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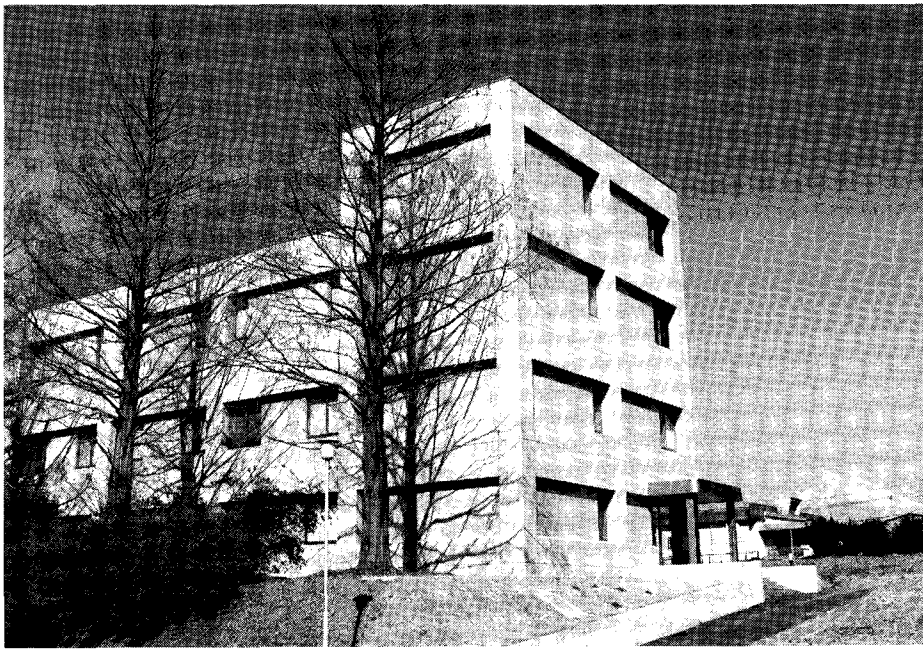

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CONTENTS

GENERAL STATEMENT

Our institute was established 47 years ago as a center for comprehensive genetics research. Outstanding accomplishments in the advancement of research in genetics, in particular population genetics, plant genetics and molecular genetics, by members of our institute have made it the premier center for genetic study in our country and a major institution with world-wide recognition. In 1984 the institute was reorganized into a National Inter-university Research Institute to promote research activities through cooperation with other institutions. Together with seven inter-university research institutes, we formed the Graduate University for Advanced Studies, in 1988. Our institute admits graduate students to the Department of Genetics of the Graduate School of Life Science. We have 27 such students at present and in addition, about ten special research students from other universities, including those from foreign countries. In addition, we have four post-doctoral fellows of the center of excellence program. I consider it vital for our research activities to have a steady flow of young scientists.

Recent rapid progress in the field of genetics using newly developed approaches has greatly shifted the course of study in biology. I consider it natural that research in our institute has recently focused more on molecular and cellular studies of various aspects of genetics, without ignoring the importance of more traditional approaches to genetics. Our institute is uniquely suited for pursuing cooperative work with scientists of various disciplines. Through interactions among various research disciplines, our institute will flourish.

We have been carrying out several research related services. The DNA Data Bank of Japan (DDBJ) is one of the three central banks in the world that gather, annotate, store and distribute information on DNA sequences. Our institute also stores and distributes various organisms with genetically characterized traits. Among them, services with mice, rice and *Escherichia coli* are particularly significant. These service activities will continue to develop in the coming years.

I hope that with guidance from people in and outside this institute and further support from governmental and private sources, I will be able to lead the institute into a more successful future.

The Center for Information Biology was established by members from the

DNA Research Center in 1995. This year the DNA Research Center was reorganized to form the Structural Biology Center with four laboratories. Nobuo Shimamoto, Molecular Biomechanism Laboratory, and Yuji Kohara, Gene Network Laboratory, were promoted to professor.

Dr. Takeshi Seno, the Vice-Director and the head of the Division of Mutagenesis, and Dr. Tsutomu Sugiyama, the Head of the Department of Ontogenetics, retired at the end of March. The past year also saw a number of changes in the staff of the institute. Promoted as associate professor, Hiroyuki Hirano was transferred to the University of Tokyo. The following twelve people joined us: as professor, Yasushi Hiromi in the Division of Developmental Genetics, Hideaki Sugawara in the newly established Laboratory of Molecular Classification in the Center for Information Biology; as associate professor, Nori Kurata in the Plant Genetics Laboratory. Hiroshi Mitsuzawa and Makoto Kimura joined the Division of Molecular Genetics, Tsutomu Ohta the Division of Cytogenetics, Hong-Wei Cai the Division of Agricultural Genetics, Toshihiko Akiba the Biomolecular Structure Laboratory, Motonori Ota the Laboratory for Gene-Product Informatics, Kaoru Fukami-Kobayashi the Gene Function Laboratory, and Satoru Miyazaki the Molecular Classification Laboratory, and Ken-ichi Nonomura the Experimental Farm.

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RESEARCH ACTIVITIES IN 1996

A. DEPARTMENT OF MOLECULAR GENETICS

A–a. Division of Molecular Genetics

(1) Molecular Anatomy of the Alpha Subunit C-terminal Domain of *Escherichia coli* RNA Polymerase: Fine Mapping of the Contact Surface with DNA UP Elements and Class-I Transcription Factors

Katsuhiko MURAKAMI, Irina ARTSIMOVITCH[1], Sudha A. CHUGANI[2], Philip E. BOUCHER[3], Yang TANG[4], Hilla GILADI[5], Martha M. HOWE[1], A. N. CHAKRABARTY[2], Scott STIBITZ[3], Pieter L. DEHASETH[4], Amos B. OPPENHEIM[5], and Akira ISHIHAMA ([1] Univ. Tennessee, Memphis, [2] Univ. Illinois, Chicago, [3] Center for Food and Drug Administration, Bethesda, [4] Case Western Reserve University, Cleveland, [5] Hebrew University-Hadassha Medical School, Jerusalem)

The carboxy-terminal one-third of *Escherichia coli* RNA polymerase α subunit plays a key role in transcription regulation by class-I transcription factors and DNA enhancer (UP) elements. The roles of individual amino acid residues within this regulatory domain of α subunit were examined after systematic mutagenesis of the putative contact regions (residues 258 to 275, and 297 to 298) for cAMP receptor protein (CRP). The reconstituted RNA polymerases containing the mutant α subunits were examined for their response to transcription activation by cAMP-CRP and the *rrnB*P1 UP element. Mutations affecting CRP responsiveness were located on the surface of the putative CRP contact helix and most of these mutations also influenced the response to the *rrnB* UP element. These observations raise a possibility that the CRP contact surface is also involved in contact with the DNA UP element, although some amino acid residues within this region play different roles in molecular communication with CRP and the UP element. Among the amino acid residues constituting the contact surface, Arg265 was

found to play a major role in response to both CRP and the DNA UP element. Judged by DNase I footprinting analysis, α mutants defective in transcription from the CRP-dependent *lac*P1 promoter showed decreased activity in the cooperative binding of CRP. Likewise, mutants defective in *rrnB*P1 transcription showed decreased binding to the UP element.

The same set of reconstituted mutant RNA polymerases containing Ala-substituted α subunit was used for mapping of the contact sites for phage Mu-encoded Mor protein for middle transcription (ref. 1), *Pseudomonas* CatR protein for transcriptin activation of the *cat* operon for catechol catabolism (ref. 4) and *Bordetella pertussis* BvgA for transcription of the virulence genes (ref. 3). Mor protein is a novel type of regulatory protein, which requires both α and σ^{70} function (ref. 1). For contact with the α subunit, Asp258 plays a key role and residues Leu262, Arg265 and Asn268 indirectly affect Mor-RNA polymerase interaction by stabilizing the ternary complex via α-DNA contact. On the other hand, the possible contact site for the CatR protein was identified to include Arg265 and Asn268 (ref. 4) and that for the BvgA protein was also mapped at Arg265 and Asn268 (ref. 3).

