nematodes. As an attempt to establish a generalized method for assigning protein coding regions in human genome DNA sequences, solely dependent on computer analyses, we calculated and characterized codon usage patterns of about 600 human genes available from the GenBank DNA Database. It was found that the G+C% at the codon first position when averaged for the 600 human genes is about 55%, that at the second position about 40% and that at the third position about 65%. Utilizing this positional difference in the codon G+C% as the criterion for searching for coding regions, a large portion of human protein coding regions in the GenBank sequences, when used for the test sample, could be successfully assigned, especially for genes whose codon third position is G+C-rich. About one fifth of the human genes, however, are known to be rather A+T-rich at the third position, and the above method turned out to be inapplicable for these A+T-rich genes. To establish a generalized method applicable for a much wider range of human gene sequences, we are now making extensive catalogs of the sequence elements (di-, tri-, or higher oligonucleotide) for exons, introns and flanking regions separately, for the purpose of introducing multiple criteria for the assignment of coding regions in human genome DNA sequences.

Purification of a DNA Supercoiling Factor from the Posterior Silk Gland of *Bombyx mori*

Tsutomu OHTA and Susumu HIROSE

The eukaryotic genome is organized into a series of looped domains that are attached to a nonhistone protein scaffolding called the nuclear matrix. DNA strands within the loops are not free for rotation, so that they can

* International Institute for Advanced Study of Social Information Science, Fujitsu Limited.

** Present Address, Department of Health & Human Services, National Institutes of Health, USA.

form a superhelical state. These chromatin loops are thought to play a role in the control of gene expression and replication by generating different levels of DNA supercoiling within the genome. In order to test this possibility, we searched for a DNA gyrase-like activity in cell extracts from various eukaryotic sources. Among these, a posterior silk gland cell extract of *Bombyx mori* produced positive results. After phosphocellulose fractionation of the posterior silk gland extract, a flow-through fraction contained a factor which introduced negative supercoils into closed circular DNA in conjunction with DNA topoisomerase II. The factor, termed DNA supercoiling factor, was purified to homogeneity by a combination of chromatography on DEAE-Sephacel, Phenyl-Sepharose CL-4B and DNA cellulose. It was a heat-stable protein of a molecular mass of 50 kD.

DNA Supercoiling Facilitates Formation of the Transcription Initiation Complex on the Fibroin Gene Promoter

Hisahiro TABUCHI and Susumu HIROSE

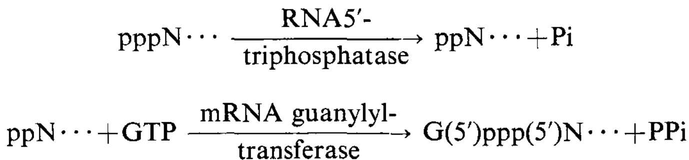
Transcription of the *Bombyx mori* fibroin gene in a posterior silk gland extract can be separated into three functional steps on the basis of sensitivity to Sarkosyl: 1. formation of an initiation complex, which is blocked by 0.025% Sarkosyl; 2. conversion of the initiation complex to an elongation complex, a step sensitive to 0.05% Sarkosyl; 3. the subsequent elongation of the RNA chain which occurs in the presence of 0.05% Sarkosyl. While the last two steps are rapid and unaffected by template topology, the first step is slow and affected by DNA conformation. In the posterior silk gland extract of *Bombyx mori*, closed circular DNA forms a superhelical state and supports more rapid assembly of the initiation complex than linear DNA does. Both DNA supercoiling and rapid assembly of the initiation complex require ATP and are abolished by the addition of a topoisomerase II inhibiter VP16. These results suggest that DNA supercoiling enhances fibroin gene transcription by facilitating formation of the initiation complex. For details, see *J. Biol. Chem.*, **263**, 15282–15287.

Mechanism of mRNA Capping Reaction

Kiyohisa MIZUMOTO

Most of the cellular as well as viral mRNAs in eukaryotes contain a 5′-terminal cap structure, m7GpppN. This cap structure is required for the efficient initiation of translation and for RNA splicing. It has also been suggested that the synthesis of the methylated cap structure may play an important role in transcription itself. The cap structure is synthesized at the initial stage of mRNA synthesis and conserved at the 5′-termini of RNAs while they are processed in the nucleus and transported to the cytoplasm. Consequently, elucidation of the mechanism of the cap structure is important for understanding the molecular mechanism of eukaryotic gene expression.

We characterized the reactions catalyzed by the mRNA capping enzyme (mRNA guanylyltransferase), the key enzyme in the cap formation, from various eukaryotic cells. The capping enzyme from animal cells consists of a single polypeptide chain with an approximate Mr of 70,000 containing two catalytic domains for each of the initial two consecutive reactions involved in the cap formation, *i.e.*, those catalyzed by mRNA guanylyltransferase and RNA 5′-triphosphatase.



$$\text{pppN}\cdots \xrightarrow[\text{triphosphatase}]{\text{RNA5}'\text{-}} \text{ppN}\cdots+\text{Pi}$$

$$\text{ppN}\cdots+\text{GTP} \xrightarrow[\text{transferase}]{\text{mRNA guanylyl-}} \text{G(5}'\text{)ppp(5}'\text{)N}\cdots+\text{PPi}$$

In contrast, the highly purified yeast capping enzyme is composed of two separate chains of 52 kDa (α) and 80 kDa (β), responsible for the activities of mRNA guanylyltransferase and RNA 5′-triphosphatase, respectively (Mizumoto, K. and Kaziro, Y. (1987), *Prog. Nucl. Acid. Res. Mol. Biol.*, **34**, 1).

To see whether the α and β chains of the yeast capping enzyme are derived from a single polypeptide or are independent polypeptides encoded by two separate genes, we attempted to isolate the gene(s) for the yeast capping enzyme. A yeast genomic expression library in λ gtll was screened with antibodies against the yeast capping enzyme. One of the positive clones, λ C3, contained a 3,500 bp yeast DNA insert. From experiments based on

an epitope selection of antibodies by antigens produced with λC3 in *E. coli*, this clone was found to contain the gene for the α subunit. The identity of the gene was further confirmed by expressing the gene in *E. coli* to produce catalytically active guanylyltransferase. The gene is present in one copy per haploid genome, and encodes a protein of 459 amino acid residues. From the primary structure of this gene as well as its mRNA size, we concluded that the α and β subunits of yeast capping enzyme are encoded by two separate genes. Gene disruption experiments showed that the α gene is essential for growth (Mizumoto, K. *et al.* (1989), *J. Cell. Biochem.*, **13D,** 209).

We characterized an RNA polymerase II transcription initiation complex formed with HeLa cell extracts and a DNA fragment containing the adenovirus major late promoter in the presence of ATP. An initiation complex with an approximate size of 50 S could be isolated by glycerol gradient centrifugation free from the bulk of RNA polymerase II and the capping enzyme. Specific transcription was detected with this complex when supplemented with the remaining nucleoside triphosphates. Analyses of the 5′-terminal structure of the transcript revealed the presence of a cap structure m^7GpppA in more than 50% of the RNA chains. These results indicate that at least two cap-related enzymes, mRNA guanylyltransferase and mRNA(guanine-7-)methyltransferase, are specifically associated with the complex. The precise step in transcription during which capping takes place and the possible interaction between the capping enzyme system and transcription factors remain to be studied.

Messenger RNA Synthesis of Sendai Virus (HVJ)

Kiyohisa MIZUMOTO

Sendai virus (HVJ), a member of the paramyxovirus group, contains a single nonsegmented negative strand RNA genome of approximately 15 kb long, encoding at least seven proteins, NP, P, C, M, F, HN and L. The genetic information of this negative strand RNA is expressed through monocistronic mRNAs that are transcribed by a virion associated RNA-dependent RNA polymerase composed of P and L proteins. The precise mechanism and the mode of transcription, however, have been unclear, since no efficient *in vitro* transcription system has previously been established. We developed an efficient and faithful *in vitro* transcription system using

purified virus particles, and found that the HVJ mRNA synthesis is almost entirely dependent on the presence of a host factor(s).

The host factor essential for the *in vitro* transcription was partially purified from mammalian cells and was shown to be separated into at least two complementary fractions, one of which, surprisingly, could be replaced by highly purified tubulin.

The analysis of the cap-containing oligonucleotide from RNase T_2 digests of *in vitro*-synthesized poly(A)$^+$RNA demonstrated that almost all RNA molecules terminate in m^{7}GpppAmpGpGp···. This indicated that the transcription of HVJ mRNA seems to start at the first U residue in the R1 consensus sequence (3′-*U*CCCNNUUNC-5′) which is found in the upstream regions of all six HVJ genes.

mRNA was synthesized *in vitro* in the presence of [α- or β-^{32}P]ATP or GTP as the labeled substrates, and the cap structure m7GpppAm was isolated after nuclease P1 digestion of RNA. The α and β phosphates of GTP and the α phosphate of ATP, but not the β phosphate of ATP, were incorporated into the cap structure. These ^{32}P-labeling data were consistent with a unique mechanism of cap formation by condensation of GDP residue from GTP with pA- at the 5′-termini of HVJ mRNAs.

Regulation of Chromosomal Replication in Bacteria

Hiroshi YOSHIKAWA

I. Regulation of initiation of chromosomal replication by DnaA-boxes in the origin region of the *Bacillus subtilis* chromosome.

A gene homologous to the *Escherichia coli dnaA* gene and two flanking regulatory regions which contain nine and four DnaA-boxes respectively, are located in the replication origin region of the *Bacillus subtilis* chromosome. Attempts to isolate an autonomously replicating fragment from these regulatory regions in order to identify *oriC* have been unsuccessful because the DnaA-box-containing regions strongly inhibited plasmid transformation, particularly when inserted into a high-copy number plasmid, pUB110. Using two plasmids differing in copy number, the two regions were subdivided into three regions, A, B and C, each containing five, four and four DnaA-boxes respectively, and differing in level of inhibition of transformation. Region C is downstream of the *dnaA* gene and inhibits transformation in high-copy, but not in low-copy number plasmids. When a part of

the DnaA-boxes were deleted from the incompatible plasmids, they became transformable and produced slow-growing transformants in which the initiation frequency of chromosomal replication was selectively reduced. Fast-growing revertants were found containing the same number of plasmids as the parent, but with single base changes in the DnaA-boxes. These mutations were in the most highly conserved bases of the DnaA-box sequence. This indicates that a sequence-specific interaction of the DnaA-box, probably with the *B. subtilis* DnaA protein, is responsible for the observed incompatibility and thus appears to be involved in the control of initiation frequency of chromosomal replication.

II. Structure of the *dnaA* region of *Pseudomonas putida*: conservation among three bacteria, *Bacillus subtilis*, *Escherichia coli* and *P. putida*.

We cloned from *Pseudomonas putida*, a gene homologous to *Escherichia coli dnaA*, and determined the sequence of the gene and its neighboring region. The *dnaA* gene and at least three other genes, *dnaA*, *recF* and *gyrB*, were found to be highly homologous to the genes in the *dnaA* regions of the *E. coli* and *Bacillus subtilis* chromosomes. A non-translatable region of some 600 bp immediately upstream of the *dnaA* gene, is also conserved in the three bacteria and contains 3, 12, and 14 DnaA-boxes (TTATCCACA and closely related sequences) in *E. coli*, *P. putida*, and *B. subtilis*, respectively. The present results confirm our hypothesis that the *dnaA* region is the replication origin region of the ancestral bacterium and that the essential feature of the *dnaA* and DnaA-box combination is conserved in most eubacteria and plays a central role in initiation of chromosomal replication.

III. Comparative anatomy of *oriC* of eubacteria.

We reported that at least six genes and their organization is conserved in three bacteria, *E. coli*, *P. putida* and *B. subtilis*. In addition, we showed that a gram positive bacteria, *M. luteus*, also had a *dnaA* and neighboring genes highly homologous with those of the above three bacteria. We therefore concluded that the *dnaA* region is conserved in most of the eubacteria. Comparison of *dnaA* genes among the four bacteria demonstrated clearly that the gene is composed of highly conserved and non-conserved domains. The remarkable feature of the *dnaA* region is the conservation of non-coding regions containing a cluster of DnaA-boxes. Gram negative bacteria contain one region upstream of the *dnaA* gene, while gram positive bacteria contain two regions flanking the *dnaA* gene.

The combination of *DnaA* protein and DnaA-box plays a central role in

the initiation of chromosomal replication in *E. coli.* We showed that the same combination is also essential in the initiation of chromosomal replication in *B. subtilis.* Thus, a temperature sensitive mutant which is defective specifically in initiation of chromosomal replication, was isolated by in vitro mutagenesis of the cloned *dnaA* gene. A single amino acid substitution was detected in the coding region of the *dnaA* gene of the mutant cell. Secondly, introduction of an excess of DnaA-boxes into *B. subtilis* cells resulted in the inhibition of initiation of chromosomal replication, probably through the titration of limited amounts of DnaA protein in the cell. The DnaA-box region upstream of the *dnaA* gene in the *P. putida* chromosome was shown to be active as *oriC.* These results show that the DnaA protein-DnaA box interaction is universal in eubacteria and plays a central role in the initiation of chromosomal replication.

II. MICROBIAL GENETICS

Gene Organization in the Region Containing a New Gene Involved in Chromosome Partition in *Escherichia coli*

Jun-ichi KATO*, Yukinobu NISHIMURA, Masao YAMADA**
Hideho SUZUKI*** and Yukinori HIROTA†

One goal of cell division is segregational transmission of a genome to daughter cells. To prevent production of chromosomeless cells, the process of cell division in *Escherichia coli*, as well as in other organisms, is placed under elabrate control, to couple it with replication and partition of chromosomes. For example, perturbation of chromosome replication arrests cell division through the induction of a division inhibitor, the *sulA* gene product, which targets the *ftsZ* (*sulB*) gene product, whose function is essential for the progression of the division process. Chromosome partition should be another process that regulates cell division. This process, although barely understood, seems to include two aspects: molecular separation (resolution of topological linkages), and topographical segregation of the replicated chromosomes. The latter aspect has been postulated as involving the attachment of replicons to the cell surface structure, the growth of which contributes to topographical segregation of the attached replicons.

Two kinds of *Escherichia coli* mutants defective in chromosome partition have been described as *parA* and *parB*. They are thermosensitive and reveal the distinctive morphology of centrally congregated nucleoids at restrictive temperatures. The *par* mutations have recently been investigated concerning their relationship to the termination of DNA replication, and *parB* is most probably allelic to *dnaG* (for primase) and located at 67 min on the genetic map (Norris, V. *et al.* 1986. *J. Bacteriol.* **168**: 494–504; Nishimura, Y. *et al.* 1988. *Ann. Rep. Natl. Inst. Genet.* **38**: 43–44). Anzlyses of such mutations are indeed expected to provide clues to the mechanism of

* Department of Bacteriology, National Institute of Health, Tokyo.
** Present address: National Children's Medical Research Center, Tokyo.
*** Faculty of Sciences, University of Tokyo, Tokyo.
† Deceased on December 23, 1986.

chromosome partition and its control over the cell division process.

We identified a new mutation, *parC*, which causes abnormal chromosome segregation, in two thermosensitive mutants of *Escherichia coli.* The thermosensitive growth of the mutants was corrected by pLC4-14 in the Clarke-Carbon collection, which is known to carry the *metC* region at 65 min (Dwivedi, C. M. *et al.* 1982. *Biochemistry* **21**: 3064–3069). The nearness of *parC* to *metC* was confirmed, and the cotransduction frequency of *parC* was 59% with *metC* and 20% with *glc*. This plasmid carries a putative gene which can suppress the cell division defect due to *ftsI* (*pbpB*) and has hence been termed *sufI* (Nishimura, Y. *et al.* 1977. *Plasmid* **1**: 67–77). The *parC-sufI* region was analyzed by subcloning the chromosome of pLC4-14. The *parC* and the *sufI* gene products were electrophoretically identified as proteins of 75 and 55 kilodaltons (kDa), respectively. The allelism of *parC*$^+$ on pLC4-14 to *parC1215* was confirmed by cloning *parC1215*. The *sufI* gene appeared to be dispensable to cell viability, and overproduction of its product caused suppression of *ftsI*. An essential gene coding for a 25-kDa protein was found between the *parC* and the *sufI* genes. These three genes were transcribed in the same direction and may be organized into an operon, with *parC* to the proximal side and with internal promoters, at least for the distal genes (Fig. 1). The localization of the gene products was examined in maxicells. The *sufI* protein was synthesized as a precursor which could be chased into a mature form. The major part of the mature form was found in the soluble fraction. The

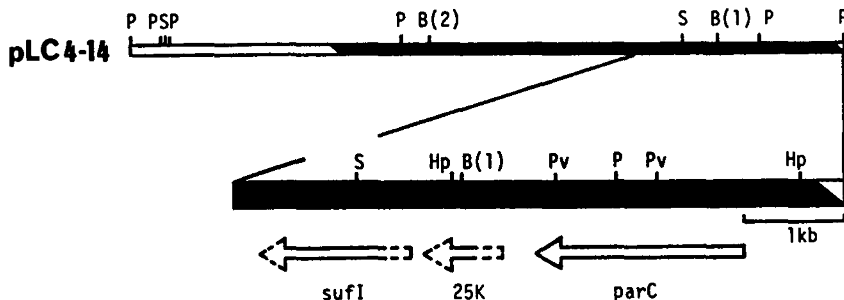


Fig. 1. Possible location of the genes for *parC*, *sufI*, and the 25 K protein. The arrows below the map indicate the roughly estimated location and direction of transcription of the *parC* gene, the *sufI* gene, and the gene for the 25 K protein. B, *Bam*HI; Hp, *Hpa*I; P, *Pst*I; Pv, *Pvu*II; S, *Sma*I.

25-kDa protein was found almost exclusively in the membrane fraction. The *parC* protein was associated with the membrane fraction in the presence of Mg^{2+}, but found in the soluble fraction when Mg^{2+} was sequestered with EDTA. The same Mg^{2+} dependent localization has been found for the *sopB* protein involved in the partition of a mini-F plasmid (K. Nagai, *et al. Annu. Meet. Mol. Biol. Soc. Jpn.* 1984, abstr. no. **7**: B-17). This similarity in Mg^{2+} dependence may suggest a property common to the proteins engaged in the partitioning of replicons.

The *parC* gene product was identified as a 75 K protein by SDS-PAGE. Proteins of similar size have been seen in *OriC*-membrane complexes and F partition-specific complexes. The process of chromosome partition presumably involves interaction of the chromosome with the cell surface structure, and the complex may be a structure for this interaction. The 75 K protein found in the F partition-specific complex was assumed to originate from the background synthesis in minicells. The *parC* product might be identical to this protein and be shared by the chromosome and mini-F for their partitioning. A genetic analysis of these proteins would be an effective approach to the problem, and analyses of *par* mutants may help in understanding the roles of the proteins contained in the DNA-membrane complex. For details, see *J. Bacteriol.* 1988. **170**: 3967–3977.

Isolation of Novel Mutants Defective in DNA Replication in *Escherichia coli*

Seiichi YASUDA

Although almost all protein factors involved in the replication of the *Escherichia coli* chromosome have been identified, the mechanism of regulation of replication remains unsolved. I have been trying to find new *dna* genes which are involved in regulation and hence, will give some clue to the understanding of this mechanism.

E. coli K12 strain SY965 was treated with N-methyl-N′-nitro-N-nitrosoguanidine, and individual clones from the mutagenized culture were tested for growth at a high temperature (42°). Clones which do not grow at the high temperature were spotted on a plate in a grid pattern of 72 clones per plate and incubated overnight at 30°. Colonies were transferred with a metallic apparatus with 72 needles arranged in a similar grid pattern onto two nitrocellulose disks (82 mm), which were placed on L broth agar plates

and incubated at 30° for 2 to 3 hrs. When visible colonies grew on the filters, one of the filters was transfered to labeling medium and the other was incubated further at 42° on a plate which had been kept at that temperature. The labeling medium was made in a fresh petri dish by adding 64 μCi of [^{3}H] thyimidine to 3.2 ml of L broth and then placing two pieces of sterile No. 2 filter paper (82 mm diameter) on it. After labeling, at 30° for 30 min. the nitrocellulose fiter was transferred to the top of a stack of four pieces of No. 2 filter paper, which had been soaked in 8 ml of 1M HCl. Colonies grown at 42° were labeled at 42° similarly, using labeling medium prewarmed at 42°. Nitrocellulose filters with labeled colonies were washed by suction with 70% ethanol, dried in vacuum, soaked in DPO solution (0.2 g/ml DMSO), dried in air and then subjected to autoradiography at −80° overnight. Colonies which showed lower incorporation at 42° than at 30° were saved as candidate *dna* mutants. Of about 800 temperature-sensitive mutants tested, 75 clones were classified as tentative *dna* mutants. 26 of 75 *dna* candidates were found to be mutants of known *dna* genes. The remaining 49 strains were analysed further to find new *dna* genes.

Two mutants, 2614 and 2618, were found to have mutations in new *dna* genes. Strain 2614 has a mutation in a gene closely linked to *aroA* and strain 2618, to *pyrC*. Both strains show residual DNA synthesis after transferring cultures to a higher temperature, suggesting that these *dna* genes function in the initiation step of chromosomal replication. Each of these mutations requires the presence of another mutation for expression of typical *dna* characters. The nature of these secondary mutations, including map positions, are not known. Crude enzyme fractions prepared from these *dna* strains had normal activity to support *in vitro* replication of *oriC* DNA. Thus, it is possible that these *dna* genes are involved in the regulation of initiation of chromosomal replication, rather than in catalytic steps, but more extensive studies are necessary.

Allelism of *Escherichia coli parA* to *gyrB* and Some Views on *par*-dependent Regulation of Cell Division

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

The *Escherichia coli parA* mutation was found originally in MFT110 and our studies on this mutant have suggested that MFT110 carries two thermosensitive (ts) mutations and the major ts mutation is not *parA* but most probably *psd* which lies at or very close to the locus where *parA* was originally mapped (Kato *et al.*, 1988, *Annu. Rep. Natl. Inst. Genet.* **38**: 41–42).

The major ts mutation in MFT110 was displaced by the wild-type allel cotransduced with a *psd*-linked Tn*10* from ECL510 (*zjd*:: Tn*10*). Thermoresistant (tr) co-transductants were isolated on L agar plates from tetracycline resistant (Tc^R) transductants selected at 30°C. The tr derivatives grew on L agar plates, but not on salt-free L agar, at 42°C, suggesting that the second ts mutation carried by MFT110 was repairable with 0.5% NaCl in the L medium. One of the tr derivatives was made Hfr by a cross with P4$\times$8 and used for mating experiments, the results of which suggested that the second ts mutation lay between *ilvA* and *xyl*. Then, *tna*:: Tn*10* was transduced from a *tna*:: *10 recF* strain (JC12334), to one of the recombinants that had the second ts mutation. Among the Tc^R transductants, five were tr and sensitive to UV (*recF*$^-$) and one was ts and UV-insensitive (*recF*$^+$). Thus, the second mutation in MFT110 should be located close to *recF* and *tna* at 83 min on the genetic map. This ts mutation was introduced into W3110 by co-transduction with *tna*:: Tn*10* and the resultant W3110ts was examined for the morphological phenotype. W3110ts manifested a typical Par phenotype with an accompanying release of nucleoidless cells at 42°C as observed with fluorescent nucleoids of DAPI (4′,6-diamidino-2-phenylindole) stained cells. Obviously, the second ts mutation was responsible for the Par phenotype as described for the original MFT110 (*parA110*). Although the ts mutation was salt-repairable as to growth, the Par phenotype was essentially the same in either the presence or absence of salt.

The chromosome region at 83 min contains a cluster of *dnaA*, *dnaN*, *recF* and *gyrB*. *parA110* was complemented by a plasmid pJB11 (*gyrB*$^+$) as well as pJM2-9, carrying the whole cluster region, but not by pSY342 (*dnaA*$^+$) and pSY504 (*dnaN*$^+$ *recF*$^+$) (Fig. 1). Therefore, *parA*110 is most

* Department of Bacteriology, National Institute of Health, Tokyo.
** Faculty of Sciences, University of Tokyo, Tokyo.

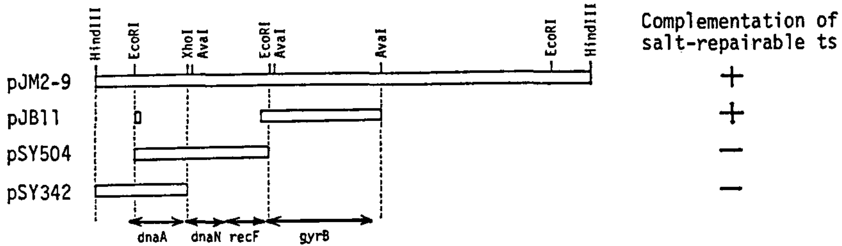


Fig. 1. Chromosome region that complements the salt-repairable ts mutation. The 13 kb *Hin*dIII fragment containing *dnaA-gyrB* region or part of this fragment was carried by the plasmids as shown with rectangles. Complementation was examined with W3110*parA110*. The plasmids were generous gifts from Drs. J. Yamagishi and S. Yasuda, to whom we are obliged.

probably an allele of *gyrB* and the ParA phenotype is likely to result from a defect in decatenation of daughter chromosomes.

Four *par* mutants have been described as *parA-D* in *E. coli.* The Par phenotype, observed typically as elongated cells with centrally congregated nucleoids, appears to result from abnormal replication or failure in decatenation of DNA: *parA* and *parB* are allelic to *gyrB* and *dnaG*, respectively; *parC* probably represents a gene for a topoisomerase-like protein that interacts with DNA-membrane (Kato *et al.*, 1988, *J. Bacteriol.* **170**: 3967; unpublished results); and ParD is due to *gyrA* (Hussain *et al.*, 1987, *Mol. Microbiol.* **1**: 259). These facts and views suggest that the primary effects of *par* mutations may be attributed to a defect in resolution of replicated chromosomes. Topoisomerases would catalyze the decatenation of replicons. Besides the catalytic activities, the structural functions in replicon partitioning have been suggested for a gyrase which binds the pSC101 *par* region (Wahle and Kornberg, 1988, *EMBO J.* **7**: 1889). Some of the topoisomerases may mediate the DNA-membrane association responsible for chromosome segreagtion in cell division. Chromosomes might then be bound to the prospective septation sites and the binding might in turn inhibit unscheduled septations and consequently the production of nucleoidless cells. In this respect, the temporal regulation of cell division might involve the structural functions of a gyrase or another topoisomerase. For details, see *Mol. Gen. Genet.* 1989, **217**: 178–181.

Nucleotide Sequence of the *sufI* Gene Responsible for the Multicopy Suppression of a Thermosensitive Cell-division Mutation in *E. coli*

Yoshihiro YAMAMOTO*, Jun-ichi KATO**, Hideho SUZUKI***
and Yukinobu NISHIMURA

The suppression of a mutation in one gene by amplified copies of a different wild-type gene has been termed "multicopy suppression" (Berg, C. M. *et al.* 1988. *Gene* **65**: 195–202). Growth thermosensitivity in an *E. coli ftsI730* mutant defective in cell division, can be corrected by a cloned *sufI* gene on a multicopy plasmid (Kato, J. *et al.* 1988. *J. Bacteriol.* **170**: 3967–3977). To elucidate the molecular mechanism of the multicopy suppression of an *ftsI* mutation by *sufI*, we determined the nucleotide sequence of a DNA fragment containing the *sufI* gene. The sequence analysis revealed that only one open reading frame allowed a region sufficient for coding for the size of SufI (55 kDa). The region of the putative structural gene for *sufI* consisted of 1,476 basepairs, coding for a polypeptide of 492 amino acid residues and of 54,178 in molecular weight, in good agreement with the size of the gene product as estimated by maxicell analysis. The hydropathy profile of the deduced amino acid sequence suggested the existence of a possible signal peptide in the N-terminal region, consistent with a previous study showing that SufI is probably a periplasmic protein, processed from the precursor into a mature form.

The amino acid sequences of SufI and FtsI were compared. Some similarity was observed between the C-terminal region of SufI and the N-terminal region of FtsI (Fig. 1A). FtsI is the penicillin-binding protein 3 participating in septal wall synthesis and its N-terminal half is supposed to comprise the peptidoglycan transglycosylase domain. Thus, SufI might be a different type of transglycosylase and its overproduction could circumvent the *ftsI* lesion by compensating for a low catalytic reaction rate. The mutation site in the *ftsI730* mutant protein is under investigation.

In addition, the N-terminal part of SufI showed some similarity to the middle part of creatine kinases of chick, rabbit, mouse and rat, which is known to be highly conserved (Fig. 1B).

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* Hyogo College of Medicine.
** National Institute of Health, Tokyo.
*** Faculty of Sciences, University of Tokyo, Tokyo.

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A) SufI  (409-467)  NGAMRSRKTEAGKIPFGLTDKRSCLFISVSLPGRTSRSTSTVRRGNGGPWL--DWATVGQS
                    .*.*** .... ... *....**   ..**.* .. .....  ...**  .   * .....
   FtsI  ( 56-116)  EGDMRSLRVQQVSTSRGMITDRSGRPLAVSVPVKAIWADPKEVHDAGGISVGDRWKALANA