An DNA UP element defines a supplementary promoter element located upstream of the promoter-35 signal and stimulates transcription by interacting with the C-terminal domain of RNA polymerase α subunit. Both P_L and P_{RM} promoters of phage λ also carry UP elements even though the activities are lower than the ribosomal RNA (*rrnB*) gene UP element (ref. 7 and 29). Mutant α with Ala-substitution at Leu260, Leu262, Arg265 and Cys269 all gave severe effect on the P_L UP element recognition (ref. 29), while the activation of P_{RM} requires Leu260, Leu262, Arg265 and Asn268 (ref. 7). The effects of Ala substitutions of αCTD on the phage λ UP elements are essentially the same as that on the *rrnB* promoter (ref. 24).

(2) Molecular Anatomy of the Alpha Subunit C-terminal Domain of *Escherichia coli* RNA Polymerase: Evidence for the Direct Contact with DNA UP Elements and Class-I Transcription Factors

Katsuhiko MURAKAMI, Olga OZOLINE, Tomofumi NEGISHI, Nobuyuki FUJITA, Jeffrey T. OWENS[1], Claude F. MEARES[1], and Akira ISHIHAMA ([1] Univ. California, Davis)

The reactivity of both isolated α-subunit and α-subunit assembled in the RNA polymerase holoenzyme against a monomercury derivative of fluorescceine acetate (FMMA) was analyzed. In both cases, the largest reactivity was identified to display Cys-269, positioned in the regulatory helix of C-terminal domain (CTD), including the contact sites for both class-I transcription factors and DNA UP elements. Substitution of Ala for both Cys269 and Cys 176 completely eliminates the reactivity of α-subunit against the fluorescent dye, supporting the prediction that another reactive amino acid is Cys176 which is positioned within or near the region important for dimerization of α-subunit and its binding of β' subunit. Mutant α-subunits, bearing only one cysteine residue at either 269 or 176, could be reconstituted into locally modified and active enzymes which provide a tool suitable for studies of intra- and intermolecular interactions of this subunit.

The modified enzyme was used to investigate RNA polymerase interaction with different promoters, either containing or lacking an upstream enhancer element. Using a single-round transcription assay, it was shown that the presence of the label decreases the efficiency of RNA polymerase interaction with promoters with UP element such as *rrnB*P1 but not with many other without the UP elements. Interaction with the *rrnB*P1 UP element leads to substantial alterations in the spectral parameters of the reporter label, which are different from those induced by interaction with promoters without UP elements. A pronounced spectral blue shift suggests that the labeled surface of αCTD closely approaches the charged UP DNA helix. Thus we propose that Cys-269 is directly involved in the contact with the UP element of promoter DNA.

Taking advantage of the recently discovered polynucleotide and polypeptide hydrolysis reaction by iron chelete Fe-BABE[(*p*-bromoacetamidobenzyl)-EDTA·Fe], the chelate was tethered to the reactive Cys residue at

position 269 and used to map its proximity to DNA UP elements. Furthermore, in order to identify possible differences in the functional roles of the two α subunits, we have developed a reconstitution method for hybrid RNA polymerases containing two distinct α subunit derivatives in a defined orientation ("oriented α-heterodimer"). The binding sites of two α C-terminal domains on the UP element DNA were determined by hydroxyl radical-based DNA cleavage mediated by Fe·BABE which was bound at Cys-269 on the UP-recognition surface of one or both α subunits (ref. 25). The results clearly indicated that the two α subunits bind in tandem to two helix turns of the *rrnB*P1 UP element, and that the β'-associated α subunit is bound to the promoter-distal region.

(3) Regulation of Intracellular Levels of Sigma Subunits in *Escherichia coli*

Miki JISHAGE, Akira IWATA[1], Susume UEDA[1], and Akira ISHIHAMA ([1] Nippon Institute of Biological Science, Ohme)

Using a quantitative Western immnoblot analysis, the intracellular levels of two principal σ subunits, σ^{70} (σ^{D}, the *rpoD* gene product) and σ^{38} (σ^{S}, the *rpoS* gene product), and of two minor σ subunits, σ^{54} (σ^{N}, the *rpoN* gene product) and σ^{28} (σ^{F}, the *rpoF* gene product), were determined in two *Escherichia coli* strains, W3110 and MC4100 (ref. 16). The results indicated that: the levels of σ^{54} and σ^{28} are maintained at 10 and 50% the level of σ^{70} in both strains growing at both exponential and stationary phases; but in agreement with our previous measurement for strain MC4100, the level of σ^{38} was undetectable at the exponential growth phase but increased to 30% the level of σ^{70} at the stationary phase. Stress-coupled change in the intracellular level was observed for two σ subunits: the increase in σ^{38} level and the decrease in σ^{28} level upon exposure to heat shock at the exponential phase; and the increase in σ^{38} level under high osmolality conditions at both the exponential and the stationary phases.

In the course of these studies, we realized that heterogeneity exists among the stock strains of *Escherichia coli* K12 strain W3110 in Japan with respect to two σ subunits, σ^{28} (σ^{F}, the *rpoF* gene product) and σ^{38} (σ^{S}, the *rpoS* gene product) (ref. 17). Five different types of W3110 were identified: A-type lineages have both σ subunits in intact forms; B-type lineages carry truncated

σ^{38} subunit and intact σ^{28} subunit; C-type lineages carry intact σ^{28} subunit but lack σ^{38} subunit; D-type lineages have only σ^{38} subunit without σ^{28} subunit; and E-type stocks lack both σ subunits. All the lineages examined, however, contain the intact forms of σ^{70} (σ^{D}, the *rpoD* gene product) and σ^{54} (σ^{N}, the *rpoN* gene product). As expected from the lack of σ^{28} subunit, cells of D-type and E-type lineages are nonmotile. The truncated form of σ^{38} subunit in B-type stocks carries two mutations near its N-terminus and lacks the C-terminal proximal region 4 due to an amber mutation. The failure of C- and E-type W3110 cells to express σ^{38} and that of D- and E-type cells to express σ^{28} was found to be due to defects in transcription even though the respective σ subunit genes remain intact. These findings emphasize the importance of paying attention to possible variations in the genetic background between laboratory stocks originating from the same strain.

(4) Regulation of the Activity of Sigma Subunits from *Escherichia coli*

Shuichi KUSANO, Tapas KUNDU, Kan TANAKA[1], and Akira ISHIHAMA ([1] Univ. Tokyo, Inst. Cell. Mol. Biol.)

Minor σ subunits are induced under various stress conditions, including the stationary growth phase. Upon exposure to such environmental conditions, the intracellular conditions are also altered even though details are not known yet. In the stationary phase cells, for instance, there are accumulations of trehalose, glycine betaine, glycogen, polyphosphate and other components in cytoplasm. In addition, the structure of nucleoids changes as to reduce superhelicity.

The effect of trehalose on transcription *in vitro* was compared between two holoenzymes, Eσ^{70} and Eσ^{38}, which were reconstituted from purified core enzyme and either σ^{70} (the major σ at exponentially growing phase) or σ^{38} (the essential σ at stationary growth phase), respectively. The optimum trehalose concentration giving maximum transcription by Eσ^{38} was higher than that by Eσ^{70}, in good agreement with the increase in intracellular trehalose concentration under certain stress conditions where σ^{38} is produced. Transcription activation by trehalose was attributed to both enhanced σ^{38} binding to the core enzyme and increased transcription initiation by Eσ^{38} holoenzyme from σ^{38}-dependent promoters. The activation of Eσ^{38} by trehalose was additive with the transcription enhancement by decreased

superhelicity of template DNA prepared from stationary-phase cells. We thus propose that the selective activation of transcription by $E\sigma^{38}$ holoenzyme takes place in the presence of specific condition(s) and factor(s) present under stress conditions.