B) SufI  ( 22- 89)  PLKASAAGQQQPLPVPPLLESRRGQPLFMTVQRAH-WSFTPGTRASVWGINGRYLGPTIRVWKGDDVKL
                    **** .. .**.*   ..* ...  **...  .*. *   *..* ..*  ...   *. ** ... .*
   CK-CM (175-235)  PLKAMTEQEQQQLIDDHFLFDKPVSPLLLASGMARDW---PDAR-GIWHNDNK----TFLVWVNEEDHL
```

Fig. 1. Similarity between SufI and FtsI or creatine kinase. Regions in the amino acid sequences of the proteins are indicated by the amino acid residue numbers in parentheses. Identical or structurally related amino acids are marked by asterisks or dots, respectively.
CK-CM: chicken muscle creatine kinase.

C-Terminal Processing of Penicillin-binding Protein 3 of *Escherichia coli*: I. Analysis of the Degradation Product that Represents the C-Terminal Half§

Youji SAKAGAMI*#, Hiromichi NAGASAWA*, Akinori SUZUKI*,
Hiroshi HARA and Hideho SUZUKI**

Penicillin-binding protein 3 (PBP 3) of *Escherichia coli* is an essential enzyme involved in cell division. It is synthesized as a precursor form distinguishable by electrophoretic mobility and then processed into a mature form. Investigations by gene manipulation for producing hybrid/truncated PBP 3 molecules have shown that the C-terminal part, rather than the N-terminal part, appears to be responsible for the processing (Hara, H. *et al.*, 1988, *Natl. Inst. Genet. Ann. Rep.* **38**: 49–51).

Preparations of purified PBP 3 contain a 40 kDa protein (40K fragment) regarded as a degradation product of PBP 3. This fragment retains penicillin-binding ability and hence is considered to represent the C-terminal half which constitutes the penicillin-binding domain of PBP 3. The 40K fragment showed reduced electrophoretic mobility in PBP 3 preparations from a mutant which is defective in the processing of PBP 3, suggesting that the processing occurred within this fragment and therefore in the C-terminal region. Thus the 40K fragment was isolated from preparative gels and

* Department of Agricultural Chemistry, University of Tokyo.
** Laboratory of Genetics, Department of Biology, Faculty of Science, University of Tokyo.
# Present Address: Department of Agricultural Chemistry, Nagoya University.
§ This and the following report in series are dedicated to the memory of the late Professor Y. Hirota who laid the foundation of these collaborative works.

analyzed for its amino acid sequence. The N-terminal part of the fragment corresponded to the deduced sequence beginning with Lys^{211}. In sequencing the peptides of PBP 3 which were generated by lysyl endopeptidase and fractioned by HPLC, we found that two peptides were identical with each other in sequence except that the N-terminal Lys was present in one and absent in the other. The identical part of the peptides corresponded to the deduced sequence beginning with Asp^{212}. Obviously, one peptide carrying N-terminal Lys invulnerable to lysyl endopeptidase was derived from the 40K fragment and the other derived from the intact PBP 3 as a result of lysyl endopeptidase cleavage between Lys^{211} and Asp^{212}. The identification of these two peptides supports the idea that the N-terminus of the 40K fragment is Lys^{211}. These results confirm that the 40K fragment represents the C-terminal half of PBP 3 and that the processing of PBP 3 occurs in the C-terminal region.

The 40K fragment was associated with insoluble cell fractions and solubilized with a detergent along with intact PBP 3 molecules. This argues that the C-terminal half of PBP 3 is associated with the membrane, although it has been suggested that the C-terminal part of PBP 3 is extruded into periplasm rather than anchored to membranes (Spratt, B. G. *et al.*, 1988, in *Antibiotic inhibition of bacterial cell surface assembly and function* (Actor, P. *et al.*, ed), American Society for Microbiology, p. 292–300).

C-Terminal Processing of Penicillin-binding Protein 3 of *Escherichia coli*: II. Determination of the Cleavage Site§

Hiromichi NAGASAWA*, Akinori SUZUKI*, Hiroshi HARA and Hideho SUZUKI**

The maturation of penicillin-binding protein 3 (PBP 3), an enzyme essential for septum formation in *Escherichia coli*, involves a cleavage of the C-terminal part of the precursor. Analyses of truncated PBP 3 molecules which lost the C-terminal part in varieties of length have shown that the region between residues 560 and 576 is essential for the processing (Hara,

* Department of Agricultural Chemistry, University of Tokyo.
** Laboratory of Genetics, Department of Biology, Faculty of Science, University of Tokyo.
§ See footnote to p. 48.

H. *et al.*, 1988, *Natl. Inst. Genet. Ann. Rep.* **38**: 49–51).

The cleavage site for processing was determined by peptide mapping and sequence analyses of the precursor and the mature PBP 3, which were purified from a processing-defective mutant and a wild type strain, respectively. HPLC peptide mapping of lysyl endopeptidase digests of the precursor and mature forms revealed the peptides that differ in retention time between the precursor and mature forms. These peptides were identified by amino acid sequencing as the C-terminal region beginning with Tyr^{540}. Each of the C-terminal peptides was cleaved into two fragments by trypsin digestion. By coupling with an aminopolystyrene resin, the resultant carboxyl-side fragment derived from the mature form was sequenced successfully to the carboxyl end, which was found to be Val^{577} with free COOH. Since it is unlikely that the Val-Ile bond was cleaved by lysyl endopeptidase or trypsin, it was concluded that the Val^{577}-Ile^{578} bond should be the cleavage site for processing. This conclusion was supported by amino acid compositions determined for the relevant tryptic peptides which suggested that the peptide from the cleavage site to the end of the deduced sequence (Ile^{578}-Ser^{588}) was present in the precursor but absent in the mature form.

The cleavage site at Val^{577} lies one residue beyond the region of residues 561 to 576 which has been suggested to be essential for the processing by analyses of the truncated PBP 3 molecules. The discrepancy might be explained by the assumption that the processing enzyme is not strict with the peptide residues to be cleaved but depends primarily on recognition of a certain sequence within residues 561 to 576.

Curiously, the Lys^{573}-Asn^{574} bond resisted both trypsin and lysyl endopeptidase and remained uncleaved in the peptides analyzed above. The cause of resistance to tryptic cleavage is probably the structure of Asp^{572}-Lys^{573}, which is known to be hardly cleaved by trypsin. However, the reason for the inhibition of lysyl endopeptidase cleavage is unclear.

C-terminal processing has been described in several proteins of eukaryotic and prokaryotic cells (*e.g.* Diner, B. A. *et al.*, 1988, *J. Biol. Chem.* **263**: 8972–8980; Yanagida, N. *et al.*, 1986, *J. Bacteriol.* **166**: 937–944). No sequence similarity in peptides around processing sites has been found among these proteins and PBP 3. The C-terminal processing of PBP 3 seems to be unique and its significance in function is not obvious at present.

Requirement of the C-Terminal Region for the Function of *Escherichia coli* Penicillin-Binding Protein 3

Hiroshi HARA and Hideho SUZUKI*

The C-terminal processing of penicillin-binding protein 3 (PBP 3) of *Escherichia coli* has been investigated by constructing the plasmids that direct the production of PBP 3 truncated at various sites in the C-terminal part. The truncated product with a normal amino acid sequence to Thr^{560} (PBP 3′-560) and the products with shorter normal sequences (PBP 3′-544 and -497) were unstable, but the product with a normal sequence to Phe^{576} (PBP 3′-576) appeared to be as stable as normal PBP 3 (Hara, H. *et al.*, 1988, *Natl. Inst. Genet. Ann. Rep.* **38**: 49–51).

To investigate the functional aspect of the truncated products, those plasmids were examined for their ability to complement a temperature-sensitive (ts) PBP 3 mutation (*ftsI*), which causes the filamentation of cells at a restrictive temperature. The ts growth of an *ftsI* mutant on agar plates was corrected by PBP 3′-576 and -560, but not by PBP 3′-544 and -497. The C-terminal region to residue 560 is considered to be essential for the function of PBP 3, while the sequence of residues 561 to 576 is apparently important to stability but dispensable in terms of function.

The morphological phenotype of the *ftsI* mutant at the restrictive temperature was normal when PBP 3′-576 was produced, showing that this truncated product was fully functional. This product was stable and processed, although at a greatly reduced rate, into the mature form which could be assumed to be identical with the normal mature PBP 3 except for a small span to the C-terminus (Nagawawa *et al.*, in this *Ann. Rep.* p. 49–50). With plasmids that directed the other truncated products, irrespective of complementation-positive or negative, filamentous cells were found even at the permissive temperature. Although PBP 3′-560 complemented the growth defect of the *ftsI* mutant, its function appears incomplete. When PBP 3′-497 or PBP 3′-544 was produced, many of the filamented cells became ghosts after 3 hr incubation at 42°C. The loss of the C-terminal peptides not only impairs the activity of the truncated PBP 3 but may produced an adverse effect on cell division and murein sacculus integrity. Such an effect would be understood if PBP 3 functions in a (division) com-

* Laboratory of Genetics, Department of Biology, Faculty of Science, University of Tokyo.

plex as suggested previously (Takeda *et al.*, 1981, *Plasmid* **6**: 86–98). PBP 3′-544 caused notable filamentation at 30°. This truncated product was so unstable that the product could not be determined for processing capacity. This product might be incorporated quite efficiently into the putative division complex, rendering the complex abortive through its rapid degradation. PBP 3′-497 was less toxic compared with PBP 3′-544. The former might be far less efficient in competition for the complex formation with the product directed by the *ftsI*^{ts} gene on a chromosome.

These results show the importance of the C-terminal region to the function of PBP 3. This region contains four hydrophobic segments bracketed by basic residues, the last of which ends at Leu^{558}. All of the four hydrophobic segments were required for the function of PBP 3, and the peptide sequence that follows the last hydrophobic segment (residues 561 to 576) is presumably required for stability and thus perfect functioning of PBP 3. The complementation was examined under overproducing conditions which might have compensated for function insufficiency. The importance of the extreme C-terminal region of the mature PBP 3 molecule is in contrast with the C-terminal region of PBP 1b, in which no processing is known and 64 residues from the C-terminus can be deleted without a detectable change in its function (Kato *et al.*, 1984, *Molec. Gen. Genet.* **196**: 449–457).

Cloning of a Gene Involved in the C-Terminal Processing of Penicillin-Binding Protein 3 of *Escherichia coli*

Hiroshi HARA and Hideho SUZUKI*

The mature form of penicillin-binding protein 3, an enzyme essential for cell division in *Escherichia coli*, is produced by proteolytic cleavage of the C-terminal part from its precursor form. We have found a mutant, JE7304, defective in this unique processing reaction (Hara *et al.*, 1988, *Natl. Inst. Genet, Ann. Rep.* **38**: 48–51). The mutation will be referred to as *prc* (*pro*cessing in the *C*-terminal part). The *prc* mutant showed temperature-sensitive (ts) growth in salt-free L broth. Temperature-resistant transductants of JE7304 obtained through P1-transduction from a wild-type strain revealed the normal processing of PBP 3 in all of the 12 clones tested, suggesting that the mutation causing the ts growth was identical or closely

* Laboratory of Genetics, Department of Biology, Faculty of Science, University of Tokyo.

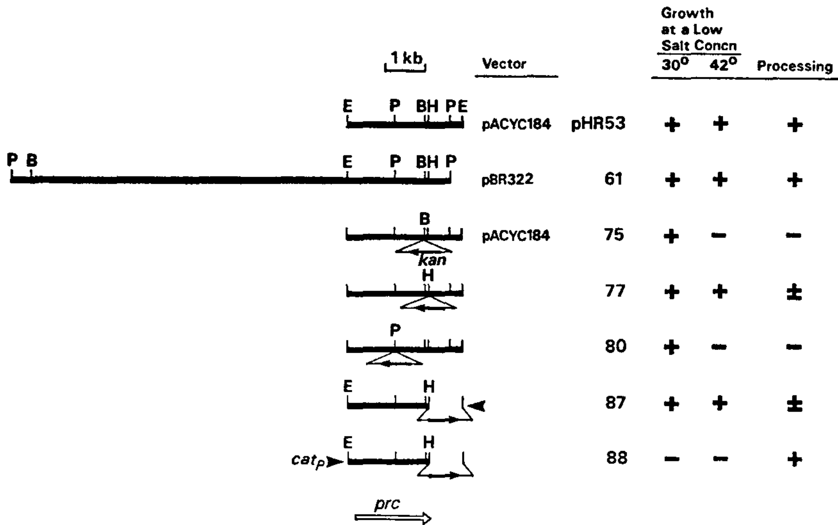


Fig. 1. Analysis of the DNA region containing the *prc* gene.
Only the cloned chromosomal regions are shown. Wedges denote the direction of the promoter of a *cat* gene, and an arrow denotes the inferred location and orientation of the *prc* gene. Growth of JE7304 transformed with each of the plasmids in salt-free L broth at 30°C and 42°C and the processing of PBP 3 in the transformants are shown by + or −. ± indicates partial correction of the processing defect. B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; and P, *Pst*I.

linked to *prc*. Therefore we tried cloning of the *prc* gene by selecting recombinant plasmids that corrected the ts growth of JE7304.

Chromosomal DNA of a temperature-resistant transductant was digested with *Eco*RI or partially with *Pst*I and the fragments were inserted into the corresponding sites of pBR322 or pACYC184. The recombinant plasmids were introduced into JE7304 by transformation and tetracycline-resistant transformants were selected at 42°C on salt-free L agar plates. All the transformants tested were found to be normal in the processing of PBP 3. Two recombinant plasmids were reserved: pHR53 containing a 2.9 kb *Eco*RI fragment and pHR61 containing an 11.1 kb *Pst*I fragment. Restriction maps of these plasmids (Fig. 1) suggested that the cloned fragments were derived from the same chromosomal region and that *prc* was contained in the 2.6 kb *Eco*RI-*Pst*I fragment. Insertion of a kanamycin resistance gene

(*kan*) into either the *Pst*I or the *Bam*HI site in this 2.6 kb fragment (pHR80 and pHR75) abolished the ability to complement the ts growth and to correct the processing defect of JE7304, indicating that *prc* covers the *Pst*I and *Bam*HI sites and is responsible for the ts growth. When *kan* was inserted into the *Hin*dIII site (pHR77) or the 0.9 kb *Hin*dIII-*Eco*RI fragment was deleted (pHR87), the ts growth was complemented but the processing defect was only partially corrected. Recloning of the 2.0 kb *Eco*RI-*Hin*dIII fragment into the same vector but in the opposite orientation (pHR88) restored the ability to completely correct the processing defect. However, this plasmid could not complement the ts growth and appeared rather inhibitory to growth: JE7304 harboring pHR88 did not grow in salt-free L broth even at 30°C. The essential region for complementing the ts growth and the processing defect is contained in the *Eco*RI-*Hin*dIII fragment. In pHR88, the *Eco*RI site of this fragment was placed to the proximal side within the *cat* gene of pACYC184. Transcription from the *cat* promoter may have affected the expression of the *prc* gene, and we infer that the *prc* gene is probably transcribed in the direction from left to right in Fig. 1 and a little beyond the *Hin*dIII site. The disagreement between the complementing abilities for the ts growth and the processing defect in pHR88 suggests that overexpression of the *prc* gene may be deleterious to cell growth, or alternatively that the two complementation phenomena may result from different genetic factors.

The ts growth of the *prc* mutant and the inhibitory effect of the promoted *prc* expression suggest that the *prc* gene is involved in an essential cellular process. It is not certain at present whether such a process is directly related or not to the vital function of PBP 3. The failure in PBP 3 processing did not always correspond with the growth defect. The *prc* mutant failed to divide normally and became elongated at 42°C in salt-free L broth, but it grew normally even without detectable processing of PBP 3 under other conditions permissive to growth.

Mapping of *prc* That Causes a Defect in the C-Terminal Processing in *Escherichia coli*

Hiroshi HARA, Atsushi HIGASHITANI*, Yukinobu NISHIMURA and Hideho SUZUKI**

The *prc* mutation of *Escherichia coli* causes a defect in the unique processing reaction which cleaves the C-terminal part of the penicillin-binding protein 3 precursor. The mutation has been found in JE7304 and appeared to be linked with the temperature-sensitive (ts) growth of this strain (Hara *et al.*, 1988, *Natl. Inst. Genet. Ann. Rep.* **38**: 48–51). Although mapping of the ts mutation had been unsuccessful possibly because of the frequent occurrence of a suppressor mutation for the ts growth but not for the processing defect of the *prc* mutant, the success in cloning the *prc* gene (Hara and Suzuki, in this *Ann. Rep.*, p. 52–54) facilitated the mapping of this gene.

The prc^+-carrying pACYC184 derivative, pHR53 (tetracycline(Tc)-resistant) was introduced into an Hfr $polA^{ts}$ strain which could not support the replication of this plasmid at the restrictive temperature. The transformants were grown at 42°C in the presence of Tc and stable Tc-resistant clones were obtained in which the plasmid was integrated into the chromosome by homologous recombination around *prc*. An Hfr cross with a multiple auxotrophic, $polA^{ts}$ mutant at 42°C suggested that the Tc-resistance (and thus the integrated prc^+ plasmid) was located around *his* at min 44 on the chromosome map. A Southern hybridization experiment with *Not*I fragments of the whole chromosome, separated by pulse-field gel electrophoresis, showed that the cloned DNA fragment in pHR53 was hybridized with a 106 kb fragment derived from a region around min 40 (Tabata, S. *et al.*, 1989, *J. Bacteriol.* **171**: 1214–1218). The restriction patterns of the cloned fragments of pHR53 and pHR61, the prc^+-carrying pBR322 derivative that contained a larger chromosomal fragment, were found to fit the pattern around map coordinate 1933 (kb) or min 40.3 on the physical map of the whole *E. coli* chromosome by Kohara, Y. *et al.* (1987, Cell **50**: 495–508). This map position was confirmed by P1-transduction experiments that located the ts mutation in JE7304 between *fadD* (min 39.9) and *eda* (min 40.7).

* Department of Biology, Faculty of Science, Nagoya University.
** Laboratory of Genetics, Department of Biology, Faculty of Science, University of Tokyo.

Accumulation of the Precursor Form of Penicillin-Binding Protein 3 in *Escherichia coli* Mutants Defective in General Protein Export

Hiroshi HARA and Hideho SUZUKI*

The extracytoplasmic proteins in *Escherichia coli* are translocated across the cytoplasmic membrane by a process that involves the *secY* and *secA* gene function and the cleavage of an N-terminal signal sequence. Penicillin-binding protein 3 (PBP 3) participates in septal murein synthesis in *E. coli* and appears to extend the bulk of its molecule into the periplasm where the murein lies. We suspected that this protein, although not an excreted protein, may use the translocation and processing mechanism in common with other extracytoplasmic proteins. However, the processing of PBP 3 was found to involve a unique reaction in which the C-terminal part, rather than the N-terminal part, is cleaved (Hara *et al.*, 1988, *Natl. Inst. Genet. Ann. Rep.* **38**: 49–51). Excretion of some extracellular proteins depends on the interaction of the C-terminus with specialized machinery (*e.g.* Gray, L. *et al.*, 1986, *Molec. Gen. Genet.* **205**: 127–133). Thus we examined whether the maturation of PBP 3 is dependent on the translocation process involving SecY and SecA proteins.

It has been shown that high-level synthesis of a MalE-LacZ hybrid protein inhibits the export of extracytoplasmic proteins, presumably by jamming the translocation machinery. A strain which carries *malE-lacZ* gene fusion and overproduces PBP 3 by plasmid pMS316 containing the PBP 3 gene was subjected to a pulse-chase experiment in the presence or absence of maltose, an inducer for *malE-lacZ*, and analyzed for the processing kinetics of PBP 3 and an outer membrane protein OmpA. When the hybrid protein was induced by maltose, the processing rate of PBP 3 as well as of OmpA was greatly reduced. Similar retardation in the processing of PBP 3 and OmpA was observed when temperature-sensitive secretion mutants ($secY^{ts}$, $secA^{ts}$ and $secA^{am}$ $supF^{ts}$) harboring pMS316 were exposed to the restrictive temperature. These results indicate that PBP 3 or the enzyme for C-terminal processing or both share the translocation machinery with other extracytoplasmic proteins. The N-terminus of PBP 3 begins with a basic and hydrophilic segment followed by a distinctly hydrophobic

* Laboratory of Genetics, Department of Biology, Faculty of Science, University of Tokyo.

segment, which is characteristic of signal sequences. This N-terminal region possibly functions as a signal for interaction with the translocation machinery although not cleaved in the following processing step. PBP 3 molecules whose N-terminal 40 residues were artificially removed failed in the translocation and accumulated in the cytoplasm (Bartholomé-De Belder, J. *et al.*, 1988, *Molec. Microbiol.* **2**: 519–525). The C-terminal processing occurs rather slowly compared with the N-terminal processing for general exported proteins, possibly after a substantial part of the molecule has been translocated through the membrane. The activity for the C-terminal processing was recovered in a membrane fraction (Hara *et al.*, 1988, *Natl. Inst. Genet. Ann. Rep.* **38**: 52–53). The processing enzyme might reside on the outer surface of the cytoplasmic membrane.

When the translocation was blocked in the secretion mutants under restrictive conditions, the cells became abnormally elongated, probably because the function of PBP 3 for cell division was impaired. If the translocation of PBP 3 is independent of SecY and SecA, the defect of the secretion mutants in PBP 3 processing should be solely due to a failure in translocation of the processing enzyme for PBP 3. In the *prc* mutant, however, PBP 3 appeared to remain unprocessed under conditions that allowed normal growth, suggesting that the processing defect did not necessarily lead to the loss of PBP 3 function. It is rather likely that PBP 3 uses the Sec translocation machinery for its proper localization in the cellular surface structure and the translocation of PBP 3 in this sense may be of primary importance to the function of septum formation.

A New Gene Controlling the Frequency of Cell Division per Round of DNA Replication in *Escherichia coli*

Akiko NISHIMURA

A novel mutant of *Escherichia coli*, named *cfcA1*, was isolated from a temperature-sensitive *dnaB42* strain, and found to have the following characteristics. Division arrest and lethality, induced by the inhibition of DNA replication, was reduced and delayed in the *cfcA1 dnaB42* strain, as compared with the parental *dnaB42* strain. Two types of inhibition of division, induced by the addition of nalidixic acid or hydroxyurea, were suppressed by the *cfcA1* mutation. Under permissive conditions for DNA replication, the colony forming ability of *cfcA1* cells was significantly re-

duced as compared with that of *cfc*$^+$ cells; conversely the division rate of *cfcA1* cells was higher than that of *cfc*$^+$ cells. The *cfcA1* mutation partially restored division arrest induced in the thermosensitive *ftsZ84* mutant at restrictive temperatures and suppressed the UV sensitivity of the *lon* mutation. The mutation was mapped at 79.2 min on the *E. coli* chromosome. Taking these properties into account, it was hypothesized that the *cfcA* gene is involved in determining the frequency of cell division per round of DNA replication, by interacting with the FtsZ protein, which is essential for cell division. For details, see *Mol. Gen. Genet.* **215**: 286–293, 1989.

A Cell Division Regulatory Mechanism Controls the Flagellar Regulon in *Escherichia coli*

Akiko NISHIMURA and Yukinori HIROTA

The formation of flagella in various thermo-sensitive (Ts) cell division mutants of *Escherichia coli* was examined at nonpermissive temperatures. The number of flagella per unit cell length, decreased sharply after shifting the culture temperature from 30° to 40°C in the following Ts mutants: *ftsC108*, *ftsD1033*, *ftsE1181*, *ftsF1141*, *ftsG29*, *ftsZ84*, *parA110*, *dnaB42*, *nrdB*, and *dnaG*. It was found that transcription of genes responsible for the formation and/or function of flagella (*hag*, *fla*, *mot*, *che*), decreased significantly at 40°C. However, in the *ftsI730* mutant at a nonpermissive temperature, or in penicillin G treated wild-type cells, cell division was blocked while formation of flagella continued. Moreover, when the *cfcA1* mutation of a gene involved in coordinating DNA replication and cell division, was introduced into the *danB42* mutant strain, inhibition of cell division and formation of flagella at 40°C was relaxed. These results indicate that the flagellar regulon is under the control of a cell division regulatory mechanism. If this kind of mechanism controls the biosynthesis of cellular constituents, it would be a fundamental reaction in the biological control mechanism that coordinates cell growth with cell division. For details, see *Mol. Gen. Genet.* **216**: 340–346, 1989.

Restriction Map of a *Bacillus subtilis* Gene Involved in Cell Division, Sporulation and Exoenzyme Secretion

Yoshito SADAIE

We established a partial restriction map of the wild type *div-341*$^+$ gene of *Bacillus subtilis* which was cloned in a temperate phage, ϕ105. The order of restriction sites was *Cfr*13I, *Apa*LI, *Mlu*I, *Stu*I, *Cla*I, *Cfr*13I. The mutation *div-341*, located between *sacU* and *uvrA* loci, results in filamentous growth at a restrictive temperature (42°C), and poor sporulation of typical *spo0* type mutants at an intermediate temperature (37°C), where vegetative growth is almost intact. The *div-341* mutant showed reductions in extracellular protease and α-amylase secretion, competence development and autolysis at 37°C. It also showed a defective spore outgrowth at 37°C. Therefore the wild type *div-341*$^+$ gene seems to be involved in a common step which is required for the excretion of extracellular proteins on the cell surface, or of those which are involved in cell division, sporulation, autolysis, and competence development. (*Mol. Gen. Genet.* **190**, 176–178, *J. Bacteriol.* **163**, 648–653., *Jpn. J. Genet.* **64**, 111–119.)

III. IMMUNOGENETICS

Novel Molecular Organization of Meiotic Recombinational Hotspots in the Murine Major Histocompatibility Complex

Toshihiko SHIROISHI[1], Tomoko SAGAI[1], Naoto HANZAWA[1], Michael STEINMETZ[2] and Kazuo MORIWAKI[1]

The physical distance between two genetic loci in the eukaryotic chromosomes is not always consistent with the genetic map distance deduced from meiotic recombination frequencies. This difference suggests that meiotic recombination may not be random, but may rather occur more frequently in certain specific regions. It has been established that meiotic recombinations within the major histocompatibility complex (MHC) of the mouse occur in clusters at restricted sites, so called, recombinational hotspots. We identified a recombinational hotspot in the wm7 haplotype of the MHC, which was derived from Japanese wild mouse, *Mus musculs molossinus*. Recombinations involving the wm7 haplotype occur at a very high frequency between H-2K and Aβ genes only in female meiosis but not in male meiosis. Recombinational break points were confined to 1 kbp of DNA between the Aβ_3 and Aβ_2 genes. Its location overlaps with a sex-independent hotspot previously identified in the CAS3 haplotype originating from another Asian subspecies, *M. m. castaneus*. We cloned the DNA fragment containing this recombinational hotspot from the wm7 haplotype and the DNA fragments of corresponding regions from standard laboratory strains, B10.A and C57BL/10J, which do not have a recombinational hotspot in this region. Nucleotide sequences surrounding this region from the wm7 and from two hotspot-negative, standard laboratory strains indicated that no significant differences are present. However, a comparison of the nucleotide sequences of this Aβ_3/Aβ_2 hotspot and a previously characterized hotspot in the Eβ gene clearly revealed that they have very similar molecular organizations. As shown in Figure 1, in each hotspot, the crossover points clustered around two molecular elements: the consensus sequence

[1] National Inst. of Genetics, Mishima, Japan.
[2] Central Res. Unit, F. Hoffmann-La Roche & Co. Ltd., Basel, Switzerland.

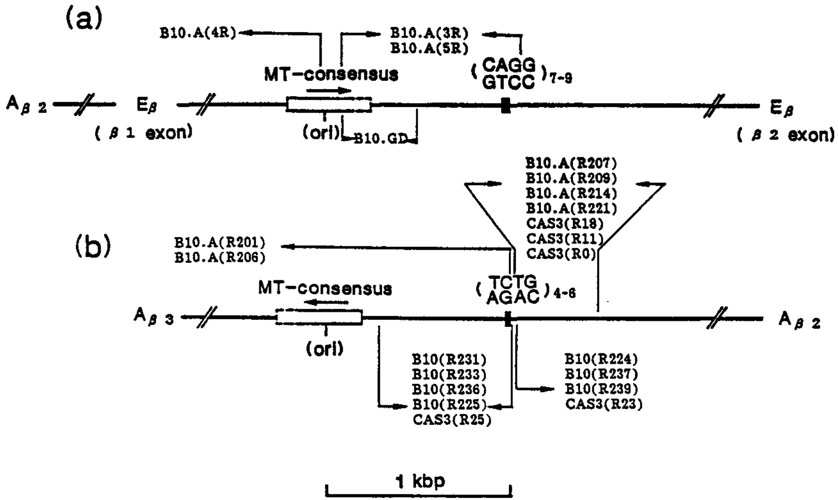


Fig. 1.

of the mouse middle repetitive MT-family, and the $(CAGA)_{4-6}$ or $(CAGG)_{7-9}$ repeat sequence. These two elements, separated from each other by 1 kbp of DNA, probably serve as the molecular basis for the site-specificity of recombination at the hotspots in the MHC.

New Evidence for Trans-Species Evolution of the *H-2* Class I Polymorphism

Tomoko SAGAI, Toshihiko SHIROISHI and Kazuo MORIWAKI

A serological survey using alloantisera specific for the H-2 class I antigens in Japanese wild mice, *Mus musculus molossinus*, revealed a high frequency of the H-2K^f antigen. This antigen has also been found in European wild mice, *M. m. domesticus* and *M. m. musculus*. In this survey, the H-2K^f antigen was characterized through the use of ten newly isolated monoclonal antibodies raised against cells of a Japanese wild mouse, and by Southern blot analysis using an H-2K locus-sepcific probe which hybridizes with the 3′ end of the gene. The serologically identified H-2K^f antigens revealed several minor variations in reactivities to the monoclonal antibodies. However, all the antigens examined could be clearly separated into two types with respect to the RFLP pattern. The first type

found together with a single, characteristic RFLP pattern, was always associated with the presence of reactivity to one particular monoclonal antibody, MS54. The second type, found representing different RFLP patterns, is associated with the absence of reactivity to MS54. This concordance between the presence of an antigenic determinant and a particular RFLP was observed not only within *M.m.* subspecies but also in a different species: *M. spretus*, carrying the same antigenic determinant, which presented an identical RFLP to that of the other MS54-positive *M.m.* subspecies. The results suggest that the antigenic determinant specific for MS54 is an ancient polymorphic structure which has survived the long period of diversification of the *Mus* species (approximately 2–3 million years) without alteration, and is associated with a stable DNA structure at the 3′ end of the *H-2K* gene.

Steroid 21-Hydroxylase Deficiency in Mice

Hideo Gotoh, Tomoko Sagai, Jun-Ichi Hata,
Toshihiko Shiroishi and Kazuo Moriwaki

The enzyme steriod 21-hydroxylase (21-OHase) plays a key role in adrenal steroidogenesis. Defects in this enzyme are responsible for one of the most common inborn defects in metabolism in humans. Duplicated genes for the enzyme are located in the class III region of the major histocompatibility complex (MHC), *HLA*. In the mouse, the genes encoding 21-OHase have been mapped to the homologous region of the *H-2* complex. We previously described an *H-2* recombinant haplotype *aw18*, in which the gene for the complement component C4 and one of the two genes for 31-OHase in the *H-2* Class III region have been deleted. We now report that newborn *aw18* homozygous mice are deficient in 21-OHase activity, and that homozygosity for the *aw18* haplotype directly cause lethality at the early postnatal stage. Morphological changes in the adrenal glands of the newborn *aw18* homozygotes are also observed. The *aw18* recombinant haplotype is expected to serve as a useful and, thus far, unique experimental system to study adrenal steroidogenesis *in vivo*, and as an animal model for the inherited human disease, congenital adrenal hyperplasia. (For details see *Endocrinology* **123**: 1923–1927, 1988).

IV. DEVELOPMENTAL AND SOMATIC CELL GENETICS

Artificial Expression of Tissue-Specific Characteristics in Embryonic Lethal Cells of *Drosophila melanogaster*

Yukiaki KURODA

In some sex-linked recessive lethal mutants of *Drosophila melanogaster*, embryos homozygous for the lethal genes die at specfiic stages in embryogenesis, due to defects in morphogenesis and in functions of specific tissues or organs. One such mutant, the *dor* mutant showed specific defects in syncytium formation of muscle cells, formation of cellular spheres and droplet formation on nerve fibers, when cells obtained from embryos at an early stage in embryogenesis are cultured *in vitro*. The *dor* gene is located in a region including six bands from 1F1 through 2A2 on the X chromosome in salivary gland cells. In the present experiment, an attempt was made to repair these defects in *dor* embryonic cells by introducing wild-type genes or their products which are responsible for these defects.

When *dor* embryonic cells were cultured in medium containing wild-type egg extract, defects in muscle cells, cellular spheres and nerve cells were found to be almost completely repaired. Embryos obtained from the mating of *dor*/*dor* females and +/Y males were allowed to develop to various stages such as blastoderm formation (3 hours after fertilization), gastrulation (5 hours after fertilization), head and trunk segmentation (9 hours after fertilization), and muscular movement (14 hours after fertilization). The extracts obtained from these embryos were tested for their ability to repair the defects of *dor* embyronic cells. An extract from embryos at the stage of blastoderm formation showed no repair activity for the defects of *dor* cells. Extracts from embryos at stages later than gastrulation were found to be effective in repairing the defects of *dor* cells.

This suggests that effective substances may be produced by the paternal wild-type gene between 3 and 5 hours after fertilization. It seems likely that the translation of the effective substances through mRNA transcribed by the paternal wild-type gene (DNA) takes place after blastoderm formation. The effective substances lost their activity after heat treatment as 80°C for 10 min, suggesting that they are enzymes or heat-labile proteins.

Further studies are under way to test the ability of DNA and mRNA, isolated from embryos at various stages, to repair the defects of *dor* cells.

Selective Infection of SR Spiroplasms in Cultured Embryonic Cells of *Drosophila melanogaster*

Yukiaki KURODA, Bungo SAKAGUCHI*, Kugao OISHI**
and Yutaka SHIMADA

Sex-ratio (SR) spiroplasms are known to selectively kill only male embryos of *Drosophila*, but do not affect female embryos. In the present experiment, how the SR spiroplasms recognize cells of male and female embryos and what types of cells or tissues of male embryos are infected by the SR spiroplasms, were examined.

SR-containing body fluid was collected from 300–600 SR-infected adult females 7–10 days after eclosion by a capillary tube, diluted with the same volume of physiological salt solution, sterilized through a Millipore filter and added to cultured embryonic cells of *Drosophila melanogaster*. After cultivation for one or two days, changes occurring in cultured cells were examined under an inverted phase-microscope and an electron microscope.

Various types of cells differentiated from undifferentiated embryonic cells in culture. In muscle cells, nerve cells and cellular spheres, no detectable changes produced by SR spiroplasms were observed under a phase-microscope. In epithelial cells, some brown necrotic changes were observed, which might have been produced through infection with SR spiroplasms.

In a control experiment, body fluid obtained from adult flies, which were not infected with SR spiroplasms, or were infected with nonkiller mutant SR spriroplasms, were not effective in producing necrotic changes in epithelial cells.

Under an electron microscope, it was observed that embryonic cells infected with SR spiroplasms contained many spiroplasm-like structures in intercellular spaces and the cytoplasm of necrotic cells. Whether cells with SR spiroplasms might be a specific type of cells, and whether SR spiroplasms might proliferate under culture conditions, are under investigation.

* Department of Agriculture, Kyushu University.
** Department of Science, Kobe University.

Antimutagenic Effects of Safrole on Chemically Induced Mutations in Cultured Chinese Hamster Cells

Yukiaki KURODA, Ajay K. JAIN* and Hideo TEZUKA

In our environment, more than two mutagens present together may act to induce mutations, at a different frequency from those estimated theoretically from the effects of the single mutagens. Also, differential exposure times of these mutagens may produce some different effects on the induced mutation frequency. In our continuing experimental series, in which the combined effects of two chemicals are being studied, the effects of safrole and cobalt chloride on the cytotoxic effects and mutations induced by ICR170 were examined in the present experiment.

ICR170 alone had a dose-dependent cytotoxic effect on survival of Chinese hamster V79 cells. The colony-forming activity of cells decreased to 50% of non-treated control cells at a concentration of 2.0 μg/ml of ICR170. Safrole alone had a weak cytotoxic effect on cell survival, producing 50% survival at a concentration of 50 μg/ml. In the presence of 50 μg/ml safrole, the survival of cells treated with 2 μg/ml ICR170 was 78%, indicating that safrole reduced the cytotoxicity of ICR170.

ICR170 at a concentration of 1 μg/ml induced 6-thioguanine (6TG)-resistant mutations at a frequency of 46.8×10^{-5}. On the other hand, safrole induced no detectable mutations at a concentration of 50 μg/ml. When cells were treated with safrole and ICR170 together, the mutation frequency of cells was similar to that induced by ICR170 alone. However, when cells were treated with ICR170, incubated for 3 hours, and then treated with safrole, the induced mutation frequency was reduced to 30.0×10^{-5}.

Cobalt chloride enhanced the cytotoxicity of ICR170 in Chinese hamster cells. On the other hand, cobalt chloride reduced the frequency of mutations induced by ICR170 to 50%, when cells were treated with these two chemicals together. The reduction of the ICR170-induced mutation frequency by cobalt chloride was also found when cells were treated with cobalt chloride 6 hours before ICR170 treatment.

These results indicate that safrole and cobalt chloride had no detectable activity in themselves for inducing mutations, but both were effective in reducing the frequency of mutations induced by ICR170.

* Central Drag Research Institute, Luchnow, India.

Injury Effect on Hydra Head Regeneration

H. SHIMIZU and T. SUGIYAMA

Three different procedures were used to remove the original head from hydra, and the regeneration processes of decapitated polyps were examined and compared. Relatively young polyps bearing the first bud protrusion of a wild-type strain of *Hydra magnipapillata* (105) were used. The head was removed by a cut made at the mid-point between the hypostome and bud protrusion.

In *standard amputation*, the head was cut off instantaneously using a sharp surgical knife, whereas in *ligation*, the head was gradually removed by ligation using a monofilament nylon fishing line. This procedure produced a much smaller wound opening in the decapitated polyp than standard amputation did. In *mirror-image-grafting*, the head was cut off as in standard amputation, followed by the grafting of two decapitated polyps in such a manner as to make the wound opening of the two polyps face and cover each other.

Polyps subjected to standard amputation restored the original number of tentacles in 2–3 days and 60–80% more in 7 days. Polyps subjected to ligation also restored the original number in 2–3 days, but only 30–40% more in 7 days (cf. Newman, 1974). In contrast, about 40% of the polyps subjected to mirror-image-grafting failed to regenerate any tentacles in 7 days.

Lateral tissue grafting was employed to examine the changes in head activation and inhibition potential in polyps subjected to standard amputationor mirror-image-grafting. No significant differences were found in the decrease in inhibition levels in polyps subjected to the two procedures. In contrast, increases in head activation levels were much smaller in polyps subjected to mirror-image-grafting than in normally amputated polyps.

These observations show that, in addition to a decrease in inhibition levels, the state of the injured tissue produced by decapitation strongly affects the increase in activation and subsequent head regeneration. This suggests that "injury effect" originally proposed by MacWilliams (1983) to explain transplantation phenomena also plays an important role in head regeneration.

Random Displacement of I-Cells into Buds and Maintenance of the I-Cell Population in *Hydra*

Toshitaka FUJISAWA

It has been believed that the interstitial cell (I-cell) population in *hydra* is maintained by regulating its self-renewal probability (Bode and David, 1978). Recently, however, Heimfeld (1986) reported that the rate of I-cell growth was relatively unchanged even when the I-cell density was lowered by hydroxyurea treatment and that the recovery of I-cells in the adult tissue was attained by increased retention at the expense of buds.

In the present study, the growth rate of I-cells and patterns of I-cell displacement into buds were examined in polyps of low I-cell densities. Such polyps were produced by introducing a small piece of tissue from a normal polyp into an epithelial polyp which lacks an I-cell lineage. The growth rate of I-cells was monitored by scoring the number of I-cells in macerated preparations. Patterns of I-cell displacement were analyzed by scoring cell numbers in adult and bud tissues and also by staining I-cells in whole mounts with Toluidine blue. The results showed that the growth rate of I-cells was 2-fold higher at low densities than at normal ones. Furthermore, it was suggested that the displacement of I-cells from an adult into buds was a random process. These results do not agree with those of Heimfeld's but rather with the prevalent concept of self-renewal regulation of I-cells.

Although there were differences in experimental procedures, the exact reason for differences in results between the present study and the Heimfeld's is not known at present.

Genetic Studies on the Silkworm Adult Lifespan
(a) Heredity of the Short Adult lifespan (*sdi*)

Akio MURAKAMI

Because the duration of adult lifespan significantly differs among various silkworm strains, it has been suggested that the adult lifetime may be genetically controlled (Murakami *et al.*, 1986). The mean adult lifespan of the insect is around 7–10 for females and 4–5 days for males. However, the adult lifespan of the Daizo strain is remarkably short (*ca.* 2 days) in both

sexes. In this experiment, using the Daizo (D) strain and the Japanese 106 (J) strain which has an average lifespan various crosses (F_1, BF_1 and F_2 of both strains) were perfomed to clarify the genetic mechanism of the adult lifespan in the Daizo strain.

Experimental conditions throughout this series were controlled at a temperature of 25°C and at various relative humidities ranging from 50 to 80% depending on the growth stage of the insect. The mean adult lifespans for each group and sex in every strain were examined. At least 100 newly emerged adult moths for each sex in every experimental group were used for observations. The death of adult moths was judged when they did not biologically respond to being poked softly by the point of a pencil.

The mean adult lifespan of F_1 hybrids between the Daizo and the J106 strains was longer than for J106 adults indicating that the adult lifespan increases through hybridization. On the basis of these results, it was inferred that the short adult lifespan in the Daizo strain may be controlled by a single recessive factor named *sdi* (short duration of the imaginal lifetime) for the convenience of the following experiments.

The lifespan of female adults of BF_1 or $(D \times J) \times D$ showed the first peak (48.4%) between the 1st and 7th days with a maximum on the 4th day and the second peak (51.6%) between the 8th and the 20th days with a maximum on the 13th day. Although this distribution of the lifespan was slightly prolonged, compared to those of the Daizo and the J106 strains (it is necessary to take into account the difference in genetic background from the pure strains), the first and second peaks of the lifespan may have originated from *sdi/sdi* and *sdi/+* individuals, respectively. A similar explanation could also be applied to the lifespan of $(J \times D) \times D$. For male moths, the distribution pattern of their lifespan corresponded to the theoretical value, although it was difficult to distinguish the distribution peaks from each other because of the possible existence of heterosis and recombinations, in addition to their generally short lifespans.

F_2 also seems to divide into *sdi/sdi*, *sdi/+* and +/+ at the ratio of 1: 2: 1. In the experiment with $(D \times J)F_2$, it was observed that the first peak (26.4%) and the second and third peaks (73.6%) were distributed between the 3rd and 4th days after emergence and between the 5th and 18th days, respectively. Since the second peak (*ca.* 25%) and the third peak (*ca.* 50%) may be produced by +/+ and *sdi/+* moths, respectively, the distribution pattern in the lifespan corresponded almost to the theoretical value.

On the basis of these experimental results, it was clear that the short adult lifespan of the Daizo strain was controlled by the single recessive *sdi* gene. In addition, it was also found the adult lifespan has a Mendelian monogenic hereditary mode and the responsible gene is located on a specific certain autosome.

Genetic Studies on the Silkworm Adult Lifespan (b) A Biological Characteristic of the Daizo (*sdi*) Strain

Akio Murakami

Although the adult lifespan of the Daizo (*sdi*) strain is quite short (*ca.* a days) regardless of sex, reproductive behaviour, and the life history throughout fertilization and embryonic, larval and pupal stages proceeds smoothly. Therefore, the *sdi* gene factor may control the short life (microbiotic imago), but not adult lethality. However, this is not certain without further investigation.

The Daizo strain moths showed no prolongation effect on lifespan with low temperature storage, which is usually found in silkworm moths of other strains or lines. However, it is of interest to note that the mutant moths showed a prolongation effect under high humidity conditions (90% or more of relative humidity), which is also a generally common feature in *Bombyx* adults. Unlike moths of ther strains or hybrids lines, death of Daizo (*sdi*) strain moths occurs immediately after oviposition, thus hardly causing a decrease in the adult fresh weight. On the other hand, a possibility that the nervous system may be concerned in the duration of adult lifespan has been indicated by the fact that the adult lifespan was shortened to about 2 days when the brain was removed at the early pupal stage (Murakami and Shimada, 1988). Taking the non-feeding habit of silkworm adults into consideration, the short adult life charactestic in the Daizo (*sdi*) strain seems to be due to a certain disorder of the nervous system caused by an abnormal nitrogen metabolism (or excretion) rather than an abnormal energy-intake.

Genetic Studies on the Silkworm Adult Lifespan
(c) Sex Differences and their Biological Significance

Akio MURAKAMI

It was reported that female adults in the silkworm *Bombyx mori* have a long lifespan compared with males in observations on sexual differences in a dozen pure lines designed to clarify the genetic mechanism of adult lifespans (or the length of moth stages) (Murakami, 1986). The present experiment was carried out to confirm the finding by using single and triple hybrids. Hybrids obtained from either dual or ternary crossbreeding with 22 combinations of the Cambodge, the Daizo, the J106 and the *od* strains were used. Rearing conditions and methods for the observation of survival days were set up in accordance with the previous communication described above. For the measurement of lifespans, at least 100 nonmated moths were used for female or male groups in each hybrid group. The number of moths that naturally ceased their biological activity was counted two times a day (10 a.m. and 3 p.m.) and the mean lifespan was calculated in each hybrid group.

The mean lifespans of male and female moths in the experiments varied from 2.7 to 6.7 days and from 4.1 to 11.6 days, respectively. When the mean lifespans of female and male moths in each experimental group were compared, it was found that the average length of the adult stage was 1.7 times longer in females than in males, although the ratio varied from 1.1 to 3.1 depending on each hybrid group. These observations corresponded well with results obtained using pure lines (Murakami, 1986). These results confirm that in the *Bombyx* silkworm, the lifespan of female moths is significantly longer than that of males.

In the silkworm, males have a higher a oxygen consumption per body-weight basis, index of metabolic activity, than females throughout the whole life cycle (Itaya, 1944). The wing-beat frequency of male moths is higher than that of female moths for the first several days after eclosion until the last day of reproduction time. After the completion of voiposition in females, their wing-beat activity is markedly reduced. In the silkworm, the nominal body-weight of females is about 1.3 to 1.5 times heavier than that of males. However, if the contribution of the total weight of eggs or oocytes in the body cavity is subtracted from the body-weight, the net body-weight of female moths may not be much heavier than that of males

or may be almost the same as males. From this it can be suggested that the metabolic rate or energy consumption may be one of the principal mechanisms for sex differences in adult lifespan rather than other possibilities.

In *Bombyx* silkworms, a member of the lepidopteran order, the male is homogametic ZZ (XX) and the female is heterogametic ZW (XY), which different from other insect orders including diptera with the XY type. In several lepidopteran species (*e.g.*, Woodruffe, 1951) other than *Bombyx* and some saturniid species (McArther and Baille, 1932), female adults also have longer lifespans than males. Rockstein and Miquel (1972) reported an adult insect lifespan list including five orders or 47 species. They indicated that females in most species surveyed live much longer than males. Thus, it becomes clear that the sexual difference in the insect adult lifespan may be not dependent on the sex-chromosome constitution of either ZW or XY types, but may be a secondary sexual characteristic. This deny the participation of sex-chromosomes, either Z(X) or W(Y), in sex differences in the adult lifespan. In addition, the possibility of involvement of certain autosomal factor(s) in the length of insect adult stages can not be excluded. In a ZW-type bird, the lifespan of both sexes is similar, or that of females is longer than that of males, but the reverse case also occurs, (Landaur and Landaur, 1931). Therefore, further studies are required to draw a final conclusion about whether ZW females have longer lifespans than ZZ males.

The Genetic Nature of Known Mutants in the Domesticated Mulberry Silkworm, *Bombyx Mori*

Akio MURAKAMI

A cursory glance over the mutant list or the linkage map of the *Bombyx* silkworm indicates that this insect abounds with what are called dominant mutations symbolized by a capital letter compared to other species, for example, *D. melanogaster* where recessive mutants are frequent. In *B. mori* approximately 85% or more of artificially-induced mutations and about 45% of spontaneous mutations are indicated as dominant characters. In addition to these features, more than a half of the induced mutations and about one-quarter of the spontaneous mutations are lethals when homozygous, suggesting that they are caused by chromosomal aberrations (either deficiency or deletion). The genetic nature of some artificially-induced

mutants symbolized by capital letters in the original paper is clearly specified as a deficiency of chromosomes. For example, a radiation-induced mutation, *Rg*, is found to be a large deficiency in chromosome 3. In the silkworm, artificially-induced recessive mutants other than lethal mutations are few, but some of them are also accompanied with lethality. In the linkage map, we have rarely witnessed translocations which are distinctly classified as chromosomal aberrations. It has been well documented that many spontaneous as well as some artificially-induced translucent (oily) skin mutations are located over a dozen linkage groups in the silkworm. Most of these manifest a weak lethality and/or sometimes inviability in the homozygous state, indicating that the genetic nature of those mutations is analogous to Minute mutations which have small deletions in the ribosomal gene loci of *D. melanogaster*. It can be said that the large part of the induced mutations is not genine gene mutations, but chromosomal aberrations cover a wide range of deficiencies.

While, for the spontaneous mutations, there are problems in an inadequate manner of the gene nomenclature for several main characters because of the unsettled wild-type in this insect and for isozyme loci which are currently marked by a capital letter regardless of the characters either dominant or recessive. The standard phenotype has been selected from a wide range of popular phenotypes available among commercial strains. It should be recalled, however, that *B. mori mandarina* Moore, a non-domesticated silkworm, is widely accepted as the possible ancestor of *B. mori* L. If we accept that *B. mori mandarina* is a wild-type of *B. mori*, then many current phenotypes for several genetic characters symbolized by the capital letter like cocoon colour, cocoon shape, larval marking pattern, moultinism and voltinism would have to be rewritten with a small letter as recessive characters. All isozyme loci documented as spontaneous mutations are currently symbolized by capital letters regardless of what are called recessive and dominant characters. This nomenclature, however, is inconventional. To avoid confusion between the conventional nomenclature system and the isozyme one, it is logical to adopt a uniform nomenclature. In conformity with the conventional nomenclature the isozyme should be redesignated as the case of a locus controlling alkaline phosphatase. For example, Aph^{n} (or Aph^{-} and Aph^{0}: a null type) should be rewritten as *aph* and the other co-dominant alleles, Aph^{S} (a slow type) and Aph^{F} (a fast type) as $+^{Aph\text{-}S}$ and $+^{Aph\text{-}F}$, respectively.