The *rpoF* gene of *E. coli* codes for σ^F (or σ^{28}) subunit of RNA polymerase which is involved in transcription of the flagellar class-III operons and the chemotaxis genes. (σ^F was overexpressed, purified to homogeneity, and compared with σ^{70}, the major σ in exponentially growing cells, with respect to the activity, specificity and response to changes in reaction conditions. The affinity of σ^F to core RNA polymerase (E) is higher than that of σ^F. In an *in vitro* transcription system, the holoenzyme containing σ^F ($E\sigma^F$) selectively transcribed the flagellar class-III and chemotaxis genes, all of which cannot be transcribed by $E\sigma^{70}$. This strict promoter recognition property is similar to those of other stress-response minor σ subunits such as σ^{32} but different from those of the principal σ subunits, σ^{70} and σ^{38}. The σ^F-dependent transcription was highly sensitive to the species and concentration of salts: the level of single-round transcription was maximum at 50 mM KCl and then decreased to negligible level at 300 mM; while the effect was quite contrasting in the case of K-acetate and K-glutamate, giving the maximum transcription level between 200–300 mM. The activity of $E\sigma^F$ was more thermolabile than that of $E\sigma^{70}$, being inhibited at high temperature (42°C). DNase I foot printing of the *fliC* and *fliD* promoters indicated that σ^F alone cannot bind to DNA but $E\sigma^F$ specifically recognizes the promoter -10 and -35 regions. Possible alteration of the promoter structure after binding of $E\sigma^F$ was suggested.

(5) Cloning and Sequencing of the RNA Polymerase II Genes from *Schizosaccharomyces pombe*

Hitomi SAKURAI, Takenori MIYAO, Hiroshi MITSUZAWA, Makoto KIMURA, and Akira ISHIHAMA

The subunit composition of RNA polymerase II (pol II) was compared between the budding yeast *Saccharomyces cerevisiae* and the fission yeast *Schizosaccharomyces pombe*. For this purpose, we partially purified the enzyme from *S. pombe*. Judging from the co-elution profiles in column chromatographies of both the RNA polymerase activity and the two large subunit polypeptides [subunit 1 (prokaryotic β' homologue) and subunit 2 (β

homologue)], the minimum number of *S. pombe* pol II-associated polypeptides was estimated to be 10, less than the proposed subunit number of the *S. cerevisiae* enzyme (ref. 28). These 10 putative subunits of *S. pombe* pol II correpond to subunits 1, 2, 3, 5, 6, 7, 8, 10, 11 and 12 of the *S. cerevisiae* counterparts.

RNA polymerase II purified from the fission yeast *Schizosaccharomyces pombe* contains ten different species of polypeptides. Previously, we cloned and sequenced both cDNA and the genes encoding the three large subunits, Rpb1, Rpb2 and Rpb3. Later the *rpb5*$^+$ gene encoding subunit 5 of RNA polymerase II was cloned and sequenced (ref. 23). The polypeptide predicted from DNA sequence of the *rpb5*$^+$ gene consists of 210 amino acids with a calculated molecular weight of 23,914. The homology of the amino acid sequence is 55% and 43% with *Saccharomyces cerevisiae* RPB5 and human hRPB25, respectively. Recently, other groups isolated the genes for Rpb6 and Rpb12 and cDNA for Rpb10. This year we cloned both cDNA and the genes encoding four small subunits, Rpb7, Rpb8, Rpb10 and Rpb11. From the nucleotide sequences, Rpb7, Rpb8, Rpb10 and Rpb11 were found to consist of 172 (Mr 19,081), 125 (Mr 14,284), 71 (Mr 8,266) and 123 (Mr 14,111) amino acid residues, respectively. All these four subunits are homologous to the corresponding subunits of *S. cerevisiae* RNA polymerase II. The *rpb7* gene contains one intron while the *rpb8*, *rpb10* and *rpb11* genes contain two introns. Taken altogether the gene organization and the predicted protein sequence have been determined for all ten subunits of the *S. pombe* RNA polymerase II.

(6) Subunit-subunit Contact Network within *Schizosaccharomyces pombe* RNA Polymerase II

Takenori MIYAO, Kiyoshi YASUI, Akira ISHIGURO, Akira IWATA[1], Makoto KIMURA, Hiroshi MITSUZAWA, Susumu UEDA[1], and Akira ISHIHAMA ([1] Nippon Institute for Biological Science, Ohme)

Eukaryotic RNA polymerase II is composed of more than 10 polypeptide chains. The minimum and essential subunits for RNA synthesis have not yet been identified. Toward this ultimate goal, we analyzed the topological arrangement of the putative subunits by analyzing the subunit-subunit contact network within *S. pombe* RNA polymerase II. Far-Western blot analysis

of *S. pombe* RNA polymerase II using ^{32}P-labeled recombinant Rpb3 or Rpb 5 fused to glutathione S-transferase (GST) as a probe, indicated that Rpb3 binds to membrane-immobilized Rpb1, Rpb2, Rpb5 and Rpb11, and Rpb5 binds strongly to Rpb1, Rpb2 and Rpb3 and weakly to Rpb5 and a 15-kDa subunit (Rpb8 or Rpb11) (ref. 23). The existence of Rpb5-Rpb3 contact was supported by detection of complexes formed between these two proteins synthesized *in vitro* using protein-immobilized beads. The contact sites on Rpb1 and Rpb2 with Rpb3 and Rpb5 are being analyzed in details using fragments of these two large subunits expressed in *E. coli*.

The same line of studies is being carried out using ^{32}P-labeled Rpb6, Rpb7, Rpb8, Rpb10, Rpb11 and Rpb12 as far-Western probes.

(7) Transcription and Replication of Influenza Virus RNA: Evidence for *de novo* Initiation of Replication

Ayae HONDA, Kiyohisa MIZUMOTO[1], and Akira ISHIHAMA ([1] Kitasato Univ., Fac. Pharm.)

Influenza virus genome is composed of eight RNA segments of negative polarity. Transcription of the influenza virus genome RNAs (vRNAs) by the virus-associated RNA-dependent RNA polymerase is initiated by using host cell capped RNAs as primers, while the mechanism of replication initiation remained unsolved. *De novo* initiation for the RNA replication was suggested because the majority of all eight vRNA segments was found to carry triphosphate at their 5′ termini based on the observations: (i) 5′ phosphorylation of vRNAs by T4 polynucleotide kinase takes place only after phosphatase treatment; (ii) capping of vRNAs can be observed with the intact yeast capping enzyme consisting of α (RNA guanylyltransferase) and β (RNA 5′-triphosphatase) subunits; but (iii) the α subunit, which exhibits guanylyltransferase activity by itself but lacks RNA 5′-triphosphatase activity, is unable to cap vRNAs.

(8) The Molecular Anatomy of Influenza Virus RNA Polymerase

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Influenza virus RNA polymerase is a multifunctional and multisubunit enzyme, consisting of three viral P proteins, PB1, PB2 and PA. Several lines of reserach have been carried out for mapping the functional sites on each P protein subunit. Radioactive 8-azido GTP (8-N_3 GTP) was photo-crosslinked specifically to the PB1 subunit. Specific crosslinking of PB1 was confirmed using oxidized GTP. The GTP analogue-binding domains were identified after proteolytic cleavage of the crosslinked PB1 with V8 protease. The cleavage pattern of PB1 was determined by analysis of the amino-terminal proximal sequence of fragments generated in the presence of increasing concentrations of V8 protease. Results indicate two GTP-binding sites in the PB1 subunit, *i.e.*, the amino terminal-proximal site I and on the carboxy terminal-proximal site II. The location of GTP-binding site I and II is close to that of sequence motif A and motif D, respectively, conserved among RNA-dependent RNA polymerases. On the other hand, radio-labeled capped RNA was UV cross-linked to the PB2 subunit. Proteolytic cleavage experiments suggested that capped RNA was cross-linked to two segments.