It thus appears that the great part of the mutations symbolized by a capital letter is not a gene mutation and some of them are not dominant types. In this connection, it is desirable to be separated then the chromosomal mutations from what we call the gene mutations in the mutant list of the *Bombyx* silkworm.

Decrease in Hatchability of Eggs Laid by Flies Reared on Excess Yeast in *Drosophila melanogaster*

Kiyoshi MINATO

In the previous study (1979), it was found that female flies laid three to four times more eggs (30–40 eggs/day/female), when reared on agar medium (agar, cornmeal, dry dead-yeast, sugar, and propionic acid) with daily changes, and with the surface partly coated with paste-like live yeast, than flies reared on agar medium without any coating with yeast. However, the hatchability of eggs decreased gradually as time went on (90–95% at a days and 50–60% at 10 days after eclosion), whereas eggs laid by flies reared on agar medium without any coating with yeast showed little decrease in hatchability. Further analyses of this phenomenon showed that when heat-treated (90°C, 10 min.) yeast was used in place of live yeast, flies laid nearly as many eggs as flies reared on agar medium coated with live yeast. The hatchability of eggs, however, decreased little during the culture of flies unlike the intense decrease in the hatchability of eggs in flies fed on live yeast.

This shows that the decreased hatchability of eggs laid by flies fed on excessive live yeast was not due to the increased rate of egg laying which may give rise to unfertilization and, hence, unhatched eggs owing to the failure of sperm to enter eggs and/or the shortage of sperm supply. On the other hand, in flies showing decreased hatchability, an insufficient supply of sperm from male flies is, also, unlikely to occur, because neither increase in the male to female ratio in population, nor exchange of aged males by young ones improved the hatchability of eggs. Since the above unhatched eggs did not show any internal morphological changes under a light microscope, they appeared to be either unfertilized due to reasons other than insufficient sperm-supply, or to be fertilized but undeveloped.

A similar phenomenon of an increase in oviposition and a decrease in hatchability of eggs was sometimes seen in stock cultures too, when the agar

medium was supplemented excessively with either paste-like or dry live yeast. The use of antibiotics in agar medium, however, improved hatchability in these cultures, suggesting that bacteria might have proliferated inside the thick sheet of paste-like live yeast and this was confirmed by examination after Gram staining. Live yeast remained non-growing and the flies fed on such agar medium might produce eggs with low hatchability. This posibility appears to be confirmed by the following findings: the hatchability of eggs decreased little when yeast was used after heat-treatment or sterilization, and agar medium coated with yeast was reserved at a low temperature (4°C), or when fresh live yeast was added to the agar medium only ujst before use. Then, it is physiologically of interest why the eggs laid by flies fed on highly infected medium often don't hatch in spite of high egg production of parent flies.

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

Miki FUJIOKA, Akira KAWAHARA, Minoru AMANO,
Kazuo WATANABE and Nobuo SHIMAMOTO

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

We found that the cells secrete autocrine growth factors. One factor was purified from conditioned medium collected from growing-phase cultures by DEAE-Sepharose column chromatography and non-denaturing polyacrylamide gel electrophoresis. The specific activity was increased 1,100-fold through purification. The purified growth factor was heat-stable, and resistant to acidic or basic treatment. Removal of the glycoside chain did not alter activity but reduction by dithiothreitol or digestion by trypsin hampered activity. These characters are similar to those of the platelet-derived growth factor (PDGF) but its molecular weight, 32 kd, is much larger than PDGF. Thus this factor might be a new family of growth

factors. The primary sequence was determined for 20 amino acid at N terminal.

The Three-Dimensional Ultrastructure of Intracellular Membranous System in Cultured Chick Skeletal Muscle Cells

Yutaka SHIMADA

The development of the transverse tubular system (T system) and the sarcoplasmic reticulum (SR) in embryonic chick skeletal muscle cells *in vitro* was studied by (1) scanning electron microscopy of freeze-polished, osmium-macerated cells and (2) transmission electron microscopy of rotary replicas of critical-point dried and physically reptured cells. Intracellular membranous organelles (T system and SR) were arranged periodically along the length of myofibers. The T system was tubular in appearance with many warts, and it followed a tortuous course. In the cortical region of the cell, a complex anastomonic network of T system tubules was observed. Occasionally, coated vesicles were associated with T system tubules. Many SR appeared to be continuous with each other and often exhibited swellings. At such dilated areas of the SR, T system tubules associated to form early triatic structures. At early stages of development, these two functionally related organelles were not arranged regularly and, thus, their distinction was sometimes difficult. Mitochondria were long rod-shaped with occasional branchings. At the cracked surface of mitochondria, mitochondrial cristae could be observed.

V. CYTOGENETICS

Molecular Analysis of Complement Factor H Genes in Wild Mice (*Mus musculus*) and Their Relatives

Yoshi-nobu HARADA, François BONHOMME*, Shunnosuke NATSUUME-SAKAI**, Kazuo MORIWAKI and Takeshi TOMITA***

Complement factor H is a plasma glycoprotein which functions as a cofactor in the conversion of C3b to iC3b by serine protease I. Three allotypes of factor H, H.1, H.2 and H.3, were reported in mice (*Mus musculus*). A serological survey of factor H allotypes in wild mice from all over the world revealed that geographical distributions of factor H allotypes were specific to the subspecies. The survey also revealed that the third allotype, H.3, was able to be found only in BFM/2Ms and BFM/1Mpl strains which were derived from *M. m. domesticus* captured in Montpellier, France. On the other hand, a serological survey of mouse relatives showed that all six strains derived from *Mus spretus* had factor H which strongly cross-reacted with H.3-specific antiserum. Consequently a molecular analysis was carried out on factor H genes from BFM/2Ms, BFM/1Mpl and *spretus* strains. RFLPs of factor H genes were detected by a Southern analysis using a 0.7 Kb cDNA probe of mouse factor H gene established by S. N.-S. A 5Kb DNA fragment of *Msp* I digestion and a 5.4 Kb fragment of *Hae* III digestion were specific to BFM/2Ms and BFM/1Mpl. All six strains derived from *M. spretus* also had these DNA fragments. No mice without the H.3 allotype had these DNA fragments. In October 1988, an additional serological survey was carried out on some wild mice captured in the Montpellier area where the original mice of the BFM/2Ms and BFM/1Mpl strains were collected. Two of the eight mice from Montbazin and all eight mice from the campus of Montpellier University had the H.3 allotype. This observation shows that the H.3 allotype of the BFM/2Ms and BFM/1Mpl strains is not a mutation which occurred in the laboratory when these strains were established.

Results of the molecular analysis and the serological survey may suggest

* Montpellier University.
** Kanazawa University.
*** Nagoya University.

that the H.3 gene in BFM/2Ms and BFM/1Mpl is derived from *M. spretus* by gene introgression after the differentiation of the genus *Mus*.

Reproductive Ability of Hybrids between *Mus musculus castaneus* and *M. m.* Subspecies in China

Michiko NIWA, Noboru WAKASUGI*, Akihiko MITA and Kazuo MORIWAKI

The reproductive performance of hybrids between *Mus musculus castaneus* and M. sub-Cht, derived from *M. m.* subspecies in Chetzu (China) which closely resembles *M. m. musculus*, was observed. The inbreeding generation of this Cht-strain was F11-F14 and the mean litter size was 4.7±0.2 (n=50, mean±sem). We made reciprocal matings between M.sub-Cht and the following 3 strains derived from *M. m. castaneus*: M.cas-Hmi, M.cas-Mal and M.cas-Bgr whose ancestors were captured in Hemei (Taiwan), Malaysia and Bogor (Indonesia), respectively. The numbers of inbreeding generations of M.cas-Hmi, M.cas-Mal and M.cas-Bgr were F2-F7, F1-F4 and F9-F13. The mean litter sizes were 5.8±0.2 (M.cas-Hmi, n=46), 6.6±0.4 (M.cas-Mal, n=33) and 5.9±0.2 (M.cas-Bgr, n=50).

Table 1. Litter size in the matings between *M. m. castaneus* and *M. m.* subspecies in China.

Matings Female × Male	No. of litters	Litter size mean±sem
Hmi × Cht	9	5.3±0.5
Cht × Hmi	4	7.5±0.9
Mal × Cht	15	5.5±0.3
Cht × Mal	4	4.5±1.2
Bgr × Cht	10	5.1±0.6
Cht × Bgr	1	5.0
(Hmi × Cht)F1 × (Hmi × Cht)F1	33	8.0±0.3
(Cht × Hmi)F1 × (Cht × Hmi)F1	38	6.2±0.3
(Mal × Cht)F1 × (Mal × Cht)F1	35	5.6±0.3
(Cht × Mal)F1 × (Cht × Mal)F1	20	6.5±0.4
(Bgr × Cht)F1 × (Bgr × Cht)F1	17	4.6±0.5
(Cht × Bgr)F1 × (Cht × Bgr)F1	3	3.3±0.7

Hmi: M. cas-Hmi, Mal: M. cas-Mal, Bgr: M. car-Bgr and Cht: M. sub-Cht.

* Faculty of Agriculture, Nagoya University.

Each reciprocal mating between the two subspecies produced progeny. F1 hybrids were fully fertile and showed no reproductive break down (Table 1), and sex ratios were normal in the progeny. Japanese wild mice, *M. m. molossinus*, are thought to be originated from hybrids between *M. m. castaneus* and a Chinese race of *M. m. musculus* (Yonekawa, H. *et al.*, 1988, *Mol Biol. Evol.* **5**: 63). The results of this study support the possibility that the intersubspecific hybrids occurred under natural conditions.

Effect of the Y Chromosome and H-2 Complex of Japanese Wild Mouse on Sperm Morphology

Dong-Sang SUH*, Józefa STYRNA** and Kazuo MORIWAKI

The spermatozoan head abnormalities in adult male mice are known to be determined by Y-linked factors and a small number of autosomal genes (Krzanowska, 1969, 1972, 1976). Two possible locations of these autosomal genes were chosen for investigation: the central portion of chromosome 4, and the proximity of the H-2 complex on chromosome 17. Both of these regions show homology to the sex-related sequences of the Y chromosome (Kiel-Metzger and Erickson, 1984).

The purpose of the present study was to investigate the relationship between sperm abnormalities and H-2 haplotypes. Segregation of sperm abnormality levels and H-2 haplotypes was investigated in F2 hybrid males obtained from reciprocal crosses involving two B10. congenic strains of Japanese wild mice carrying H-2 and the Y chromosome: B10.MOL-OHM ($H\text{-}2^{wm4}$, 23.1% of sperm abnormalities) and B10.MOL-OKB ($H\text{-}2^{wm8}$, 11.1% of sperm abnormalities). In both types of crosses mean levels of abnormal spermatozoa were significantly higher for males typed as $H\text{-}2^{wm4}$/$H\text{-}2^{wm4}$ than for heterozygous $H\text{-}2^{wm4}$/$H\text{-}2^{wm8}$ or homozygous $H\text{-}2^{wm8}$/$H\text{-}2^{wm8}$. These results suggest that the gene for high sperm abnormality is linked to the H-2 complex of the B10.MOL-OHM strain. For details, see *Genet. Res.* **53**: 17–19 (1989).

* Genetic Engineering Center, Korea Advanced Institute of Science and Technology, Seoul, Korea.

** Department of Genetics and Evolution, Institute of Zoology, Jagiellonian University, Kraków, Poland.

DNA Sequencing Analysis of rDNA in Robertsonian Mice

Yasuyuki KURIHARA, Hitoshi SUZUKI*, Heinz WINKING**,
François BONHOMME*** and Kazuo MORIWAKI

Previously we reported DNA restriction fragment length polymorphisms (RFLPs) of ribosomal RNA genes (rDNA) in wild mice with Robertsonian translocations (Rb). As Rb mice carrying RFLP patterns specific to each wild mouse subspecies, *M. m. domesticus*, *M. m. brevirostris*, *M. m. bactrianus* and *M. m. musculus*, were observed, we assumed that Rb mice originated from intersubspecies hybrid between each of the above four subspecies. In order to confirm this hypothesis, the following rDNA from Rb and normal wild mice were cloned to analyse their nucleotide sequences; 6.5 Kb EcoRI fragment from *M. m. domesticus* (SK), 5.5 Kb EcoRI fragment from *M. m. brevirostris*, 5.1 Kb EcoRI fragment from *M. m. bactrianus*, 5.5 and 5.1 Kb EcoRI fragments from Rb mice (WMP and Ta, respectively). DNA sequence analysis demonstrated that European wild mouse subspecies, *M. m. domesticus* and *M. m. brevirostris*, seem to be the closest to the two Rb mice. The origin of Rb mice suspected from this finding did not coincide with the RFLP data mentioned above. Further study is needed to specify the origin of Rb mice.

Geographical Survey of Wild Mouse Populations in Eastern Asia Based on Restriction Analysis and Sequence Analysis of Mitochondrial DNA

H. YONEKAWA, N. MIYASHITA and K. MORIWAKI

Analyzing mtDNA polymorphisms detected by restriction enzyme digestion, we plotted the geographical distribution pattern of the *Mus musculus* subspecies in China. The Chinese populations consist mainly of two subspecies, *M. m. castaneus* and *M. m. molossinus*. *M. m. castaneus* is bound in Southern China, whereas *M. m. musculus* is found in Northern China. The subspecies boundary lies along the Yang-tzu river (Yonekawa *et al.* 1989).

To confirm these results we collected 36 additional wild mice from 5 new

* Jikei Medical School.
** Medizinische Hochschule Lübeck, Federal Republic of Germany.
*** Universite Montpellier, France.

localities in China such as Guilin, Manasi, Mohe, Menhai, Xinin and one locality, Urmuchi, which has been examined before. We found that the haplotype from Urmuchi mice is identical to that reported previously and also identical to that from the Xinin mice. Furthermore, the Xinin mice also have two other different haplotypes.

Only one exception was found on the geographical distribution pattern; the mice trapped at Menhai, which was expected to be *castaneus* territory, have the mtDNA haplotype specific to *M. m. domensticus*.

The mice trapped at Mohe showed the following interesting features; their morphologies showed several characteristics specific to *M. m. castaneus* such as tail/body ratio and coat color, suggesting that the mice belong to *M. m. castaneus*. Allozyme analysis also supported this idea with some diagnostic markers such as Hbb^{d} which is specific to *castaneus* mice in Eastern Asia (Miyashita *et al.* 1984). The restriction analysis of mtDNA from the mice, however, showed that they had the *musculus* haplotype. This suggests that cytoplasmic gene flow occurs in the mice of Northern China and it should be noticed that this is reverse of the situation observed in Japan (Yonekawa *et al.*, 1989).

Although restriction analysis is useful, as mentioned above, to deduce what subspecies of *Mus musculus* the wild mice collected belong to, it is not suitable for examining the method nucleotide and amino acid substitutions during subspeciation of *M. musculus*. To examine this subject, we sequenced entirely, three haplotypes of mtDNA; two mtDNAs from *M. m. domesticus* and one from *M. m. molossinus* (H. Yonekawa, E. Harmel, C.-W. Wang, K. Fischer Lindahl, unpublished). Comparing the entire sequence among the three mtDNA and another one which has been already published (Bibb *et al.*, 1981), we found that there were not many base substitutions in the D-loop regions within *domesticus* mtDNA. This, however, was not true when we compared *molossinus* and *domesticus* mtDNA. We found two distinct hot spots of base substitutions in the D-loop regions. We are now proceeding with sequence analysis of the D-loop regions among the various kinds of subspecies which we have so for collected.

VI. MUTAGENESIS AND RADIATION GENETICS

Tumor Suppression Gene in Asian Wild-derived Strains of Mice

Nobumoto MIYASHITA and Kazuo MORIWAKI

We determined susceptibility to urethane-induced pulmonary adenoma for 8 strains derived from wild mice. The animals were sacrificed in the 5th month after a single subcutaneous injection of 1.5 mg urethane/g body weight. After the lungs were fixed in ethanol/formaldehyde (9: 1), the number of adenoma foci was assessed. Since all of the wild-derived strains had a mean of less than 1 adenoma per mouse, it is apparent these strains are resistant to the development of pulmonary adenoma.

In order to analyze the genetic factor for tumor suppression, we crossed the adenoma-resistant BGR strain, which was derived from wild mice (*Mus musculus castaneus*) collected in Indonesia, with adenoma-sensitive A/Wy mice. The F1 progeny between these strains exhibited adenoma induction similar to that of the BGR parent. This finding suggests that the resistant BGR phenotype was completely dominant to the sensitive strain A/Wy phenotype. Because A/Wy-backcross progenies showed a statistically significant difference from the 1: 1 distribution, the possibility of multiple genes' being dominant for the suppression to adenoma formation was apparent. A two-gene model can be proposed from the results with F2 progenies in which the predicted ratio of 9: 6: 1=resistant: intermediate: sensitive was found. These findings indicate that the suppression of adenoma formation is regulated by at least two genes in the BGR strain.

VII. POPULATION GENETICS

Further Simulation Studies on Evolution by Gene Duplication

Tomoko OHTA

In order to understand the origin of multigene families, Monte Carlo simulations were performed to see how a genetic system evolves under unequal crossing-over, mutation, random genetic drift and natural selection, starting from a single gene copy. Both haploid and diploid models were examined. Beneficial, neutral, and detrimental mutations were incorporated, and "positive" selection favored those chromosomes (haploid) or individuals (diploid) with more beneficial mutations than others. The same model for haploids was previously investigated with special reference to the evolution of gene organization, and the ratio of numbers of beneficial genes to pseudogenes was found to be a rough indicator of the relative strengths of positive and negative (against deleterious alleles) natural selection.

In the present study the evolution of gene organization and sequence divergence among genes in the multigene family was examined. It was found that positive selection accelerates the accumulation of arrays containing different beneficial mutations, but that total divergence, including both neutral and beneficial mutations, is not very sensitive to positive selection, under this model. The proportion of beneficial mutations to total mutations accumulated is a better indicator of positive selection than is total divergence. Results indicated that various observed examples in which amino-acid substitutions are accelerated, when compared with synonymous substitutions in duplicated genes, reflect the effect of selection in a manner similar to that of the present scheme. The diploid model was shown to be more efficient for accumulating beneficial mutations in duplicated genes than the haploid one, and the relevance of this finding to advantages for sexual reproduction was discussed. For details, see *Evolution* **42**: 375–386.

Time for Acquiring a New Gene by Duplication

Tomoko OHTA

In view of the widespread occurrence of gene families in eukaryotic genomes suggesting the importance of gene duplication in evolution, a population genetics model incorporating unequal crossing-over was formulated. By using this model, the time needed for acquiring a new gene was investigated by an approximate analytical method and by computer simulations. The model assumes that natural selection favors those chromosomes with more beneficial genes than other chromosomes in the population, as well as random genetic drift, mutation, and unequal crossing-over. Starting from a single gene copy, it was found that the time for acquiring another gene with a new function is dependent on the rates of occurrence of unequal crossing-over and mutation. Within a realistic range of parameter values, the required time was at least several times $4N$ generations, where N is the effective population size. Interchromosomal unequal crossing-over at meiosis was found to be more effective than intrachromosomal (between sister chromatids) unequal crossing-over for obtaining a new gene, provided that other parameters were the same. However, the genetic load for acquiring a gene is larger under the model of interchromosomal crossing-over. The relevance of this finding to advantages for sexual reproduction was discussed. For details, see *Proc. Natl. Acad. Sci. USA* **85**: 3509–3512.

Evolution by Gene Duplication and Compensatory Advantageous Mutations

Tomoko OHTA

Relaxation of selective constraint is thought to play an important role for evolution by gene duplication, in connection with compensatory advantageous mutant substitutions. Models were investigated by incorporating gene duplication through unequal crossing-over, selection, mutation and random genetic drift into Monte Carlo simulations. Compensatory advantageous mutations were introduced, and simulations were carried out with and without relaxation, where genes were redundant on chromosomes. Relaxation was introduced by assuming that deleterious mutants have no effect on fitness, so long as one or more genes free of such mutations remain

in the array. Compensatory mutations are characterized by an intermediate deleterious step in their substitutions, and therefore relaxation by gene redundancy is important. Through extensive Monte Carlo simulations, it was found that compensatory mutant substitutions require relaxation in addition to gene duplication, when mutant effects are large. However when mutant effects are small, such that the product of selection coefficient and population size is around unity, evolution by compensatory mutation is enhanced by gene duplication even without relaxation. For details, see *Genetics* **120**: 841–847.

More on the Episodic Clock

Naoyuki TAKAHATA

Previously, I (Genetics 1987, **116**: 169–179) quantitatively studied three conceivable causes for elevated variance in the rate of molecular evolution: (1) multiple substitutions, (2) deleterious mutations and changing population sizes, and (3) changes in the degree of selective constraints against a gene through successive substitutions. In addition, I made a cautionary note for a fourth case, varying mutation rates among lineages, because this case violates the usual assumption that all lineages under study share common rules for molecular evolution. Based on statistical models for (1)–(4), I found (1) and (2) to be unsound, but (3) and (4) as likely causes. Gillespie (1988), however, raised question on the motivation for (1), the evaluation of (2), and the strong model dependence of (3). I replied to these questions as a letter to the Editor in Genetics. For details, see Genetics **118**: 387–388.

The Coalescent in Two Partially Isolated Diffusion Populations

Naoyuki TAKAHATA

The n_0 coalescent of Kingman (1982) describes the family relationships among a sample of n_0 individuals drawn from a panmictic species. It is a stochastic process resulting from n_0-1 independent random events (coalescences) from each of which n ($2\leqq n\leqq n_0$) ancestral lineages of a sample are descended from $n-1$ distinct ancestors for the first time. A similar genealogical process was studied for a species consisting of two populations with migration between them. The main point of interest is with the probability density of the time length between two successive coalescences and

the spatial distribution of $n-1$ ancestral lineages over two populations when the n to $n-1$ coalescence takes place. These are formulated based on a non-linear birth and death process with killing, and are used to derive several explicit formulae in selectively neutral population genetics models. To confirm and supplement the analytical results, a simulation method was proposed based on the underlying bivariate Markov chain. This method provides a general method for solving the present problem even when an analytical approach appears very difficult. It becomes clear that the effects of the present population structure are most conspicuous with a 2 to 1 coalescence, and to a lesser extent with a n to $n-1$ ($3 \leqq n$) coalescence. This implies that in a more general model of population structure, the number of populations and the way in which a sample is drawn are important factors in determining the n_0 coalescent. For details, see *Genet. Res. Camb.* **52**: 213–222.

Dubious Maternal Inheritance of Mitochondrial DNA in *D. simulans* and Evolution of *D. mauritiana*

Yoko SATTA, Nobue TOYOHARA, Chiaki OHTAKA, Yumi TATSUNO, Takao K. WATANABE, Etsuko T. MATSUURA, Sadao I. CHIGUSA and Naoyuki TAKAHATA

Within-line heterogeneity was found in the mitochondrial DNA (mtDNA) in two isofemale lines of *D. simulans*. The co-existing types, S and M, were typical of the mtDNA in *D. simulans* and in *D. mauritiana*, respectively, with their nucleotide divergence per site being *ca.* 2.1%. Segregation analysis confirmed that some individuals in these lines were heteroplasmic and suggested incomplete maternal inheritance of mtDNA in *Drosophila*. Examination of other lines of *D. simulans* revealed that the M type of *D. mauritiana* occurs with frequency 71% in Réunion, 38% in Madagascar and 0% in Kenya. This finding and interspecific sequence comparisons of both M types indicate that *D. mauritiana* diverged from *D. simulans* probably less than 240,000 years ago. For details, see *Genet. Res. Camb.* **52**: 1–6.

Diffusion Model of Population Genetics Incorporating Intergroup Selection and Epistatic Interaction in Fitness

Motoo Kimura

In order to treat Wright's "shifting-balance theory" of evolution in quantitative terms, I propose a diffusion model (diffusion equation method) in which intergroup selection (or competition between demes) and epistatic (*i.e.*, non-additive) interaction in fitness are incorporated.

Let us assume a hypothetical population (species) consisting of an infinite number of competing subgroups (demes) each with N_e breeding diploid individuals (or $2N_e$ haploid individuals) mating at random. Consider two independent gene loci and assume that a pair of alleles A and A' are segregating in the first locus, and alleles B and B' are segregating in the second locus. Let x and y be respectively the relative frequencies of A' and B' within a deme. We shall denote by $\phi(x, y; t)$ the distribution function of x and y at time t such that $\phi(x, y; t)\Delta x\Delta y$ represents the fraction of demes whose frequencies of A' and B' lie in the ranges $(x, x+\Delta x)$ and $(y, y+\Delta y)$ respectively, where $0<x, y<1$.

Mutation is assumed to occur at the first locus at the rate v_1' per generation from A to A', and in the reverse direction at the rate v. Similarly, at the second locus, the mutation rate from B to B' is v_2', and in the reverse direction v_2. With respect to natural selection within demes, we assume "genic selection" and denote the relative fitnesses (in terms of Wright's selective values) of the four haploid genotypes AB, $A'B$, AB' and $A'B'$ respectively by w_{11}, w_{21}, w_{12} and w_{22}. Migration is assumed to occur following Wright's island model so that in each generation, individual demes contribute emigrants to the entire gene pool of the species at the rate m, and receive immigrants from the pool at the same rate.

As to intergroup selection, we denote by $c(x, y)$ the fitness, relative to inter-deme competition, of the deme with gene frequency pair (x, y). This means that during a short time interval Δt, the change of $\phi(x, y; t)$ by intergroup selection is

$$\Delta\phi = \{c(x, y) - \bar{c}\}\phi\Delta t\,, \qquad (\Delta t \to 0)\,,$$

where $\bar{c}$ is the mean of $c(x, y)$ over the whole population (species).

Then, we have the following diffusion equation for $\phi(x, y; t)$.

$$\frac{\partial \phi}{\partial t} = L(\phi) + \{c\,(x, y) - \bar{c}\}\phi$$

In this equation ϕ stands for $\phi(x, y; t)$ and

$$L = \frac{1}{2}\frac{\partial^2}{\partial x^2} V_{\delta x} + \frac{1}{2}\frac{\partial^2}{\partial y^2} V_{\delta y} - \frac{\partial}{\partial x} M_{\delta x} - \frac{\partial}{\partial y} M_{\delta y}$$

is the two dimensional Kolmogorov forward operator, where

$$V_{\delta x} = \frac{x(1-x)}{2N_e}, \quad V_{\delta y} = \frac{y(1-y)}{2N_e},$$

$$M_{\delta x} = v_1'(1-x) - v_1 x + \frac{x(1-x)}{w}\frac{\partial w}{\partial x} + m(\bar{x} - x)$$

and

$$M_{\delta y} = v_2'(1-y) - v_2 y + \frac{y(1-y)}{w}\frac{\partial w}{\partial y} + m(\bar{y} - y)$$

in which $w = w_{11}(1-x)(1-y) + w_{21}x(1-y) + w_{12}(1-x)y + w_{22}xy$.

If the competitive ability of a deme is proportional to the mean fitness of the deme, then

$$c(x, y) = kw(x, y)\,,$$

where k is a positive constant. This means that genotypes which are advantageous for individual selection are also favorable for group selection, and vice versa.

Of particular interest, in relation to Wright's shifting-balance theory, is the case in which single mutants are deleterious while the double mutant is advantageous so that $w_{11}=1$, $w_{21}=1-s_a'$, $w_{12}=1-s_b'$ and $w_{22}=1+s_{ab}$, where $s_a'>0$, $s_b'>0$, $s_{ab}>0$. Assuming that these selection coefficients are much smaller than unity, and neglecting higher order terms, we have $x(1-x)(-s_a'+\varepsilon y)$ and $y(1-y)(-s_b'+\varepsilon x)$ respectively for the changes of the frequencies of A' and B' per generation due to intra-deme selection, where $\varepsilon = s_a' + s_b' + s_{ab}$. The numerical analysis of the diffusion equation for such a case is under way.

A Sexual Selection Model for the Evolution of Imitative Learning of Song in Polygynous Birds

Kenichi AOKI

While normal song development in oscines depends on imitative learning, it presumably was innately determined (directly inherited, non-modifiable) in the ancestral species. Evolution of imitative song from innate song was investigated theoretically, based on a genetic model of sexual selection by female choice in a polygynous population. Since song is usually limited to sexually mature males, and females exhibit preferences with respect to various aspects of song, sexual selection may well have been involved. Specifically, it was assumed that imitative learning may give rise to a variant song that some females prefer over innate song; and also that female preference is not affected by early song experience. However, the assumption of polygyny restricts the applicability of the results obtained here, since most living bird species are monogamous. It was found that coevolution of imitative song and female preference from low initial frequencies requires that the preference be strong. The available data, although not of direct relevance, suggest that such strong preference may sometimes occur. When females that show preference are more common than those that mate indiscriminately, a two-fold difference in preference may still be necessary for imitative song to be favored. If female preference is assumed to be fixed, the effect of various modes of song transmission can be compared. It was shown that transmission from father to son is critical and must be efficient, unless compensated for by strong female preference. Moreover, an initial increase in imitative song is less likely to occur if paternal song is forgotten to be replaced by a new song learned from another adult male. These predictions may be difficult to reconcile with the assumption of polygyny, which may imply limited opportunity for interaction between father and son. They would certainly seem to rule out the possibility of the evolution of imitative song in a lekking species. Finally, the conditions for the evolution of imitative song were compared with those for the evolution of human speech. The latter were obtained previously on a model assuming that speech serves to communicate adaptive cultural traits from parent to child. The conditions are highly analogous in spite of the disparity in the selective forces assumed. For details, see *Amer. Natur.*, in press.

Pleiotropy and Preadaptation in the Evolution of Human Language Capacity

Kenichi AOKI and Marcus W. FELDMAN

The capacity for spoken language in the human is a genetic trait, but the information communicated by this means is to a large extent culturally determined. Using a gene-culture coevolutionary approach, we modeled the hypothesis that speech evolved as a channel for the communication of adaptive cultural traits from parent to offspring. The motivation for this work was a condition obtained previously, that an initial increase in communication would require at least a two-fold advantage for the transmitted trait. In the present study, we showed that under reasonable assumptions the invasion condition becomes less stringent. In Model 1, we assumed that two adaptive cultural traits could be transmitted. A gene that permits communication of one adaptive trait modifies the expression of another gene that permits communication of the second adaptive trait. In Model 2, we assumed that a related function such as greater memory capacity was a prerequisite for speech, and that this function conferred an advantage independent of its association with speech. In both models we assumed haploid sexual genetics and a simple scheme of vertical transmission. The stability properties of all corner and edge equilibria of the models were analyzed. The two models taken together suggest a possible scenario for the initial stages of the evolution of speech. For details, see *Theor. Popul. Biol.* **35**: 181–194.

VIII. EVOLUTIONARY GENETICS

Algorithmic Analysis of the Computer Program for Likelihood of a Human Pedigree

Takashi GOJOBORI and Norikazu YASUDA

The computer program "LIPED", for computing the likelihood of a human pedigree, was originally made by J. Otto. The program is based on the so-called "Elston-Stewart algorithm," in which the conditional probabilities of a phenotype, given a particular genotype, are computed. LIPED can be a powerful tool for linkage analysis, since in the analysis, the likelihood of a human pedigree must be computed for a given value of the recombination rate. In particular, LIPED is very useful for the study of human genome mapping, because it can be used for the linkage analysis of genes responsible for genetic diseases. However, it is quite difficult to manage this program because too many input parameters are involved. To avoid this difficulty, we modified LIPED by incorporating a menu system into the computer program. Since the menu system can work as a friendly man-machine interface, our modified version of LIPED is more manageable than the original one.

Molecular Evolution of Human and Simian Immunodeficiency Viruses

Takashi GOJOBORI, Etsuko N. MORIYAMA and Shozo YOKOYAMA

To construct phylogenetic trees of human immunodeficiency viruses (HIVs) and simian immunodeficiency viruses (SIVs), we estimated numbers of nucleotide substitutions between viral isolates. From these numbers, phylogenetic trees were constructed by unweighted pair-group (UPG) and neighbor-joining (NJ) methods. The trees obtained clearly show two major groups of viruses; the HIV-1 and HIV-2 groups. The HIV-1 group contains virus isolates from North America and Central Africa. The HIV-2 group consists of HIV-2 isolates from West Africa and all SIV isolates presently available. The divergence times at all branching points of the tree were estimated, using rates of nucleotide substitution. In particular, the divergence time between the HIV-1 and HIV-2 groups were estimated

to be 150–200 years. Using the obtained trees, we also estimated the patterns of amino acid (and nucleotide) substitutions. This may be useful for vaccine development using prediction of the direction of amino acid substitutions. For details, see IV International Conference on AIDS, Book 1, p142.

Molecular Evolution of the Human Immunodeficiency and Related Viruses

Shozo YOKOYAMA, Lyinn CHUNG, Etsuko N. MORIYAMA
and Takashi GOJOBORI

A phylogenetic tree for different human immunodeficiency viruses types 1 (HIV-1) and types 2 (HIV-2), lentiviruses, and oncoviruses has been constructed by comparing the nucleotide sequences of the two regions of their *pol* genes which encode reverse transcriptase and endonuclease/integrase. The results obtained indicate that (1) different HIV-1 isolates form one cluster and their common ancestor diverged from the ancestor of HIV-2, (2) the common ancestor of HIV-1 and HIV-2 isolates diverged from that of the lentiviruses, and (3) the lentivirus group and the oncoviruses diverged from a common ancestor. In particular, the divergence time between the HIV-1 and HIV-2 groups suggests that AIDS has existed for several hundred years but went undetected. For details, see *Mol. Biol. Evol.* **5**(3): 237–251 and "*Gann* '88" pp 55–62.

Evidence for Species-to-Species Transfer in Evolution of Pathogenic Determinants of *Escherichia coli* and *Vibrio cholerae* 01

Tatuo YAMAMOTO, Etsuko N. MORIYAMA and Takashi GOJOBORI

To clarify the origin and evolution of the pathogenic determinants in bacteria, particularly enterotoxigenic *Escherichia coli* and *Vibrio cholerae* 01, we constructed a phylogenetic tree for heat-labile toxin (LT). LT has structural and functional features in common with cholera toxin (CT) produced from *Vibrio cholerae 01* which is the causative agent of cholera. Moreover, LT is subdivided into two types (LTh and LTp) having different epitopes; LTh is found only in human isolates of enterotoxigenic *E. coli*, whereas LTp is found only in porcine isolates. The phylogenetic tree

showed that the divergence between the CT gene and the two LT genes occurred much more recently than the species divergence between *V. cholerae 01* and *E. coli*. The species divergence was inferred from the phylogenetic tree for 5S rRNA genes. Moreover, the codon usage of the LT gene is similar to other genes of *V. cholerae* rather than those of *E. coli*. It suggests that the LT genes might have resulted from the species-to-species transfer of the CT gene from *V. cholerae 01* to *E. coli*.

For details, see Advances in Research on Cholera and Related Diarrheas 06, Pp 195–212.

Mitochondrial DNA Polymorphism among Five Asian Populations

Shinji HARIHARA, Naruya SAITOU, Takashi GOJOBORI
and Keiichi OMOTO

Mitochondrial DNA (mtDNA) polymorphisms were examined using 13 different kinds of restriction enzymes on the total DNA obtained from blood samples of five Asian populations: Japanese, Ainu of northern Japan, Koreans, Negritos (Aeta) of the Philippines, and the Vedda of Sri Lanka. As a result, 28 restriction-enzyme morphs were detected and combinations of the morphs enabled us to classify the mtDNAs of 243 individuals into 20 mtDNA types. We constructed a phylogenetic tree for these mtDNA types. It showed that the Japanese, Ainu, and Korean populations were closely related to each other. Although the Aeta were found to show a relatively close relationship to these three populations, the Vedda were quite different from the other four populations.

For details, see *Am. J. Hum. Genet.* **43**: 134–143.

Gametic Disequilibrium for Isozyme Genes Found in Wild and Cultivated Rices

Hiroko MORISHIMA

Isozyme data in rice cultivars (*Oryza sativa*) and their wild relatives (*O. rufipogon*), accumulated with the collaboration of a number of my colleagues, were analyzed in relation to multilocus association patterns. Nonrandom association of alleles at two loci ordered within gametes, usually called "linkage disequilibrium", is termed "gametic disequilibrium" (GD), as this

phenomenon is affected by many factors other than linkage. In the present study, deviation from random association was measured by $D'=D/D_{max}$ ($D=X_1-p_1q_1$), assuming two loci A and B; X_1, the gamete frequency of A_1B_1; p_1 and q_1, the frequencies of alleles A_1 and B_1, and D_{max}, the maximum D possible for a given set of gene frequencies. The following results were obtained:

1) Among cultivated strains, a strong GD was frequently found in 12 loci assayed. The D′ values did not show relationships to the degree of chromosomal linkages between the relevant loci. This nonrandom association results in differentiation into two major enzymatic groups corresponding to the Indica and Japonica types.

2) Sixty wild strains assayed in the same manner showed generally smaller values of D′ than cultivars, as expected from their mating system of partial outbreeding. Yet, certain loci-combination (*Cat-1 vs. Pgi-2*, *Cat-1 vs. Est-2*, *Cat-1 vs. Acp-1*, etc.) showed nonrandom association in the same direction as in cultivars, suggesting that Indica-Japonica differentiation at the isozymic level occurred in wild progenitors though not as conspicuous as in cultivars. Further, a locus *Pox-1* which is polymorphic in wild rices but monomorphic in cultivated rices, showed GD with some loci. This seemed to reflect ecotypic differentiation into perennial and annual types occurring within this species.

3) GD observed among individual plants within natural populations was inconsistent in different populations. It was not certain whether intrapopulational GD is in a stable state or not.

4) In three experimental populations obtained from crosses between wild rice strains, it was observed that D′ values between two loosely linked loci, *Pox-1* and *Acp-1*, tended to increase as generations proceeded.

A part of the GD observed in rice cultivars possibly originated from random drift or the founder effect and has been maintained by the system of predominant inbreeding. However, the increasing trend of GD at certain loci over time and GD which are found consistently in different regions characterizing ecotypes, shows that the possibility of GD being molded by the coadaptation of interacting loci can not be ruled out.

Tropical and Temperate Types in Asian Rice Cultivars

Lin-Hua TANG*, Yo-Ichiro SATO and Hiroko MORISHIMA

It is generally accepted from variation studies on characteristics and isozymes in rice that Asian rice cultivars tend to be differentiated into two major varietal groups, Indica and Japonica. To examine whether or not differentiation towards a direction other than the Indica-Japonica line occurs in Asia, isozyme variations were extensively surveyed, using 226 cultivars considered to be a random sample of Asian rices. Data of 12 polymorphic loci were analyzed by a multivariate analysis called "Quantification III", with each allele being treated as a variate. The first axis distinguished two varietal groups. This division as expected, coincided with the Indica and Japonica types classified by three diagnostic characteristics, with a few exceptions. The variation revealed by the second axis showed a trend of differentiation between cultivars according to tropical and temperate origins and it was found in Indicas as well as in Japonicas. The isozyme loci which contributed much to this axis of variation were *Amp-3*, *Est-2* and *Pgd-1*. Reexamination of our old data of 11 characteri stics (Morishima & Oka 1981, *Jpn. J. Breed.* **31**: 402–413) for Asian rices revealed a similar trend in differentiation between tropical and temperate varietal groups in Indica as well as in Japonica types along a third axis of the principal component analysis.

Previously, Oka (1958, *Ind. J. Genet. & Pl. Breed.* **8**: 79–89) argued that Japonica types can be further divided into tropical and temperate types by a characteristic association pattern. More recently, Nakagahra (1978, *TARS* **11**: 77–82) reported that Chinese Indicas are genetically different from Indicas in tropical countries. From the results obtained in the present study, it may be concluded that differentiation into tropical and temperate types was found in Asian rices, and that it occurred within both Indica and Japonica types, bringing about some genetic differences at the isozymic and phenotypic levels common to both types.

* Food Crop Inst., Jiangsu Acad. of Agric. Sci., China.

Plant Opal Analysis: its Significance to the Origin and Evolution of Rice

Yo-Ichiro SATO, Hiroshi FUJIWARA* and Tetsuo UDATSU*

The origin of cultivated rice (*Oryza sativa* L.) has been disputed for many years, beginning with de Candolle. Its differentiation into two ecogeographic races, *indica* and *japonica*, has also been controversial, because bio-archaeological evidence is scanty. In this study, the silica body (accumulated SiO_2 in particular cells) removed from living rice plants, and that excavated from soil (plant opal) were discussed as evidence for support of such arguments. The plant opal has been used for assessment of past vegetation, and as evidence for rice cultivation at archaeological sites, based upon species-specific morphology. It has not been however, used for studies of intraspecific variation. We showed *indica* and *japonica* were distinguishable by the shape of the silica body removed from 96 cultivars, with a few inconsistencies. Studies on plant opal from excavated samples may enable us to indicate whether they were *indica* or *japonica* cultivars. Accumulated data on the shape of plant opals would be helpful in assessing the origin and evolution of *indica* and *japonica* types.

On the Maximum Likelihood Method for Estimating Molecular Trees: Uniqueness of the Likelihood Point

Kaoru FUKAMI** and Yoshio TATENO

Studies were carried out on the uniqueness of the stationary point on the likelihood surface for estimating molecular phylogenetic trees, yielding proof that there exists at most one stationary point, i.e., the maximum point, in the parameter range for the one-parameter model of nucleotide substitution. The proof is simple, yet applicable to any type of tree topology with an arbitrary number of taxa. The poof ensures that any valid approximation algorithm is able to reach the unique maximum point under the above mentioned conditions. An algorithm developed incorporating Newton's approximation method was then compared with the conventional one by means of computer simulation. The results showed

* Faculty of Agriculture, Miyazaki University.
** Ochanomizu University.

that the newly developed algorithm requires less computer time than the conventional one, whereas both algorithms lead to identical molecular phylogenetic trees in accordance with the proof. (*J. Mol. Evol.*, **28**: 460, 1989)

Is Molecular Evolution Parsimonious? Theoretical Approach to the Problem

Yoshio TATENO

The parsimony concept in phylogeny was reviewed first, which confirmed that no scientific basis had been established to support or deny the concept. Nevertheless, it is also true that the concept is quite useful in a practical sense for constructing phylogenetic trees. Next, a method for constructing molecular phylogenetic trees which was developed on the basis of the parsimony concept was introduced. It directly makes use of nucleotide sequences. The parsimony concept is incorporated in the method in such a way that it always chooses the tree which, on the whole, requires the fewest possible nucleotide changes among the candidates examined. The performance of the method was then examined by conducting computer simulation that imitates an evolving gene along a model phylogenetic tree. The results quantitatively confirmed the usefulness of the parsimony concept under the simulation model. There are, however, cases in which the parsimony criterion gives plural tree topologies including the correct one. There seems to be no way to single out the correct topology among them, unless employing other criteria. (In: *Population Biology of Genes and Molecules*—Proceedings of the Fourth Symposium for the International Prize for Biology—Edited by J. F. Crow and N. Takahata, in press)

Different Rates of Evolution of Autosome-, X Chromosome- and Y Chromosome-linked Genes: Hypothesis of Male-driven Molecular Evolution

Takashi MIYATA, Kei-ichi KUMA, N. IWABE, Hidenori HAYASHIDA and T. YASUNAGA

The relationships between different numbers of germ-cell divisions between males and females, and different mutation frequencies between autosomes and sex chromosomes, were examined under the assumption that errors in DNA replication are the major factor in mutations con-

tributing to molecular evolution. The expected mutation frequency of the X chromosome relative to that of autosomes (R(X/A)) was shown to be 2/3 in the XX female/XY male system, if the male to female ratio of the number of germ-cell divisions (a) is very large. Similarly, for the relative mutation frequency R(Y/A) of the Y chromosome, we have R(Y/A)=2 for $a \gg 1$. To test this, we compared nucleotide sequences of autosome- and X-linked genes for human and mouse (or rat), and analysed the evolutionary rate of silent substitutions, a weakly constrained component that would reflect mutation frequency directly. The rate of silent substitution for X-linked genes relative to that for autosome-linked genes (R′(X/A)), was shown to be 0.60, being close to the expected value 2/3. Similarly, for the relative evolutionary rate R′(Y/A) of the Y-linked gene, we observed R′(Y/A)=2.2, which again is close to the expected value 2. The marked correlation between the observed rates R′ and the expected rates R for a—1 suggests that males serve as mutation generators. The relative mutation frequency of the ZW female/ZZ male system was shown to the reverse of that of the XX/YY system: For $a \gg 1$, $R(Z/A)=4/3 \gg R(W/A)$. Surprisingly small value of R(W/A) is expected. An analysis of avian genes would provides clear-cut proof for the present model.

Immunoglobulin-like Sequences in the Extracellular Domains of Proto-oncogene *fms* and Platelet-derived Growth Factor Receptor

Hidenori HAYASHIDA, Kei-ichi KUMA and Takashi MIYATA

The McDough strain of feline sarcoma virus (SM-FeSV) carries the transforming gene, v-*fms*, which belongs to the family of *src*-related oncogenes with tyrosine-specific protein kinase activity. The sequence analyses of the products of the v-*fms* and its cellular homologue (c-*fms*), revealed structural properties of a cell-surface receptor consisting of an extracellular domain, a transmembrane region and a cytoplasmic tyrosine kinase domain. Furthermore, c-*fms* was shown to be identical or related to, the receptor for macrophage colony-stimulating factor (CSF-1). The platelet-derived growth factor (PDGF) receptor previously has been shown to be closely related in sequence to the c-*fms* protein over the entire sequence and has been identified as a member of the *src* family. We found that the extracellular domains of the c-*fms* protein and the PDGF receptor show marked sequence

homologies with known immunoglobulin-related sequences. Thus c-*fms* and the PDGF receptor belong to two distinct gene families; the cytoplasmic coding sequence belongs to the *src* family and the extracellular coding sequence to the immunoglobulin gene superfamily. This unique structure of the c-*fms* and PDGF receptor possibly evolved through the shuffling of primodial genes from distinct gene families. *Proc. Jpn. Acad.* **64B**: 113–118, 1988.

Male-Driven Molecular Evolution: A Model and Nucleotide Sequence Analyses

Takashi MIYATA

There are several lines of evidence suggesting that errors in DNA replication are the major source of mutations contributing to molecular evolution. If this is really the case, there is another important factor which should be considered explicitly in relation to the rate of molecular evolution. It is generally thought that the number of germ cell divisions in sperm differs from that in eggs. Inclusion of this factor with possible sources of mutations may be important for a comprehensive understanding of mechanisms underlying molecular evolution.

Under the assumption that errors in DNA replication are the major factor in mutations contributing to molecular evolution, we showed that different numbers of germ-cell divisions between males and females result in different mutation frequencies between autosomes and sex chromosomes. The expected mutation frequencies $R_{X/A}$, of the X chromosome and $R_{Y/A}$, of the Y chromosome, relative to the mutation frequency of autosomes, were respectively shown to be 2/3 and 2 in the XX female/XY male system, if the male to female ratio of the number of germ-cell division (α) is very large. To test this, we compared nucleotide sequences of autosome- and X-linked genes, human and mouse, (or rat) and analyzed the evolutionary rates of silent substitutions. The average rate of silent substitutions of X-linked genes relative to that of autosome-linked genes ($R'_{X/A}$) was shown to be 0.58, being close to the expected value 2/3. For the relative evolutionary rate, $R'_{Y}/_{A}$, of the Y-linked gene, we observed $R'_{Y/A}=2.2$, which again is close to the expected value 2. From the strong correlation between the observed R′ and the expected R for $\alpha \gg 1$, we proposed that males possibly serve as a mutation generator. We also found that, in X-linked genes, the

rate of pseudogene evolution is 1.54 times higher than the functional counterpart and rodents evolve with a rate 2.3 times higher than those of non-rodent mammals. These properties quantitatively agree well with those found in autosome-linked genes, suggesting a uniformly reduced mutation frequency over the whole X chromosomal region. The relative mutation frequencies of the ZW female/ZZ male system was shown to be the reverse of those of the XX/XY system: For $\alpha \gg 1$, $R_{Z/A}(=4/3) > 1 \gg R_{W/A}$. Surprisingly small value of $R_{W/A}$ is expected. An analysis of avian genes would provide clear-cut proof for the present model.

IX. HUMAN GENETICS

Cytogenetic Studies on Chromosome 18 Polyploidy Syndrome by Non-radioactive in Situ Hybridization Techniques and Probe L1.84

Takashi IMAMURA, Tomoko HASEGAWA, Hitoshi NAKASHIMA and Asao FUJIYAMA