By transfection of two P protein genes, one intact gene for one P protein and the other trauncated gene for another P protein, and immunoprecipitation of binary complexes, we determined the subunit-subunit contact sites among three P proteins. The PB1 is the core subunit of RNA polymerase, to which both PB2 and PA bind independently (ref. 30).

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A–b. Division of Mutagenesis

(1) A Ubiquitin Pathway Essential for Onset of Anaphase in Mitosis

Hiroaki SEINO, Fumio OSAKA, and Fumiaki YAMAO

A cDNA encoding a novel ubiquitin-conjugating enzyme designated as UbcP4 in fission yeast was isolated. Disruption of its genomic gene revealed that it was essential for cell growth. In vivo depletion of the UbcP4 protein demonstrated that it was necessary for cell cycle progression at two phases, G2/M and metaphase/anaphase transitions. The G2-arrest of UbcP4-depleted cells was dependent upon *chk1* which mediates checkpoint pathway. UbcP4-depleted cells arrested at metaphase had duplicated and condensed chromosomes but were defective in separation. However, septum formation and cytokinesis were not restrained during the metaphase arrest. Overexpression of UbcP4 specifically rescued the growth defect of *cut9*^{ts} cells at a restrictive temperature. *cut9* encodes a component of the anaphase promoting complex (APC) which is required for chromosome segregation at anaphase and moreover is defined as cyclin-specific ubiquitin ligase. Cdc13, a mitotic cyclin in fission yeast, was accumulated in the UbcP4-depleted cells. These results strongly suggested that UbcP4 is a ubiquitin-conjugating enzyme working in conjunction with APC and mediates the ubiquitin pathway for degradation of "sister chromatid holding protein(s)" at the onset of anaphase and possibly of mitotic cyclin at the exit of mitosis.

(2) CDC34 Dependent-Ubiquitin Pathway: Role of GRR1 in the Ubiquitination of G1 Cyclin Cln2

Tsutomu KISHI and Fumiaki YAMAO

In *S. cerevisiae*, Cdc34, Cdc4 and Cdc53 form a complex, and are required for the G1-S transition. *CDC34* encodes a ubiquitin-conjugating enzyme, suggesting that Cdc34-dependent proteolysis is necessary for the cell cycle progression. Sic1, an S-phase CDK inhibitor, has been identified as an essential target of this pathway. Other essential target proteins could be elucidated since a *cdc34 sic1* double mutant still arrests in G2.

We isolated extragenic suppressors of a *cdc34-1 sic1* mutant. One group of suppressors was recessive, and was found to be allelic to *GRR1*, a gene shown to be involved in degradation of G1 cyclins (Cln1 and Cln2) as well as glucose repression. Conversely, overproduction of Grr1 in *cdc34 sic1* cells impaired colony formation at 30℃. We isolated *MGO1* (*M*ulticopy suppressor of *G*rr1 *O*verproduction defect) which suppressed this growth defect when introduced on a multicopy vector. Sequence analysis revealed that *MGO1* is identical to *SKP1*, which has been shown to be required for the ubiquitin-mediated proteolysis of Cln2 and Sic1. Using GST pull-down assay, we found that *in vitro* translated Grr1 bound to both bacterially produced Gst-Skp1 and Gst-Cln2. Furthermore, *in vitro* translated Cln2 bound to Gst-Skp1, depending upon the presence of Grr1. Association of Grr1 and Gst-Cln2 did not require the carboxyl-terminal PEST sequences. These data suggest that Grr1 serves as a bridge between ubiquitination machinery and Cln2.

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A–c. Division of Nucleic Acid Chemistry

(1) Host Proteins Involved in Transcription of The Sendai Virus Genome

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The Sendai virus, a member of the Paramyxoviridae, contains a single, nonsegmented RNA genome of 15 kb long with negative polarity, which encodes six viral proteins, nucleocapsid protein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin-neuraminidase (HN), and large protein (L). The NP, P, and L proteins are associated with the genome to form a nucleocapsid. The genetic information of this RNA genome is expressed through at least six monocistronic mRNA species transcribed by a virion-associated RNA-dependent RNA polymerase, which is thought to be composed of the P and L proteins.

In vitro mRNA synthesis by The Sendai virus is almost entirely dependent on the addition of cellular proteins (positive host factors), one of which could be tubulin. We investigated the distribution of host factors in various rat organs. Extracts from the brain, thymus, heart, lung, testis, ovary and uterus all supported *in vitro* Sendai virus transcription, among which the highest activity was obtained with the brain extract. On the other hand, little or no activity was detected in the liver, spleen, and kidney extracts. An inverse correlation between the apparent host factor activity to stimulate mRNA synthesis and RNase activity that hydrolyzes Sendai virus mRNAs was found, except in the liver extract. However, when a transcription initiation complex was isolated and subjected to RNA chain elongation reaction, all of the extracts including those from liver, spleen and kidney, were active.

Immunoblotting showed that tubulin molecules were integrated in these initiation complexes, supporting the notion that tubulin is involved in initiation complex formation. We alsoidentified transcription inhibitory activity without any detectable RNase activity in the liver extract. This negative host factor seemed to act on RNA chain elongation. It is likely that Sendai virus transcription is regulated by both positive and negative regulatory factors. (For details, see Ref. 1.)

(2) Crystal Structure of the *Escherichia coli* Replication Terminator Protein Complexed with *Ter* DNA

Katsuhiko KAMADA and Kosuke MORIKAWA (Biomolecular Engineering Research Institute)

Replication is terminated at defined sequences of DNA in prokaryotic chromosomes. In *Escherichia coli*, this termination is mediated by the site-specific DNA-binding protein designated as Tus. The Tus protein (mw: 36 kDa) is a monomeric molecule that binds six specific sequences (*Ter*) within the replication terminus region, and the Tus-*Ter* complex prevents the passage of DnaB helicase, which is a constituent of replication machinery. The three-dimensional (3D) structure at the atomic level is required to gain insight into the mechanism by which the Tus protein recognizes the *Ter* sites and blocks the replication fork in a polar manner.

The most suitable crystals for X-ray analysis were grown from a mixture of the Tus protein and a 16 base long *Ter* DNA using the microdialysis method in PEG solution (ref. 3). The crystal structure was resolved at 2.7Å resolution and refined (ref. 4). The Tus protein shows a very unique structure as compared with other DNA binding proteins. The protein embraces the *Ter* element from both sides of the DNA with two protruded α-helical regions. The most remarkable feature of the complex is an extensive protein-DNA interface which involves many direct and indirect polar interactions. Two β-sheets are responsible for the recognition of the DNA in the major groove.

A genetic approach allowed the isolation of the Tus mutant. Most of these single mutation sites are mapped on the β-sheet region in the 3D-structure. These mutants that affect the efficiency of replication arrest, partially or completely impair the binding of the Tus protein with DNA. This suggests

that replication arrest and Tus-DNA binding might be inseparable.

The overall structural feature of the complex also implies that the α-helical regions on the fork-blocking side act as a directional DNA clump and prevent the passage of the DnaB helicase. Presumably, a mechanism that enables the replication fork from the opposite side to pass through, would rely upon the structural disturbance of the β-sheet region induced by the unwinding motion of DnaB. This interpretation agrees with the physical collision model that shows the intrinsic structure of the complex dominating the directional arrest of the replication fork.

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B. DEPARTMENT OF CELL GENETICS

B–a. Division of Cytogenetics

(1) Localization of Mouse Rad51 and Lim15 on Meiotic Chromosomes at Late Stages of Prophase 1

Tomoatsu IKEYA and Tomoko OGAWA

Studies on the localization of Rad51 and Lim15 proteins on meiotic chromosomes of lily showed that these proteins participate in searching for homologous chromosomes, which is initiated on the leptotene stage and leads to pairing in the zygotene stage. However, the roles of these proteins in chiasma formation and chromosomal exchanges are not known. Taking advantage of the much shorter lengths of mouse chromosomes which facilitates the examination of on an entire chromosome with a chiasma region through optical methods, we studied the localization of Rad51 and Lim15 homologues on the mouse chromosomes.

In the chromosomes of mouse testis during the late stages of meiotic prophase 1, the Rad51 and Lim15 proteins were present in the core regions of the chromosomes which remained after removal of the chromatin by DNase II. Rad51 was located along the core of the synaptonemal complexes (SC) in the pachytene stage and in the chiasma regions in the diplotene stage. The protein was not present on separated homologous cores in the diplotene stage. On the other hand, the Lim15 protein was present almost exclusively at both ends of the core of chromosomes from the pachytene to diplotene stages and even in diakinesis. Differences in the roles of these proteins in the late stage of recombination were evident from these findings. Ref. 1

(2) Characterization of DNA Ligase from the Fungus Coprinus Cinereus

Shimako MATSUDA

DNA ligase was highly purified from the fungus Coprinus cinereus at the

meiotic recombination stage, pachytene. The pachytene DNA ligase showed three polypeptides with molecular masses of 88, 84 and 80 kDa, as estimated by α-[^{32}P]AMP-labeling assay. These three polypeptides were susceptible to reaction with mAb against a 16-amino-acid sequence in human DNA ligase I, which is conserved in the C-terminal regions of mammalian, vaccinia virus and yeast DNA ligases. Since rapidly purified preparations from fresh pachytene cells exhibited a single polypeptide of DNA ligase with a molecular mass of 88 kDa, the smaller polypeptides seemed to be limited-degradation products of 88-kDa polypeptide during the isolation and purification procedures. Km values for ATP and $(dT)_{20}$ hybridized with $(dA)_n$ were 1.5 mM and 90 nM, respectively. This enzyme was capable of joining $(dT)_{20}\cdot(rA)_n$ and $(rA)_{12\text{-}18}\cdot(dT)_n$ as well as $(dT)_{20}\cdot(dA)_n$, and able to ligate blunt-end DNA in the presence of poly(ethylene glycol) 6000. DNA ligases were also partially purified from zygotene cells at the meiotic pairing stage and mitotic mycelium cells. In their molecular mass, immuno-reactivity, *Km* value and substrate specificity, they were indistinguishable from pachytene DNA ligase. These results suggest that the fungus C. cinereus in the pachytene stage contains DNA ligase with a molecular mass of 88 kDa as a main or a single species, which is quite similar to DNA ligases from the zygotene and mycelium cells in molecular and catalytic properties. Ref. 2

(3) Genetic Characteristics of New recA Mutants of *Escherichia coli* K-12

Andrei ALEXSEYEV (ALEXEEV)

To search for functionally thermosensitive (FT) recA mutations, as well as mutations which differently affect RecA protein functions, seven new recA mutations in three different regions of the RecA protein structure proposed by Story *et al.* were constructed. Additionally, the *recA2283* allele responsible for the FT phenotype of the *recA200* mutant was sequenced. Five single mutations (*recA2277*, *recA2278*, *recA2283*, *recA2283E* and *recA2284*) and one double mutation (*recA2278-5*) generated, respectively, the amino acid substitutions L-277→N, G-278→P, L-283→P, L-283→E, I-284→D, and G-278→T plus V-275→F in the alpha-helix H-beta-strand 9 region of the C-terminal domain of the RecA protein structure. According to recombination, repair, and SOS-inducible characteristics, these six mutations fall into four phenoty-

pic classes: (i) an FT class, with either inhibition of all three analyzed functions at 42 degrees C (*recA2283*), preferable inhibition at 42 degrees C of recombination and the SOS response (*recA2278*), or inhibition at 42 degrees C of recombination only (*recA2278-5*); (ii) a moderately deficient class (*recA2277*); (iii) a nondeficient class (*recA2283E*); and (iv) a mutation with a null phenotype (*recA2284*). The *recA2223* mutation generates an L-223→M substitution in beta-strand 6 in the central domain of the RecA structure. This FT mutation shows preferable inhibition of the SOS response at 42 degrees C. The *recA2183* mutation produces a K-183→M substitution in alpha-helix F of the same domain. The Lys-183 position in the Escherichia coli RecA protein was found among positions which are important for interfilament interaction. Ref. 3

(4) Chromatin Structure of the Yeast URA3 Gene at High Resolution Provides Insight into Structure and Positioning of Nucleosomes in the Chromosomal Context

Shigeo TANAKA

To characterize nucleosome structure and positioning in the chromosomal context, the chromatin structure of the whole URA3 gene was studied in the genome and in a minichromosome by testing the accessibility of DNA to micrococcal nuclease and DNase I. The cutting patterns and hence the chromatin structures were almost indistinguishable in the genome and in the minichromosomes. The only notable exception was enhanced cutting between nucleosomes U3/U4 and U4/U5 in the minichromosomes. The results demonstrate that there is no severe constraint acting from outside the URA 3 gene in chromosomes and minichromosomes. While low-resolution mapping showed six regions with a positioned nucleosome (U1 to U6), each region resolved in a complex pattern consistent with multiple overlapping positions. Some regions (U1, U4, U5 and U6) showed multiple positions with a dominant rotational setting (DNase I pattern), while U2 showed positioning within 10 bp but with no defined rotational setting, demonstrating that nucleosome positions were not in phase and not coordinately regulated. Reduced DNase I cutting from about 50 bp form the 5′ end towards 3′ end was common to all nucleosome regions. This polarity has been observed on isolated core particles. The results demonstrate that the DNase I pattern

observed in vitro indeed reflects a structural property of nucleosomes in the chromosomal context. It is emphasized that despite the local heterogeneity revealed by high-resolution mapping, the low-resolution map is a reasonably accurate representation of the chromatin structure. Ref. 4

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Oral presentaions

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2. Ogawa, T., Ikeya, T., and Terasawa, M.: Dual nature of Rad51 and Lim15 proteins in meiotic recombination. EMBO Meeting: Recombination, France, May.
3. Ikeya, T., Sato, S., Tabata, S., and Ogawa, T.: Localization of mouse Rad51 and Lim15 proteins on meiotic chromosomes at late stage of prophase 1. Gordon Research Conference: Meiosis, USA, June.
4. Ogawa, T. and Shinohara, A. Biochemical activities of Rad52 protein in homologous recombination. Gordon Research Conference: Meiosis, USA, June.

B–b. Division of Microbial Genetics

(1) Minus-Strand Origin of Filamentous Phage *versus* Transcriptional Promoters in Recognition of RNA Polymerase

Atsushi HIGASHITANI, Nahoko HIGASHITANI, and Kensuke HORIUCHI

Replication of complementary strand DNA in filamentous phages is in-

itiated by a primer RNA, which is synthesized at the minus-strand origin on the viral single-stranded DNA by *E. coli* RNA polymerase holoenzyme containing the σ^{70} subunit. We demonstrated that the affinity of RNA polymerase *in vitro* to the origin was about 16-fold higher than that to the *lac*UV5 promoter. We also showed that the temperature dependence of the primer RNA synthesis was much lower than that of *lac*UV5 transcription. The high affinity of RNA polymerase to the origin depended on the single-strandedness of the "-10 region". The nucleotide sequence of the non-template strand in the "-10 region" was found to be important for the function, while that of the template strand was not. These observations suggest that the σ^{70} subunit directly interacts with the single-stranded non-template strand containing adenine residue(s) at the -10 region of the promoter. For details, see Ref. 1.