The development of improved non-radioactive in situ hybridization techniques with chromosome-specific probes for cytogenetic analysis, has made this method an increasingly important molecular tool for directly detecting specific nucleic acid sequences within cells or genomes. These probes, now available for over half of the human chromosomes, hybridize to repeated sequences on compact regions of the target chromosome near the centromere. This permits the rapid identification of chromosomes in metaphase spreads, determination of copies of selected chromosomes in interphase nuclei, and the determination of the relative positions of chromosomes in interphase nuclei. We used highly sensitive methodology for the localization of individual DNA sequences using fluorescence detection of biotinated probes hybridized in situ. Because of the unusually low background and high hybridization efficiency achieved, this methodology allows direct, high resolution localization of single sequences not only on chromosomes, but, more importantly, within the interphase nucleus. We used this approach to investigate the origin and structure of a tiny extra chromosome in five patients who showed syndrome of chromosome 18 polyploidy.

All five (two males and three females) examined showed psychomotor retardations, dolichocephalies, delicate, but only slightly anomalous, facial appearances, and small, low set ears. Limited joint movements were observed in four patients. Chromosomal studies by the G-banding method revealed one tiny extra-metacentric chromosome in all cases. Both arms of each extra-metacentric chromosome were very similar to the short arm of chromosome 18. Biotin-labeled probe L1.84 (Devilee, 1986), specific for the pericentric region of chromosome 18, was hybridized in situ to metaphase chromosomes and interphase nuclei of lymphoblast cells from

these patients, which resulted in three bright fluorescein spots in each metaphase figure and nucleus. All exhibited labelling on the centromere of both chromosomes 18 and that of the minute extra-chromosome. Thus, the minute extra-metacentric chromosome could be i(18p). One female patient without joint limitations had a mosaicism of 46, XX/47, XX, +i(18p). By the combined use of the procedures of chromosome microdissection and polymerase chain reaction, we are cloning for use as probes, a number of unique DNA sequences from the tiny extra-metacentric chromosome. Our goal is to identify the genes that cause tri- or tetrasomy 18 syndromes.

Population Studies of Gene Conversion within the Duplicated Human α-Globin Gene Family

Hitoshi NAKASHIMA, Asao FUJIYAMA and Takashi IMAMURA

The identification of individuals possessing one or three adult α-globin genes on a single chromosome instead of the normal two α-gene haplotype provides strong genetic evidence for the occurrence of unequal crossing-over in the human α-gene complex. The alignment of the restriction maps of the α-loci residing on the one-, two-, and three-gene chromosomes reinforces this hypothesis. Additional evidence that sequence homology in the α-gene cluster promotes unequal recombination, is the production of deletions, indistinguishable from those found in human populations, through the propagation of the cloned α-gene region in Escherichia coli.

Although the duplicated human α-globin genes encode identical polypeptides, this and other previous studies have established that the $\alpha 1$ and $\alpha 2$ genes are not identical at the DNA level. Whereas the 5′-untranslated regions, the three coding blocks, all of the first and the 5′ four-fifths of the second intervening sequences (IVS1 and IVS2) are highly homologous, the 3′-ends have markedly diverged. This finding must be reconciled by any mechanism purported to affect the concerted evolution of the α-globin genes. We studied frequencies of gene conversions producing single or triplicated α-gene genotypes in the Japanese population. To precisely identify the 5′-homology boundary of the α-genes, we cloned and sequenced a naturally occurring product of unequal crossing-over and recombination between the normal α-globin genes, in an attempt to define the molecular mechanism which underlies this process in the Japanese population.

High molecular weight nuclear DNAs were prepared from white blood cells of 645 unrelated individuals residing in West Kyushu, followed by digestion with restriction endonucleases, BamHI and EcoRI. The first screening was carried out by electrophoresis of digests on an agarose gel plate and samples showing an abnormal digesting pattern were examined further by restriction with BglII, HpaI and SacI. We found that 10 of the 645 samples showed a triplicated $\alpha\alpha\alpha$ loci on a single chromosome, but none showed the presence of a single α haplotype. Thus, the gene frequency of the triplicated α-gene cluster in a Japanese population might be assessed as 0.008 (10/1290), while that of the single gene locus is less than 0.0008. This study provides the opportunity to compare the frequencies of α-globin gene deletion (α^+-thalassemia), as well as those of triplication, in the Japanese with those in Melanesians exposed to different malaria endemicities. Although the mechanism involved remains obscure, epidemiological data provide some indications of the magnitude of the apparent selective advantage conferred by α-globin gene deletion with respect to malaria. It is notable that while the trplicated $\alpha\alpha\alpha$ genotype was present at a gene frequency of 1%, irrespective of the region, there is a strong association between the endemicities of malaria and the frequencies of α-gene deletion in different parts of melanesia. Thus far, the situation in Japan suggests that the genes responsible for the hemoglobin variants encountered here do not confer a marked selective advantage, but are approximately neutral and owe their low frequency to a simple mutation process and random genetic drift which has been occurring in a relatively small number of founders in this population.

Analysis of β-Globin Gene Mutation in Japanese Patients with β-Thalassemia Reveals a Substitution of Thymine for Cytosine in the Second Intron

Yuji NARITOMI, Yutaka CHIFU, Hitoshi NAKASHIMA
Asao FUJIYAMA and Takashi IMAMURA

We identified the substitution of a thymine for a cytosine at nucleotide position 654 in the second intron of the β-globin gene that causes β-thalassemia in two Japanese families. The molecular basis for the thalassemia syndrome in our Japanese patients can be explained by aberrant m-RNA splicing in IVS-2. One patient was apparently homozygous for the thalas-

semia gene, but analysis of both of the allelic β-globin genes revealed that the patient was a compound heterozygote for different β-globin gene mutations. Another allele had a normal sequence in the region of 1,994 bases of the β-globin locus so far examined. Therefore, we thought that the other mutation occurred outside the region sequenced at the site controlling the gene expression, which remained to be studied further. This mutation was reported to occur rather frequently in patients of Chinese origin, but has rarely been found in other ethnic groups.

β-Thalassemia is a heterogeneous inherited disorder of β-globin gene expression and one of the most common single gene defects. Although various mutations have been identified in defective β-globin genes, a limited number of specific mutations are prevalent in a given ethnic group in which the thalassemia occurs rather frequently. In the Japanese population, heterozygous β-thalassemia occurs with a frequency of approximately one in 1000 individuals, while only a few patients with homozygous β-thalassemia have been observed so far. This is the first molecular identification in Japanese patients of the β-globin gene mutation. This mutation in IVS-2 has only been found in Chinese patients. The β-thalassemia gene in this Japanese family and those in Chinese may be of the same origin as they are both framework 1 genes. The spread of the mutation to the present day geographical pattern has been explained on the basis of population migration. However, since the family record indicated no apparent evidence of this family being related to Chinese, the possibility of an independent occurrence of specific mutations cannot be excluded.

Regulation of ras Activity on Membranes by Post-Translational Processing Modification

Asao FUJIYAMA, Susume TSUNASAWA and Fumio SAKIYAMA

The *ras* oncogene was first identified as a transforming gene of Harvey/ Kirstein sarcoma viruses. The identification and isolation of *ras* genes from the DNA of human cancer tissue and the analyses of those gene sequences, along with that of normal tissue, revealed that a single point mutation changed the normal *ras* gene into a transforming *ras* gene. This finding of the difference between normal and transforming *ras* genes is the first experimental evidence that cellular transformation is induced by a single mutation on one gene. Utilyzing recombinant DNA technique,

further biochemical analyses have been carried out using p21 proteins produced in *E. coli* cells. Although the *E. coli* product exhibits biochemical functions such as GTP binding and GTPase, these proteins are non-functioning as transforming proteins. We as well as others have reported that ost-translational modification, including modification by fatty acid acylation, is essential for the transforming activity of ras proteins. Our goal is to understand the mechanism of cellular transformation by *ras* through the analyses of post-translational regulation of *ras* protein.

Molecular Phylogeny and Evolution of Primate Mitochondrial DNA

Kenji HAYASAKA, Takashi GOJOBORI and Satoshi HORAI

We determined nucleotide sequences of homologous 0.9 kb fragments of mitochondrial DNAs (mtDNAs) derived from four species of old-world monkeys (Japanese, rhesus, crab-eating, and Barbary macaques), one species of new-world monkey (common squirrel monkey), and two species of prosimians (Philippine tarsier and ring-tailed lemur). Using these nucleotide sequences and homologous sequences for five species of hominoids (human, chimpanzee, gorilla, orangutan, and gibbon), a phylogenetic tree for the four groups of primates was constructed. The phylogeny obtained is generally consistent with evolutionary trees constructed in previous studies, except for the position of the tarsier. Although most molecular studies have suggested that the tarsier is more closely related to anthropoids than to prosimians, our analysis of mtDNA showed that the tarsier is more closely related to the lemur than to anthropoids.

To estimate the rate of nucleotide substitution, we examined the relationship between between-species numbers of nucleotide substitutions and divergence times. The estimated rate of nucleotide substitution for mtDNAs in hominines (human, chimpanzee, and gorilla) is $(0.53\text{–}1.05)\times 10^{-8}$/site/year/lineage and that in old-world monkeys is $(1.12\text{–}2.24)\times 10^{-8}$/site/year/lineage. A relative rate test using the squirrel monkey as a reference species also revealed that more nucleotide substitutions have occurred in the lineage of old-world monkeys than in hominoids. These results may suggest that the rate of nucleotide substitution for mtDNA has slowed down in hominines compared with that for old-world monkeys. This evolutionary feature of mitochondrial genes is similar to one found in

nuclear genes.
For details, see *Mol. Biol. Evol.* **5**: 626–644, 1988.

Maternal Inheritance of Deleted Mitochondrial DNA in a Family with Mitochondrial Myopathy

Takayuki OZAWA*, Makoto YONEDA*, Masashi TANAKA*,
Ikuya NONAKA** and Satoshi HORAI

Skeletal muscles from a mother and her daughter, both with chronic progressive ophthalmoplegia, were analyzed. Histological and biochemical analyses of their muscle samples showed typical features of this type of mitochondrial myopathy. Southern blot anlysis revealed that, in both patients, there were two species of mitochondrial DNA (mtDNA): a normal one and a partially deleted one. The sizes of the deletion were different; the mutant mtDNAs from the mother and the daughter had about 2.5- and 5-kilobase deletions, respectively. The two mutant mtDNAs shared a common deleted region of 1.2-kilobases. However, both the start and the end of deletion were different between them, implying a novel mode of inheritance. This is the first report that mutant mtDNA is responsible for the maternal inheritance of a human disease. For details, see *Biochem. Biophy. Res. Commun.* **154**(3): 1240–1247.

A Mutation-Epidemiologic Study on Sporadic Retinoblastoma

Ei MATSUNAGA and Kensei MINODA***

Whereas our knowledge about the molecular basis of genetics and genesis of retinoblastoma has increased tremendously during the past years, few epidemiologic studies have been conducted to investigate possible environmental factors associated with germinal or somatic mutations leading to heritable or nonheritable retinoblastoma. Obviously, the rarity of this disease makes it difficult to collect a sufficiently large number of sporadic bilateral or unilateral cases to provide an epidemiologically sound conclusion. Of particular interest are the possibilities of a viral etiology

* Department of Biomedical Chemistry, Faculty of Medicine, University of Nagoya, Nagoya.
** National Institute of Neuroscience, Tokyo.
*** Teikyo University Ichihara Hospital.

for the somatic mutations and a paternal age effect upon the germinal mutations. A viral etiology has been suggested from time to time but has never been tested by epidemiologic studies or by an examination of patients for antiviral antibodies. A paternal age effect may suggest an accumulated and overall effect of various kinds of environmental mutagens.

Based on medical records of more than one thousand patients with retinoblastoma as ascertained by a nationwide registry, we were able to collect data concerning dates of births for 753 sporadic unilateral cases; about 90% of them can be assumed to be due to somatic mutations which occurred some time during the fetal or early postnatal periods. These children were born in the period from 1965 to 1982, and the control was constructed on the basis of the vital statistics data for all Japan, adjusted by the year of births of the patients. The frequency of these cases fluctuates to some extent by months of births, but there was no statistically significant deviations from the control. Thus, the occurrence of non-heritable retinoblastoma is not likely to be associated with certain viruses such as human adenovirus 12 whose activity varies markedly with season.

We also analyzed the paternal ages for 225 patients with sporadic bilateral retinoblastoma, which can be assumed to have arisen from germinal mutations. These patients were born in the periods from 1965 to 1968 and from 1975 to 1982, for which the vital statistics data provided paternal age distributions for all legitimate live-births in Japan. The mean paternal age of the patients was 30.2 years, which was close to the 30.1 for the control population as adjusted by the year of births of the patients. Thus, there was no evidence for a paternal age effect. This finding may be due, in part, to a diminishing variance in parental ages during the last 30 years in this country. However, it does suggest that paternal exposure to ionizing radiation or chemical mutagens does not play a major role in the production of germinal mutations for retinoblastoma.

In conclusion, our epidemiologic survey based on a large body of data failed to catch any clue for environmental risk factors in sporadic retinoblastoma. The negative results, together with fairly uniform pattern in the incidence of this tumor among different populations (Parkin et al. 1988, *Int. J. Cancer* **42**: 511), suggest that most, if not all, cases of sporadic retinoblastoma are caused by some intrinsic biological mechanisms common to all individuals, and not by mutagens found in our daily environment that are variable from place to place and from time to time.

Ethnic Differences in the Incidence of Childhood Malignancies: Comparison of Japanese and U. S. Data

Ei MATSUNAGA and Hirokazu NISHIHIRA*

It is generally accepted that the first step in the carcinogenic process is a genic or chromosomal mutation in a somatic cell. In fact, in a number of malignancies, specific cellular changes have recently been identified at the chromosomal or DNA level. In order to search for genetic and/or environmental factors that are responsible for the induction of these mutations, it is imperative to know the incidence of individual malignancies in the general population. This, however, is not always easy. In Kanagawa Prefecture, with a population of nearly 7 million, a new approach to childhood cancer registry was initiated in 1972 by making use of an official record. This was made possible because since 1971, childhood malignancies have been among a number of "designated diseases" for which the cost of medical treatment is defrayed, upon application, by central and local governments. By reviewing the application forms submitted by patients to the prefectural government, it was possible to achieve nearly complete ascertainment of new cases of childhood malignancies within the prefecture.

Table 1. Incidence rates (per million per year) of malignant neoplasms in children under 15 years of age. U.S. data are from Young and Miller (1975).

Diagnosis	Kanagawa (1975–'82)	U.S. (1969–'71)	
		White	Black
Leukemia	39.7	42.1	24.3
Lymphoma	7.2	13.2	13.9
Brain tumors	24.4	23.9	23.9
Retinoblastoma	4.2	3.4	3.0
Wilms' tumors	3.5	7.6	7.8
Neuroblastoma	8.3	9.6	7.0
Liver tumors	2.1	1.9	0.4
Bone tumors	4.7	5.6	4.8
Testis tumors	2.1	1.0	0.4
Soft tissue sarcomas	3.3	8.4	3.9
Others	8.0	7.8	8.4
Total	107.5	124.5	97.8

* Kanagawa Children's Medical Center

Analysis was made of data (published by Nishihira in Japanese in a local journal) concerning new cases of malignancies in children under 15 years of age for which the diagnosis was first made within the respective years from 1975–'82. The number of all malignancies that occurred in each year ranged from 168 to 196, with the ratio of male to female patients being 1.30. As shown in Table 1, the most frequent malignancy was acute leukemia, followed by brain tumors, neuroblastoma, lymphoma, bone tumors, retinoblastoma, Wilms' tumors, soft tissue sarcomas, liver tumors and testis tumors. Compared with U.S. data for white and black children (Young, J. L. and R. W. Miller, 1975, *J. Pediat.* **86**: 254), no ethnic difference can be seen in the incidence rates for brain tumors, retinoblastoma, neuroblastoma, and bone tumors. Leukemia is as common among Japanese as in U.S. white children but in blacks it is less common. The rates for lymphoma and Wilms' tumors in Japan are about one-half that in U.S. white and black children. The rate for liver tumors in Japan is close to that in U.S. white children, but in U.S. black children it is much lower. The rate for testis tumors is the highest in Japan and it is lowest in U.S. black children. Soft tissue sarcomas seem as common in Japan as in U.S. black children but it is much more common in U.S. white children. The overall incidence rate was 107.5 in Japanese children, which is intermediate between U.S. white (124.5) and black children (97.8). These differences

Table 2. Incidence rates (per million per year) of lymphoma and soft tissue sarcoma in children under 15 years of age. U.S. data are from Young and Miller (1975).

	Japan (1975–'82)	U.S. (1969–71)	
		White	Black
Lymphoma	7.2	13.2	13.9
Lymphosarcoma	2.6	4.7	5.2
Reticulosarcoma	1.2	0.8	1.3
Hodgkin's disease	0.8	5.8	6.1
Malignant lymphomas*	2.7	1.1	0.4
Soft tissue sarcomas	3.3	8.4	3.9
Rhabdomyosarcoma	2.0	4.5	1.3
Fibrosarcoma	0.2	0.9	
Hemangiosarcoma	0.2	0.1	0.4
Others c. unspecified	0.9	2.9	2.2

* Not otherwise specified.

Table 3. Incidence rates (per million per year) of retinoblastoma and Wilms' tumor by laterality in children under 15 years of age.

	Japan (1975–'82)	U.S. white (1969–'71)
Retinoblastoma	4.2	3.4
Bilateral	1.4	1.2
Unilateral	2.8	2.2
Wilms' tumor	3.5	7.6
Bilateral	0.1	0.4
Unilateral	3.4	7.2

appear to be largely genetic.

With regard to lymphoma and soft tissue sarcomas, it is interesting to investigate which subcategories differ in incidence among the three ethnic groups. Because our original data did not provide information about the histological classification of tumors, we estimated the incidence of respective subcategories according to the relative frequencies in the All Japan Children's Cancer Registration (Vol. II, edited by N. Kobayashi, 1982). As shown in Table 2, Hodgkin's disease is rare in Japan; its incidence is about 1/7 that in U.S. white or black children, and lymphosarcoma also appears less common in Japan. Regarding soft tissue sarcomas, both rhabdomyosarcoma and fibrosarcoma appear less common in Japanese than in U.S. white children.

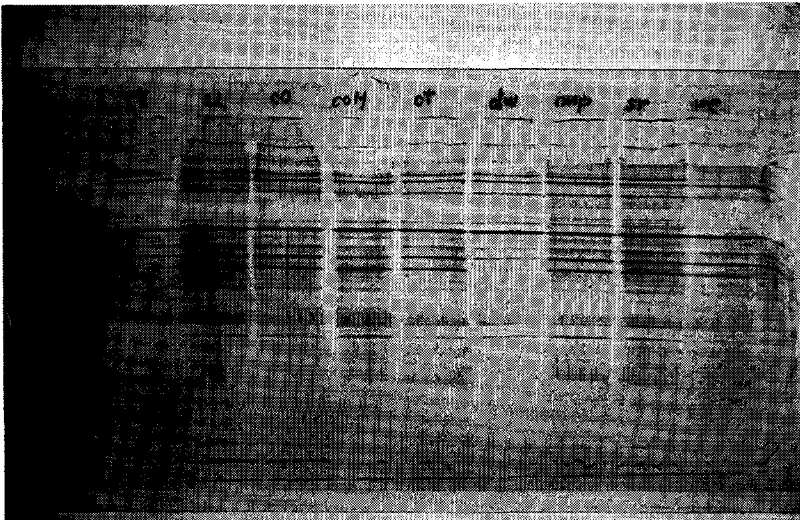
It is well known that for sporadic retinoblastoma or Wilms' tumors, bilateral cases almost always arise from *de novo* germinal mutation, while most unilateral cases are due to somatic mutations. For sporadic cases of retinoblastoma, about 1/3 are bilaterally affected and there are no ethnic differences in this respect. For Wilms' tumor, the proportion of bilateral cases is about 3% in Japan (Hanawa, Y., 1988, *Gann Monog.* **36**: 71) and about 5% in the U.S. (Breslow, N. E. and Beckwith, J. B., 1982, *JNCI* **68**: 429). We partitioned the overall incidence rates of both tumors into rates for bilateral and unilateral cases respectively and compared them between Japanese and U.S. white children (Table 3). With respect to retinoblastoma locus, there are no ethnic differences in the germinal or somatic mutation rate, but for Wilms' tumor locus both germinal and somatic mutations appear to occur more frequently in U.S. white children than in Japanese children. This seems to be the first case in human genetics suggesting an ethnic difference in mutation rates.

X. APPLIED GENETICS

Protein Bands in Morphological Mutants of Japanese Morning Glory Detected by Electrophoresis in Immobilized pH Gradients

Toru ENDO

A number of horticulturally valuable morphological mutants in Japanese morning glory have been recognized since 16th century. Several loci of these mutants were used in the present study, acuminate (*ac*), cordate (*co*), Hederacea (co^{H}), dwarf (*dw*), maple (*m*), polymorph (*py*), side-reduced (*sr*), weeping (*we*) and a standard type, Tokyo-Kokei (TK), with the first six mutants being detectable at the cotyledonary stage. They were successively backcrossed with TK for at least three generations, and semi-isolines of them were obtained. Dry seeds of each line were crushed to 100 mesh powder, 200 mg of which was washed with 100 ml of a mixture of ethoxyethanol/2-mercaptoethanol, 4:1 (v/v), to remove polysaccharides. This pretreatment was essential to apply the seed protein to electrophoresis with immobilized pH gradients (IPG). After pretreatment, the seed protein



was extracted with a 100 ml buffer of 10 mM ammonium carbonate containing 0.1 M 2-mercaptoethanol (pH 8.6), freezed dried, dissolved in 1 ml of 8 M urea and then 20 μl of the suppernatants was analysed. The regions of IPG used were a wide type, pH 4–10, as well as narrow types, pH 4–5, pH 5–6 and pH 6–7. The former showed about fifty bands and each of the latter three about thirty, with most of them extremely fine. Bands were inclined to become wider or more obscure in regions with pH 7–8 or pH 8–9. In the photograph shown (pH 6–7 region), the dominant mutant strain of co^{H} showed a unique band near pH 6.7, which was not located in the other other strains.

Distribution of *Wx* Alleles in Rice

Yoshio SANO

The *waxy* (*wx*) locus specifies a starch-bound glucosyl transferase that is responsible for the production of amylose in the endosperm. Amylose content in the endosperm affects grain quality and it varies among nonglutinous rice cultivars. As reported already, multiple alleles at the *wx* locus occur in rice. Wx^{a} and Wx^{b} as well as *wx* were found in Asian cultivars (*Oryza sativa*). Wx^{a} and Wx^{b} control the quantitative level of both amylose and the gene product (called *Wx* protein) in the endosperm, indicating that the allelic difference is a major factor regulating amylose content among nonglutinous cultivars. In addition, some cultivars showed an intermediate amount of both amylose and *Wx* protein between Wx^{a} and Wx^{b}. Backcrossing experiments using these cultivars revealed that an additional *Wx* allele contributes to an amount of amylose and *Wx* protein intermediate to that observed in the original strains. Thus, the *wx* locus gives an opportunitiy to look into the evolutionary significance of gene regulation and dynamic changes of allelic frequency in response to farmers' selection of cultivated rice.

Modern rice cultivars in Japan mostly carry Wx^{b} while all wild relatives with the AA genome carry Wx^{a} only. Indica type rice predominantly carries Wx^{a}, showing a higher amylose content as do its wild relatives. The intermediate allele between Wx^{a} and Wx^{b} seems to be frequent in Javanica type rice as recognized by the quantitative level of the *Wx* protein. Such an intermediate allele was not found in five wild species (66 strains surveyed in total). All allelic states except for Wx^{a} seem to be specific to

Asian cultivars. The distribution of *Wx* alleles was surveyed in traditional old cultivars from southern parts of Japan (16 from Kyushu and 20 from Okinawa) in addition to 8 weedy strains from Korea and Japan. Results indicated that Wx^a was not detected in those from Kyushu but was frequently detected in those from Okinawa and the weedy strains. In contrast, traditional cultivars from Kyushu frequently carried an intermediate allele as well as Wx^b, suggesting that the fixation of Wx^b took place recently in modern Japanese cultivars after the elimination of Wx^a. Wx^a itself appeared to have no adverse effect, since weedy strains often carried Wx^a. Thus alleic changes might have been brought about through artificial selection by farmers.

Molecular Cloning of Ribosomal RNA Genes from Two Cultivated Rice Species, *Oryza sativa* and *O. glaberrima*

Yoshio SANO

Ribosomal RNA (rRNA) genes are organized as families of tandemly repeated genes, which may comprise the nucleolar organizer (Nor) regions. The intergenic spacer region between rRNA coding sequences shows extensive sequence divergence suggesting that the intergenic spacer region might have rapidly evolved. In rice, differential expression of rRNA genes have been suggested since the number of Nor regions varies among different species or taxa. Therefore, the intergenic spacer region might be useful for reexamining complex relationships among rice species. In order to compare the molecular organizations of rRNA genes, the repeat unit was cloned from two cultivated rice species; *Oryza sativa* and *O. glaberrima.* Two strains, Taichung 65 (Japonica type of *O. sativa*) and W025 (*O. glaberrima*) were used for cloning of rDNA repeats. Taichung 65 carried homogeneous rDNA repeats of 8.3 kb, and W025 homogeneous repeats of 7.95 kb. BamHI cleavage yields two fragments of DNA from each rDNA repeat unit as detected by southernblotting hybridization. Although a BamHI fragment of 3.8 kb was constant in size, the other fragment which included the intergenic spacer region was highly variable among species. Taichung 65 had a 4.5 kb BamHI fragment of DNA while W025 had a 4.15 kb fragment in addition to an invariable fragment of 3.8 kb.