(2) The Active Center of the Initiator Protein of Filamentous Phage f1

Satoshi ASANO, Atsushi HIGASHITANI, and Kensuke HORIUCHI

The gene II protein (gpII) of filamentous phages (f1, fd, M13) is a multifuctional protein that plays several key roles in phage plus-strand DNA replication. First, it binds to the plus-strand origin and introduces a specific nick in the plus-strand of supercoiled replicative form DNA (RF I). The 3′-hydroxyl end of the nick serves as the primer for the rolling-circle type replication. Binding of gpII induces DNA bending and duplex melting around the nicking site. The melting requires the negative superhelicity of DNA, and the nicking reaction requires the Mg^{2+} ion. Upon completion of a round of replication, gpII functions in termination by cleaving and circularizing the displaced single strand.

We showed that a small fraction of the nicked DNA product was covalently attached at the 5′ end to gpII. The covalent bond was extremely unstable in the presence of active gpII. The residue that bridged gpII to the 5′ end of DNA was phospho-tyrosine.

There are 15 tyrosine residues in gpII. We therefore constructed 15 mutant gpII, in each of which a tyrosine residue was replaced by phenylalanine. Complementation tests of a gene II amber phage were carried out using these mutant proteins provided in trans from plasmids. Three mutants only

weekly complemented the amber phage. Three other mutants were temperature sensitive. Only one mutant(gpII Y197F) was totally incapable of complementing the amber mutant. The remaining mutants strongly complemented the amber phage. We purified gpII Y197F gpII and tested for nicking activity and sequence-specific DNA binding activity for the origin. The results indicated that gpII Y197F could specifically bind to the origin and bend DNA normally. However, it could not introduce the nick.

In order to directly determine the tyrosyl residue of gpII to which the nicked DNA product is covalently attached at the 5′ end, the covalent complex was digested with trypsin, and the peptide-DNA complex was isolated by ion-exchange chromatography and gel filtration. The results of peptide sequencing of the isolated peptide-DNA complex indicated that the tyrosine residue at position 197 from the N-terminus was covalently attached to the DNA.

We concluded that the tyrosine residue at position 197 is involved in the nicking reaction as the active center.

In contrast to the case of the cisA protein of lytic phage phiX174, the covalent bond between gpII and the 5′ end of DNA was quickly cleaved by gpII itself. The instability of the covalent complex may be related to the unique mode of DNA replication in filamentous phages which allows coordinated growth of the phage and the host bacteria.

(3) A New Class of Mutations of Penicillin-Binding Protein 3 of *Escherichia coli*

Hiroshi HARA and Kensuke HORIUCHI

Penicillin-binding protein 3 (PBP 3) of *E. coli* is a peptidoglycan-crosslinking enzyme essential for formation of the division septum. The crosslinking activity is carried by the C-terminal penicillin-binding domain. We suspected, from the following findings, that PBP 3 acted as an oligomer or a complex with other cell division proteins and that the function for the oligomer/complex formation was carried by the N-terminal region: (i) A mutant PBP 3 with an altered active site for the crosslinking activity (S307A) showed a dominant lethal effect, and the effect was reversed by replacement of the N-terminal 40 residues with an uncleavable signal peptide. (ii) Overproduction of N-terminal fragments caused a dominant negative effect.

We mutagenized the N-terminal region and selected mutations that reversed the dominant lethal effect by S307A. Such intragenic suppressors in the N-terminal region were then combined with the wild-type crosslinking activity domain in the C-terminal region. Among them we found PBP 3 mutants that were non-functional in septum formation. One of these mutants was found to have a deletion of the 4 residues Arg-Val-Ala-Trp, at the 41st–44th positions (R41-W44) from the N terminus.

In a separate experiment to exchange the N-terminal region of PBP 3 with other sequences, we inserted a synthetic 12-base oligonucleotide containing an *Eco*RI-recognizing sequence into the *Mlu*I site within the PBP 3 gene. The resultant mutant PBP 3, which had a 4-residue insertion of Asn-Ser-Arg-Val after V42, was also found non-functional in septum formation. When combined with the S307A C-terminal domain, it suppressed the dominant lethal effect.

These mutants (i) retained penicillin-binding ability, indicating normal crosslinking activity, and (ii) were C-terminally processed by a periplasmic protease Prc as was wild-type PBP 3, indicating normal translocation across the cytoplasmic membrane. In contrast to crosslinking-defective mutations isolated so far by monitoring penicillin-binding ability, these mutations are of a novel class, probably defective in the oligomer/complex formation. The deletion and insertion were within the N-terminal hydrophobic sequence F24-I48. We converted R41, the sole charged residue in the sequence, to 19 other kinds of amino acid residues, but the septum-forming ability was not lost. A residue(s) within V42-W44 or the length of the hydrophobic sequence may be important for PBP 3 function.

(4) The First Two Genes of the Cell Envelope Biosynthetic/Cell Division Gene Cluster in the *Escherichia coli* Chromosome 2-Minute Region

Hiroshi HARA and Kensuke HORIUCHI

In the 2-min region of the *E. coli* chromosome, cell envelope biosynthetic and cell division genes are tightly clustered in the same orientation. A promoter, P_{mra}, at the beginning of this *mra* cluster is required for expression of the first nine genes, up to *ftsW*. We concluded this because a P_{mra} :: P_{lac} strain in which the promoter was displaced by the *lac* promoter grew in the

absence of a *lac* inducer IPTG, only if it harbored a plasmid containing a chromosomal fragment ranging from P_{mra} to *ftsW* or further downstream.

The first two genes of the cluster, not known for their function, have been suspected to be involved in cell division, because their homologs are well conserved in the homologous clusters of many other bacterial species including *Micoplasma genitalium*, a peptidoglycan-free bacterium. However, when a plasmid expressing seven genes ranging from *ftsL*, the third gene of the cluster, to *ftsW* under a promoter in the vector region was introduced into the P_{mra} :: P_{lac} strain, the strain grew normally in the absence of IPTG. The third and fourth genes, *ftsL* and *ftsI*, have been proven essential for cell division, and a plasmid expressing five genes ranging from *murE*, the fifth gene, to *ftsW* did not support the growth of the P_{mra} :: P_{lac} strain in the absence of IPTG. When the first two genes on the chromosome were destroyed by displacing a *Pvu*II fragment covering most parts of them with an antibiotic resistance gene, the resultant strain showed no defect in growth or division. We concluded that these two genes are dispensable at least under laboratory conditions.

(5) The *spr* Gene of *Escherichia coli*

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Escherichia coli Δ*prc* mutants lacking Prc, a periplasmic proteolytic enzyme involved in the C-terminal processing of penicillin-binding protein (PBP) 3, show thermosensitive growth at low osmolarity. Suppressor mutations that reversed the thermosensitivity were all mapped at 47 minute on the chromosome map. These *spr* mutations caused thermosensitivity at low osmolarity in the prc^+ background.

From the Kohara λ phage genomic library we found a clone that could transduce *spr* into an spr^- recipient expressing the *c*I repressor from a plasmid. We subcloned the *spr* gene and determined its nucleotide sequence. The predicted product was a 21-kDa lipoprotein. When the *spr* gene was overexpressed, a 21-kDa protein was detected in the envelope fraction. This product seemed to be degraded by Prc protease, because the protein band on SDS-gel was more intense in a Δ*prc* mutant than in the wild type. Primer

extension experiments revealed two promoters for the *spr* gene, the upstream one being stronger than the other. Two *spr* mutations were analyzed for their nucleotide sequences: one had an insertion of IS*1* between the two promoter sequences, reducing the expression a great deal; and the other had a one-base insertion, truncating the product to about one third of the wild-type product.

Hyperexpression of *spr* using the λ p_L promoter caused cell lysis. A Δ*prc* mutant, in which the Spr protein is not degraded by the protease, lysed from simultaneous thermal and osmotic stresses. Such stresses caused *spr* mutants to cease growing. Hyperproduction of Prc protease, which would reduce the amount of intact Spr, also caused growth cessation. Thermosensitivity on a low osmolarity medium of an *spr* mutant was suppressed by overproduction of PBP 7, a peptidoglycan DD-endopeptidase. This multicopy suppression was not observed by mutant PBP 7 lacking the DD-endopeptidase activity. The deduced amino acid sequence of Spr was found to show significant homology to peptidoglycan endopeptidase II of *Bacillus sphaericus* and to the C-terminal region of p60 proteins of many *Listeria* species, which seems involved in cell separation after division. Thus we suppose that Spr may function in cell wall formation as a peptidoglycan-hydrolyzing enzyme.

(6) Peptidoglycan Polymerase with no Crosslinking Activity in *Escherichia coli*

Hiroshi HARA

Penicillin-binding proteins (PBPs) 1a and 1b of *E. coli* are bifunctional peptidoglycan-synthesizing enzymes with a glycan-polymerizing domain in their N-terminal halves and a peptide-crosslinking domain in their C-terminal halves. Their activity is essential for growth: a mutant lacking both PBP 1a and 1b is not viable, although a mutant lacking eitherof them is viable. More than 10 years ago we discovered a monofunctional enzyme that possess glycan-polymerizing, but no crosslinking activity, and purified and characterized it. It was to be shown responsible for a considerable part of the total in vitro glycan-polymerizing activity measured.

Recent progress in the genome project led to identification of a gene, at 72 minute on the chromosome map, whose predicted product shows homology to the N-terminal glycan-polymerizing domain of the bifunctional PBPs, but lacks a region corresponding to the C-terminal domain. A Swiss group

cloned this *mgt* gene, and showed a significant increase in in vitro peptidoglycan synthesis with overexpression of the gene. I disrupted this gene by inserting an antibiotic resistance gene, and found such a mutant strain grew normally. This insertion mutation could be transduced into mutants defective in either PBP 1a or 1b without causing any apparent growth defect. These results indicate that *mgt* is not an essential gene.

(7) Functional Dissection of Cell-Division Inhibitor SulA of *Escherichia coli* and Its Negative Regulation by Lon

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E. coli SulA is induced by SOS response and inhibits cell division through interaction with FtsZ. To determine which region of SulA is essential for the function of cell division inhibition, we constructed a series of N-terminal and C-terminal deletions of SulA and a series of alanine substitution mutants. Arg 62, Leu 67, Trp 77, and Lys 87 in the central region of SulA were found essential for the inhibitory activity. N-terminal residues of SulA ranging from the 3rd to the 27th amino acid and C-terminal 21 residues were dispensable for the activity. The mutant protein lacking N-terminal residues from the 3rd to 47th was inactive as was that lacking C-terminal 34 residues. C-terminal deletions of 8 or 21 residues increased the growth-inhibiting activity in *lon*$^+$ cells, but not in *lon*$^-$ cells. The wild-type and mutant SulA proteins were isolated in a form fused to *E. coli* maltose-binding protein (MBP), and tested *in vitro* for sensitivity to Lon protease. Lon degraded wild-type SulA and its deletion mutant lacking N-terminal 93 amino-acids, but did not degrade it if 21 residues at the C-terminus were deleted. Futhermore, the wild-type SulA and the N-terminus-deleted mutant formed a stable complex with Lon, while the C-terminus-deleted mutant did not. Only 20 C-terminal residues fused to MBP formed a stable complex with, but were not degraded by, Lon. The LacZ protein that was fused at its C-termnus to 8 or 20 amino-acid residues from the C-terminus of SulA was stable in *lon*$^+$ cells. These results indicate that the C-terminal 20 residues of SulA are sufficient for recognition by, and for complex formation with, Lon. They are necessary, but not sufficient, for degradation of SulA by Lon. For

details, see Ref. 3.

(8) Search for Homologs of Human ATM Gene in *C. elegans*

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We are interested in check point controls after meiotic recombination and mitotic DNA damage. In the last year the human ataxia telangiectasia mutated (ATM) gene was isolated and characterized by Savitsky, K. *et al.* (*Science*, **268**, 1749–1753, 1995). The ATM protein contains a PI-3 kinase domain in its C-terminal region. This feature is shared with a group of proteins including rad3 in *S. pombe*, MEC1(ESR1) and TEL1 in *S. cerevisiae*, MEI-41 in *D. melanogaster*, and human DNA-dependent protein kinase.

From a blast search of a *C. elegans* database (ACEDB), a homolog of ATM was identified in cosmid T06E4.3. We named the gene atl-1 (ATM like 1). The primary structure of the cDNA was determined by sequencing the RT-PCR products of *C. elegans* mRNA and the yk76g7 cDNA clone. The total length of the cDNA is 7711bp and the putative gene product consists of 2514 amino acid residues. Comparison of amino-acid sequences of the PI-3 kinase motif indicated that the ATL-1 sequence is much closer to MEC1 (ESR1), TEL1, MEI-41, than to bovine p110 which is the catalytic subunit of PI-3 kinase. By using RT-PCR, it was found that the level of mRNA of atl-1 was increased about 3-5 fold in the adult stage, while it was expressed in all stages. We have carried out injection experiments of atl-1 anti-sense RNA, whose length was about 2 kb starting from the 3′ poly A terminus, into the N2 wild-type worm. Surprisingly, the phenotype of the f1 progenies was extremely pleiotrophic. We could classify the phenotypes into various categories such as dead egg, L1 lethal (irregular L1), protruding vulva, abnormal gonad, irregular oocyte, sterile, dpy or small size, burst from vulva, abnormal intestine, unc, and high incidence of males. These results may suggest that atl-1 is involved in a check point control of mitosis and meiosis, as ATM is. The defect of the gene function seems to result in premature segregation of meiotic chromosomes and/or in increase of mutations in several different cells.

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B–c. Division of Cytoplasmic Genetics

(1) Cell-cycle-dependent Expression of the STK-1 Gene Encoding a Novel Murine Putative Protein Kinase

Hitoshi NIWA, Kuniya ABE, Takahiro KUNISADA, and Ken-ichi YAMAMURA

We cloned a novel putative serine/threonine kinase-encoding gene, designated STK-1, from murine embryonic stem (ES) cell and testis cDNA libraries. The kinase most closely related to STK-1 is Xenopus laevis XLP46 protein kinase which shows 71% amino acid identity to STK-1 between their kinase domains. Nevertheless, STK-1 is conserved throughout the phylogeny with hybridizing sequences being detected in DNA from mammals, amphibians, insects and yeast. STK-1 mRNA is detected in testis, intestine and spleen, tissues that contain a large number of proliferating cells, but not in other tissues. All cell lines tested expressed STK-1 mRNA with levels being dependent upon proliferation rates. In NIH 3T3 cells, STK-1 is expressed in a cell-cycle-dependent fashion. These findings suggest a role for STK-1 in cell growth. For details, see Ref. 1.

(2) Endoderm-Specific Gene Expression in Embryonic Stem Cells Differentiated to Embryoid Bodies

Koichiro ABE, Hitoshi NIWA, Katsuro IWASE, Masaki TAKIGUCHI, Masataka MORI, Shinichi ABE, Kuniya ABE, and Ken-ichi YAMAMURA

Mouse embryonic stem (ES) cells can differentiate into various cell types within cell aggregates called embryoid bodies (EBs). This structure consists of ectodermal, mesodermal and endodermal tissues, and resembles embryos of the egg-cylinder stage. After 8–10 days in culture, about half of the EBs expand into large cystic structures homologous to the visceral yolk sac of postimplantation embryos. To study endoderm differentiation at the molecular level, we examined expression of endoderm marker genes during the process of embryoid body (EB) development. a-fetoprotein (AFP) and transthyretin (TTR) transcripts increased at the stage when embryoid bodies began to form yolk sac like structures and were expressed strongly thereafter. Expression of HNF4 (HNF; hepatocyte nuclear factor), a variant form of HNF1 (vHNF1, also called HNF1β), and HNF3β started before the onset of AFP and TTR expression. HNF1 (also called HNF1α) expression began a few days after the onset of the expression of the transcription factors described above. The serum albumin (ALB) transcript was only found in late large cystic EBs. Also AFP gene expression preceded ALB gene expression. These results suggest that the patterns of endoderm gene expression during EB development reflect the order found during mouse development in vivo, and EB formation may serve as an in vitro system for studying these differentiation processes. For the details, see Ref. 2.