DNA was isolated from 10-day etiolated seedlings by phenol-chloroform

extraction and was banded in a CsCl-ethidium bromide gradient. Purified DNA was partially digested with Sau3A, then 15–20 kb fragments of DNA were separated in a sucrose gradient and ligated to λEMBL4 DNA cleaved with EcoRI and BamHI. After *in vitro* packaging, recombinant phages were screened by plaque hybridization of ^{32}P labelled RNAs (a mixture of 17S RNAs and 25S RNAs from rice seedlings). Then, rDNA repeats were subcloned from the λEMBL4 genomic clones into the BamHI site of pUC13. Finally, four plasmids, pRY12, pRY18, pRY42 and pRY44 were obtained, pRY12 and pRY18 carry a 4.5 kb insert from Taichung 65 and a 4.15 kb insert from W025, respectively, which contain the intergenic spacer region. pRY18 and pRY44 carry invariable fragments of 3.8 kb from the two species. The intergenic spacer region in pRY12 and pRY42 was extensively compared by means of a series of single and double digests using KpnI, XbaI, SmaI and SalI since the coding region seemed to be highly conservative. Further, TaqI, SmaI, SalI and HinfI sites were mapped by partial digests of end-labeled DNA. Partial digests of end-labeled DNA revealed that HinfI and SalI sites greatly differed in the middle of the intergenic spacer region between the two species. Thus, comparisons of the intergenic spacer region might be useful for reevaluation of species' relation ships at the molecular level.

Ribosomal DNA Spacer-Length Polymorphisms in a Wild Rice Population from Dongxiang, China

Yoshio SANO, Haoxiong X. YI[1], Qiqan Q. SHAO[2] and Shinya IYAMA

The northernmost population of wild rice (*Oryza rufipogon*) occurs in Dongxiang (28.1°N), Jiangxi, China. The plants are perennial and give fertile progenies when crossed with cultivars. Interestingly, they survive temperatures below 0°C in winter and regrow the following season. In order to compare their genetic constitution with those from tropical and subtropical areas, we examined rDNA spacer-length (sl) variations in the Dongxiang population.

Southernblotting analysis revealed that the intergenic spacer is highly variable in size and is differentiated among morphological and geographical groups of wild and cultivated rices, indicating its rapid change in evolution.

[1] Jiangxi Univ., Nanchang, Jiangxi, China.
[2] Inst. Genet., Beijing, China.

Among mouy sl variants detected so far, two (4.5 kb and 5.0 kb BamHI fragments) were found to predominate in Japonica but not in Indica rice. The two variants were seldom found in wild rice strains from tropical and subtropical areas, but they frequently occurred in the Dongxiang population. No difference was observed in the subrepeat structure within the intergenic spacer between Dongxiang wild rice and Japonica, suggesting that they carry rDNA of the same origin.

Selection Strategies Based on Two Different Measurements of Selection Efficiency, Expected Genetic Advance and Chance of Success

Katsuei YONEZAWA

Selection methodology in plant breeding has been discussed in terms of the genetic advance expected to be attained by selection and a selection method (selection index, field design, etc.) expected to give the largest genetic advance has been regarded as the most efficient. The size of the genetic advance, however, is not always the most appropriate criterion for measuring the efficiency of selection. However, a selection method which does not produce the greatest genetic advance may provide the largest chance of success of obtaining genotypes with sufficient desirability as a new cultivar. The chance of success rather than the expected genetic gain may fit the breeder's concern in many situations, especially in breeding of self-pollinating crops. The optimal selection strategy varies according to whether efficiency of selection is measured by expected genetic advance or chance of obtaining desirable genotypes.

As an example, a very simple modelled population as presented in Table 1 was assumed to be a target population in which selection is carried out. Now suppose that N plants of this population are grown and assessed for

Table 1. Genetic constitution of a target population for selection.

	Genotype	
	Desirable	Undesirable
Expected frequency	f	$1-f$
Genotypic value	1	0
Population mean	$f\cdot 1+(1-f)\cdot 0=f$	

selection, with plants showing phenotypic value (assessment scores) rated as desirable as or more desirable than a critical value, being selected. When the amount of resources such as time and labour is limited, as usually is the case in practical breeding projects, the number of plants triable, N, should be small if an assessment with high precision is applied. Introducing the following variables,

p_d=the probability that a desirable genotype is selected, i.e., shows a phenotypic value (assessment score) surpassing the critical value for selection,

p_u=the probability that an undesirable genotype is selected due to mistaken assessment,

and assuming that genetic segregation does not occur in the next generation (as assumable for a genetically fixed population of a self-pollinating crop), the expected genetic advance, which for simplicity is expressed here by the average genetic value of the selected plants, is formulated as,

$$M=\sum_{i=0}^{N}\binom{N}{i}f^i(1-f)^{N-i}\left(\frac{i}{N}p_d\right)\Big/\left(\frac{i}{N}p_d+\frac{N-i}{N}p_u\right)$$

The chance of success, i.e., the probability that at least one desirable genotype is obtained by the selection, is presented as,

$$Pr=1-\sum_{i}\binom{N}{i}f^i(1-f)^{N-i}(1-p_d)^i$$

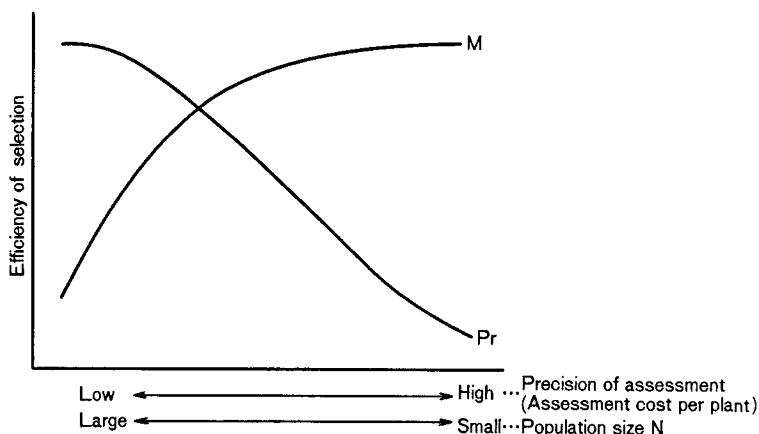


Fig. 1. Relationship between efficiency of selection and precision of assessment with a fixed amount of resources expendable for selection.

The probabilities p_d and p_u were formulated, though not presented here, in terms of the precision of assessment of plants. The probability p_d becomes larger, and in contrast, p_u becomes smaller, as the precision of the plant assessment gets higher. Assessments with a higher precision, require greater expense of resources.

For many probable situations, relationships, as shown in Fig. 1, between the precision of assessment and the efficiency of selection as measured by *M* and *Pr* were determined. *M* incerases, while in contrast, *Pr* decreases with the increasing precision of the assessment. Thus, when measured by *M*, an assessment with high precision, i.e., high-input assessment, produces a better result. When measured by *Pr*, an assessment with a rather simple method, i.e., low-input assessment, is recommended.

XI. DATABASE

Activities of the DNA Data Bank of Japan

Sanzo MIYAZAWA and Hidenori HAYASHIDA

The primary task of the DDBJ is, of course, DNA sequence collection. However, in addition to that, we have a wide range of activities; 1) DNA data collection and data entry in collaboration with other databanks, 2) data distribution, including the secondary distribution of the GenBank and EMBL databases in Japan, 3) to provide on-line access to DNA and related databases, 4) to develop research tools for sequence analysis, 5) to regularly publish newsletters to inform people of the activities of the DDBJ, and 6) to provide training courses for users of the DDBJ computer system. We have been developing a data entry system to manage data collection, a search/retrieval system for sequence databases, and research tools for DNA and protein information analysis. To let people know of such activities of the DDBJ, we published newsletter No. 7, and had a training course in 1988. Newsletters contained articles that described the state of international collaboration among databanks and how to submit data to databanks as well as matters of interest specific for Japanese scientists such as available databases at the DDBJ, how to access the DDBJ computer system and how to use the databases. The activities of the DDBJ are also conveyed through the on-line service of the DDBJ computer system. All these activities provided by the DDBJ are open to anybody irrespective of whether one works for a non-profit organization or not. In the following, I will briefly report this year's activities of the DDBJ.

1) Data Entry and Management

Sanzo MIYAZAWA and Hidenori HAYASHIDA

Our data collection began in December, 1986 and is carried out in collaboration with the GenBank and the EMBL Data Library. The collaboration includes projects of designing a new feature table and rebuilding a DNA database. Data is currently entered in the GenBank format and fully annotated. Since we released the first version of the DDBJ database in July, 1987, our database has been released every half year; version 3, which included 345,850 bases in 230 entries, was released in July, 1988, and version

4 including 535,985 bases in 302 entries was released in January, 1989. The DDBJ collected about 240,000 bases in the year from July 1987 to July 1988. About 8,000,000 bases were collected during the same period by the EMBL Data Library and the GenBank. In other words, the DDBJ processed about 1/30 of the total collection of DNA sequences in a year; this number may be reasonable, if the number of staff of the DDBJ is compared with those of GenBank and the EMBL Data Library. Each release included a coding sequence database and a peptide sequence database that were extracted and translated from the original DNA sequence database. Release 2 and later releases included the files of journal index, accession number index, short directory, and data submission form.

2) FLAT Database and Sequence Analysis System for DNA and Proteins: Release 1.0 β. Sanzo MIYAZAWA

We have been developing a search and retrieval system for flat file databases in order to provide simple tools for using DNA and protein sequence databases. This system called FLAT consists of primitives, most of which perform a single operation and work as filters in the UNIX system; a filter program reads a line from standard input, processes it and then writes some output into standard output. Some basic commands available in FLAT perform single operations such as 1) extracting specified types of records from database files, 2) searching for strings in each entry of database and, if found, outputing those entry names, 3) performing "and", "or", and "xor" in respect of entry names, and 4) extracting specified entries from database files. These filters may be combined with the UNIX pipe to perform complicated jobs; one may search and retrieve entries from databases by key words such as author name, journal name, title, organism name, source name, and any combination of such items. This is a typical approach for designing programs in the UNIX system. Strings for these programs are specified in the regular expression, so that one can search and retrieve entries in databases by fuzzy key words and entry names. The "seqgrep" program also allows users to use the regular expression to specify sequence patterns to be searched for in databases. Some of these filters were programmed in the Bourne shell and use UNIX tools such as sed, egrep, sort, and awk, so that they are flexible enough to support many formats of databases and to easily keep up with format changes which often occur. At present, the Genbank, EMBL, PIR (Protein Identification Resource) and PRF (Protein Research Foundation) data formats are sup-

ported. However, this approach tends to trade computational speed for flexibility, and so applications whose processing speed is critical are written in the C language. A program "getgb", which extracts specified entries from databases, uses a pseud index file to quickly find the location of entries in a flat database file. This FLAT search/retrieval system for sequence databases is designed to be portable among UNIX systems which are available for a wide range of computers from super to micro computers. Its β version of release 1.0 was released in 1988.

3) The Qanalys Sequence Analysis System for Molecular Evolution: Version 1.0 β Hidenori HAYASHIDA

Programs for sequence analysis were developed to study molecular evolution, including programs for the calculations of base or amino acid composition and similarity between sequences, a sequence alignment program, and a display program for postprocessing. The β version of release 1.0 was released in 1988.

4) A Guide to the DDBJ Computer System: the "getinfo" Command
Sanzo MIYAZAWA

An online help program called "getinfo" was made to provide databank staff and users an easy way to get necessary information. One may use the "getinfo" to learn how to submit DNA data and to which databank the data should be submitted, and even to get a data submission form. The "getinfo" apparently mimics the help utility of the VAX/VMS system. However, unlike the VMS help utility, each help information is stored as a flat file and organized into a tree-like structure, if necessary, by using symbolic links or pseud symbolic links. The pseud symbolic link was devised because the symbolic link is not available in the System V UNIX. The "getinfo" displays a specific topic and, if available, a list of help items at the next level and prompts users to choose one of them. A pager program, pg or less, which is available in the UNIX system is used to print files on a terminal, so that one may read a file page by page, and may save it in a file, if necessary. A hard-copy version of the guide to the DDBJ computer system was published in 1988.

For details, see "DNA Data Bank of Japan: present status and future plans" by Sanzo Miyazawa in "*The interface between Computational Science and Nucleic Acid Sequencing, Santa Fe Institute Studies in the Sciences of Complexity*, Eds. G. Bell and T. Marr (Reading, MA: Addison-Wesley), vol. VIII, 1989".

Estimation of the Average Energy Increments of Protein Native Structures due to Amino Acid Exchanges and Its Use in Evaluating a Substitution Probability Matrix for Homology Search

S. MIYAZAWA and R. L. JERNIGAN*

The energy change due to an amino acid exchange in protein structures was estimated on average, and used to evaluate a substitution probability matirix, which was then used to measure the similarity between protein sequences. In a statistical sense, each type of amino acid residue is found at a particular location in the three dimensional structure of proteins; non-polar residues are more often found in the non-polar environment of the protein core and polar residues on the protein surface. Residues surrounding an amino acid in protein structures are specific to the type of amino acid. We consider a typical or average protein which satisfies statistical features observed in a large set of protein structures, and the average energy increment due to an amino acid exchange in such an average protein. In a previous study (Miyazawa & Jernigan, 1985), we estimated the effective inter-residue contact energy of each type of amino acid pair for proteins in solution from 18,192 residue-residue contacts observed in 42 globular proteins, and also compiled the types of residues and their average numbers in contact with each type of amino acid. By using their contact energies and statistical data of surrounding residues, we evaluated the average energy increment due to an amino acid exchange in the average protein for each pair of the 20 kinds of amino acid residues. These estimates show reasonable characteristics of physico-chemical similarities of amino acids. The average energy increment due to an amino acid exchange would be a good measure of structural instability caused by an amino acid exchange. We assume that the fitness of an amino acid exchange in the evolutionaly process is proportional to the Boltzmann factor of the average energy increment due to the amino acid exchange. The substitution process of codons consists of two steps, mutation and selection at the DNA level, and selection at the protein level. The process of codon substitution is assumed to be in equilibrium and for simplicity its rate at the DNA level is assumed to be proportional to the equilibrium frequency of the codon. Thus a transition matrix of amino acid substitution for a long time interval which corresponds to 250 PAM (accepted point mutations per 100 residues) is generated from

* National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, USA.

that of codon substitution for a short time interval, and the log of each element of the 250 PAM matrix divided by the amino acid frequency is calculated as a scoring matrix in the same way as Dayhoff et al. (1978). The correlation coefficient between this scoring matrix and Dayhoff's scoring matrix (MDM_{78}), which corresponds to the 250 PAM substitution probability matrix calculated from amino acid substitutions observed in closely related proteins, is about 0.55 for all off-diagonals but 0.82 when infrequent amino acids of met, trp, cys and tyr are excluded. The poor correlation for all off-diagonals may result from statistical errors due to small numbers of substitutions. This scoring matrix has demonstrated the same degree of detection power of sequence homology as the Dayhoff's scoring matrix. These results indicate that the average energy increments due to an amino acid exchange as estimated here reflect the structural instability caused by the amino acid exchange. This manuscript is in preparation.

Publications from Genetic Resources Section, Genetic Stocks Reseach Center

Shin-ya IYAMA

The following publications were released from the Genetic Resources Section this year.

1. "Silkworm Strains in Japan". (Compiled by S. Iyama, H. Doira and A. Murakami) 181 pages (in Japanese). This was completed in cooperation with the Silkworm Genetic Resources Subcommittee, the Japanese Society of Sericulture. The catalogue listed 943 entries from 18 locations in Japan, with special emphasis on experimental strains. Each stock was described with its strain name, location kept, genotype, historical record of establishment and acquisition, genetic characteristics, and information on maintainance and distribution. A list of gene symbols with the description of their characteristics was added for the convenience of research workers in using materials.

2. "Rice Genetics Newsletter Vol. 5, 1988". 162 pages. (in English). This volume contained the following:

(1) Report of the Committee on Gene Symbolization, Nomenclature and Linkage Groups, describing 8 newly registered genes, 13 newly adopted gene symbols and 16 marker genes newly confirmed for their linkage relations.

(2) Two reports from coordinators describing 1) current status of analysis and symbols for male sterile cytoplasms and fertility-restoring genes and 2) gene symbols for leaf and culm traits.

(3) List of 73 recently published papers on rice genetics.

(4) 60 research notes of various fields of rice genetics, the first five of which concerned chromosome studies related to the chromosome numbering for linkage groups.

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ABSTRACTS OF DIARY FOR 1988

Biological Symposium

269th	meeting	Mar. 18	Restriction endonucleases for pulsed field mapping of chromosomes (Michael McClelland)
270th	—	Apr. 26	Translational control by phosphorylation of mammalian initiation factors (John W. B. Hershey)
271st	—	Apr. 27	Molecular genetics in mouse development (William F. Dove)
		Apr. 27	Molecular genetics of the t-region of the mouse genome (Alexandra Shedlovsky)
272nd	—	May 26	How sick are we genetically-speaking? Genetic and partially-genetic diseases in man (K. Sankaranarayanan)
273rd	—	May 27	Mouse gene mapping using R.F.L.P.'s: recent data (Guénet Jean-Louis)
274th	—	May 30	Mammalian X-chromosome: Some comparative aspects (James R. Miller)
275th	—	July 15	Recent developments and insight in the anti-HIV activity and metabolism of novel purine and pyrimidine nucleoside analogues (Ir. Jan Balzarini)
276th	—	Aug. 11	Mutation affecting the extra chromosomal replication of the *Tetrahymena* ribosomal RNA gene (E. Orias)
277th	—	Oct. 17	Algal/hydra symbioses—a model for host-symbiont interactions (Menashem Rahat)
278th	—	Oct. 19	Nucleic acid databases—Vision for the future (James C. Cassatt)
279th	—	Oct. 24	EMBNet: Network for molecular biology in Europe (Chris Sander)
280th	—	Oct. 31	Neurons and neuropeptides in hydra and other coelenterates

			(C. J. P. Grimmelikhuijzen)
281st	—	Nov. 1	Identification of a novel *E. coli* sigma factor involved in high temperature gene expression (James W. Erickson)
282nd	—	Nov. 25	Antimutagenesis in microbial systems (Delbert M. Shankel)
283rd	—	Dec. 12	Col El DNA replication (Jun-ichi Tomizawa)
284th	—	Dec. 14	Regulation of ribosome synthesis in *E. coli*: translational regulation, retroregulation, feedback loops and cooperativity in assembly (Masayasu Nomura)
285th	—	Dec. 25	Statistical analysis of DNA sequences (Kotoko Nakada)
286th	—	Dec. 28	Molecular basis of mutagenesis (Laurence A. Loeb)

Mishima Geneticists' Club

333rd	meeting	Jan. 6	Function of *env*-related leukemogenic gene encoded by Friend spleen focus-forming virus (Yoji Ikawa)
334th	—	Jan. 8	Interactions between repressor and operator (Akira Sarai)
335th	—	Jan. 14	Molecular genetics of urea biosynthesis (Masataka Mori)
336th	—	Jan. 20	Gene regulation and evolution of highly repetitive and transcribable sequence in the salmon genome (Kenichi Matsumoto)
337th	—	Feb. 22	Expression of the storage-protein gene of soybean in transgenic petunia (Ikuo Nakamura)
338th	—	Apr. 13	Gene regulation in teratocarcinoma stem cells: Analysis using recombinant retrovirus (Makoto Taketo)
339th	—	Apr. 22	Somatic cell genetic study on the mammalian thymidylate synthase gene (Dai Ayusawa)
340th	—	Apr. 30	Study on the structure and function of human thymidylate synthase gene (Sumiko Kaneda)
341st	—	May 9	Purification of a transcription factor for

			immunoglobulin genes and transcriptional activation from HIV promoters (Kiyoshi Kawakami)
342nd	—	May 9	New insight into mechanisms of DNA enzymes (Samuel Wilson)
343rd	—	May 25	Mechanisms of speciation in historical perspective (William B. Provine)
344th	—	May 31	Intracellar concentration and cooperative binding of single-stranded DNA binding protein (Nobuo Shimamoto)
345th	—	July 25	Chromosomal recombination of eukaryote (Kenzo Sakaguchi)
346th	—	Oct. 14	Control of ribosome synthesis in *E. coli* and *S. pombe* (Masahiro Yamagishi)
347th	—	Oct. 19	Studies on transposon-like elements and repetitive sequences in plant genomes (Hiroyuki Hirano)
348th	—	Oct. 19	Low temperature stress and gene expression in rice (Yasunori Ban)
349th	—	Oct. 25	Molecular evolutionary analysis of *Drosophila* genes (Etsuko Moriyama)
350th	—	Nov. 26	Regulation of transcription by cAMP (Koji Aeba)

FOREIGN VISITORS IN 1988

April 9, 1984–	Pascale Barbier, Université des Sciences et Techniques du Languedoc, Montpéllier, France
April 1, 1987–March 31, 1988	Dong Sang Suh, Genetic Engineering Center, Korea Advanced Institute of Technology, Korea
August 4, 1987–	Ling Hua Tang, Institute of Food Crops, Jiangsu Academy of Agricultural Sciences, China
September 1, 1987–February 10, 1988	Sam-Eun Kim, Sericultural Experiment Station, Rural Development Administration, Korea
December 4, 1987–	Xiao Mei Wu, Lanzhou Institute of Biological Product, Ministry of Public Health, China
December 4, 1987–	He Zhao, Lanzhou Institute of Biological Product, Ministry of Public Health, China
February 20, 1988	Song Wang, Animal Research Institute, Chinese Academy of Sciences, China
March 18–19	Michael McClelland, University of Chicago, U.S.A.
April 14	Makoto Taketo, The Jackson Laboratory, U.S.A.
April 19	Debao Li, National Rice Research Institute, China
April 19	Zhingmin Xiong, National Rice Research Institute, China
April 19	Guowen Hu, National Rice Research Institute, China
April 19	Yi Du, National Rice Research Institute, China
April 19	Xiaoan Zhang, Ministry of Agriculture, Animal Husbandry and Fishery, China
April 26	John W. B. Harshey, University of California, U.S.A.
April 27	William F. Dove, University of Wisconsin, U.S.A.
April 27	Alexandra Sheldlovsky, University of Wisconsin, U.S.A.
May 2–30	William B. Provine, Cornell University, U.S.A.
May 9	Samuel Wilson, National Institutes of Health, U.S.A.
May 9	Kiyoshi Kawakami, The Rockefeller University, U.S.A.
May 25	K. Sankaranarayanan, State University of Leiden, The Netherlands

May 27	Jean-Louis Guénet, Institut Pasteur, France
May 30	James R. Miller, University of British Columbia, Canada
June 2	Kazutoshi Maeda, Wayne State University, U.S.A.
July 15	Ir. Jan Balzarini, Rega Institute, Belgium
August 11	Edward Orias, University of California, U.S.A.
September 2–December 20	Delbert M. Shankel, University of Kansas, U.S.A.
September 2	Marney Thone, CSIRO Division of Animal Production, Australia
September 9	Usha Goswami, National Institute of Oceanography, India
September 16–November 16	Menashem Rahat, The Hebrew University, Israel
September 17–19	James F. Crow, University of Wisconsin, U.S.A.
September 27–	Guan Cheng Sun, Sericultural Research Institute, Chinese Academy of Agricultural Sciences, China
October 14	Masahiro Yamagishi, University of California, U.S.A.
October 19	James C. Cassatt, National Institutes of Health, U.S.A.
October 24	Chris Sander, Europe Molecular Biology Laboratory, West Germany
October 31	C. J. P. Grimmelikhuijzen, Universität der Hamburg, West Germany
November 1	James W. Erickson, University of Wisconsin, U.S.A.
November 4	Amauri B. Simonetti, University of São Paulo, Brazil
November 14	Jin-ming Qian, Wenzhou Agricultural Research Institute, Zhejiang Province, China
November 14	Rolando Saborit Reyes, Rafael Sanzo Mancebo Rice Experimental Station, Cuba
November 14	Wagdy Mohamed Khalil Elswaidy, Agriculture Department, Dumiat Governorate, Egypt
November 14	Emmanuel Tetteh-Bio, Kpong Farms' Project, Ghana
November 14	Md. Jasin Bin Ab. Rahman, Muda Agricultural Development Authority, Malaysia
November 14	Agustin Antonio Martinez López, National Institute for Agricultural Research, Mexico
November 14	Rafiqullah Shah, Agricultural Research Institute,

	Pakistan
November 14	Saúl Antonio Orrego Puelles, Enterprise of Rice Commercialization, North-Coast, Peru
December 2	James F. Crow, University of Wisconsin, U.S.A.
December 2	W. G. Hill, Institute of Animal Genetics, Scotland
December 2	Charles Langley, National Institute of Environmental Health Sciences, U.S.A.
December 2	Masatoshi Nei, University of Texas, U.S.A.
December 2	Montgomery Slatkin, University of California, U.S.A.
December 2	Geoffrey A. Watterson, Monash University, Australia
December 2	B. S. Weir, North Carolina State University, U.S.A.
December 2	Shozo Yokoyama, University of Illinois, U.S.A.
December 7	Abraham Eisenstark, University of Missouri, U.S.A.
December 12	Jun-ichi Tomizawa, National Institutes of Health, U.S.A.
December 14	Masayasu Nomura, University of California, U.S.A.
December 24	Kotoko Nakada, National Cancer Institute, U.S.A.
December 28	Laurence A. Loeb, University of Washington, U.S.A.

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電　話　代表 (0559) (75) 0771

to Gotenba
Nihon Univ.
to Hakone
to Shizuoka
Mishima Station
New Tōkaido Line
Tōkaido Line
National Highway 1
to Tokyo
Rakujuen
Izuhakone Line
Mishima Shrine
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