(3) Nuclear Organization in Fission Yeast Meiosis

Yasushi HIRAOKA

We found that in fission yeast meiotic prophase, telomeres are clustered near the spindle pole body (SPB; a centrosome-equivalent structure in fungi) and take the leading position in chromosome movement while centromeres are separated from the SPB. The movement of chromosomes that preludes meiosis was observed in living cells of fission yeast by fluorescence microscopy. Further analysis by in situ hybridization revealed that the telomeres remain clustered at the leading end of premeiotic chromosome movement,

unlike mitotic chromosome movement in which the centromere leads. While it is generally believed that the centromere is a unique chromosomal site that leads the chromosome movement, our finding demonstrates a striking example in which telomeres can lead chromosome movement.

The meiotic telomere position contrasts with mitotic nuclear organization in which centromeres remain clustered near the SPB and lead chromosome movement. Thus, nuclear reorganization switching the position of centromeres and telomeres must take place upon entering meiosis. We analyzed the nuclear location of centromeres and telomeres in genetically well-characterized meiotic mutant strains. An intermediate structure for the telomere-centromere switching was observed in haploid cells induced to meiosis by a synthetic mating pheromone; fluorescence in situ hybridization revealed that in these cells, both telomeres and centromeres were clustered near the SPB. Further analyses in a series of mutants showed that the telomere-centromere switching takes place in two steps; first, association of telomeres with the SPB and second, dissociation of centromeres from the SPB. The first step can occur during the haploid state in response to a mating pheromone, but the second step does not take place in haploid cells and probably depends on conjugation-related events. In addition, a linear minichromosome was also colocalized with authentic telomeres instead of centromeres, suggesting that telomere clustering plays a role in organizing chromosomes within a meiotic prophase nucleus. (Chikashige *et al.*, EMBO J., **16**, 193–202, 1997)

(4) Fluorescence Imaging of Living Mammalian Cells

Yasushi HIRAOKA

Cellular events are accomplished by a coordinated interaction of molecular components within a cell. Continuous observation of such interaction in living cells can be essential to the understanding of the temporal and spatial relationships of those molecular components. With its molecular selectivity in imaging, fluorescence microscopy has become of increasing importance for molecular cell biology, especially since the advent of green fluorescent protein. Toward this end, we have developed fluorescence microscopy techniques to examine the dynamics of specific molecules in living mammalian cells. Multple-wavelength imaging in living cells enabled visualiztion of

dynamic interactions of cellular structures during mitosis. Continuous observations provide a unique oppotunity to detect transient, ephemeral events which can not be detected in fixed specimens. (Hiraoka and Haraguchi, Chromosome Res., **4**, 173–176, 1996)

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C. DEPARTMENT OF ONTOGENETICS

C–a. Division of Developmental Genetics

(1) Control of Neuronal Identities by the *Drosophila* Nuclear Receptor, SEVEN-UP

Yasushi HIROMI and Steven R. WEST[1] ([1] Department of Molecular Biology, Princeton University)

Our major research goal is to understand the molecular mechanism by which neuronal diversity is generated. While much has been learned about how a cell becomes a neuron, little is known about how each neuron adopts its identity as a specific neuron. We have previously shown that the *seven-up* gene controls photoreceptor neuronal identities in the *Drosophila* retina; loss of *seven-up* function results in the transformation of one neuronal type to another. This is one of the clearest examples of genes that control the decision between different neuronal types. SEVEN-UP is a member of the nuclear receptor family and is likely to act by regulating transcription of its target genes. Expression of *seven-up* is confined to the neurons whose fate is affected in *seven-up* mutants, thus SEVEN-UP is likely to be an autonomous factor that specifies the fate of individual neurons. SEVEN-UP is highly conserved throughout evolution. The human and *Drosophila* homologs of SEVEN-UP exhibit more than 90% amino acid identity in both the DNA binding domain and the ligand binding domain. Vertebrate homologs of SEVEN-UP are also expressed in a spatially restricted pattern during nervous system development, suggesting that they may also play a role in neuronal specification.

Through a series of misexpression studies, we showed that expression of SEVEN-UP in any cell type within the ommatidium that normally does not express SEVEN-UP results in an alteration of its cell fate. Each cell has a unique response and unique responsive period, suggesting that misexpression of SEVEN-UP interferes with a molecular machinery that is used in many cell fate decisions. Surprisingly, generation of these phenotypes does not require the presence of the DNA binding domain of SEVEN-UP; expression

of the chimeric molecules containing a heterologous DNA binding domain and the SEVEN-UP ligand binding domain causes many of the same phenotypes in the eye observed following misexpression of the intact SEVEN-UP protein. The presence of a conserved DNA binding domain in SEVEN-UP implies that the endogenous protein acts by binding to its target DNA. On the other hand, production of similar phenotypes by misexpressing the intact protein and the protein lacking its DNA binding domain suggests that the mechanism by which misexpressed SEVEN-UP acts is by titrating another molecule, presumably a protein (which we call protein X). We expect protein X to be an evolutionarily conserved molecule that is involved in many cell fate decisions. Currently we are conducting genetic and molecular screens to identify protein X.

(2) *biparous*, a Novel bHLH Ggene Expressed in Neuronal and Glial Precursors in *Drosophila*

Andrew BUSH[1], Yasushi HIROMI, and Michael COLE[1] ([1] Department of Molecular Biology, Princeton University)

Genetic studies have uncovered many genes that are involved in the first steps of neuronal development in *Drosophila*. Less is known about the intermediate steps during which individual precursor cells follow either the neuronal pathway or the glial pathway. We have identified a novel bHLH gene, *biparous*, that is expressed in neuronal and glial precursors in the *Drosophila* embryo. Unlike most bHLH genes whose expression is confined to early neurogenesis, *biparous* expression continues towards the final stages of embryonic development. Expression of *biparous* is not observed in postmitotic neurons and precedes the expression of *repo*, a gene activated in later stages of glial differentiation. The bHLH domain is sufficiently different from previously described bHLH domains to imply a novel function. See ref. 1.

(3) KLINGON, a New Member of the *Drosophila* Immunoglobulin Superfamily, Participates in the Neuronal Differentiation of the R7 Photoreceptor Neuron

Samantha J. BUTLER[1], Sandip RAY[1], and Yasushi HIROMI ([1] Department of Molecular Biology, Princeton University)

Of the eight photoreceptor neurons of the *Drosophila* compound eye, the R7 neuron is the only UV-sensitive neuron and also synapses in the layer of the optic lobe that is different from all other photoreceptor neurons. Starting from an enhancer trap line, we identified a transcription unit, *klingon*, that is expressed in a restricted pattern of neurons during embryonic neurogenesis and in the R7 photoreceptor precursor throughout its development. *klingon* is a member of the Immunoglobulin superfamily and encodes a putative protein of 528 amino acids and contains three C2-type Immunoglobulin-like domains followed by one fibronectin type III repeat. When KLINGON is expressed in S2 tissue culture cells, it is associated with the cell membrane by a glycosyl-phosphatidylinositol linkage and can mediate homophilic adhesion. Genetic analysis has revealed that *klingon* is an essential gene that participates in the development of the R7 neuron. Ectopic expression of *klingon* in all neurons in a genetic background that lacks the R7 neuron causes the rhabdomere of the R8 neuron to project more apically, suggesting that KLINGON may be involved in positioning of the photoreceptor rhabdomere. See ref. 2.

(4) Cell Lineage Analysis of the Mushroom Body

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During neurogenesis, a large number of neuron and glia are generated from neuroblasts, bipotential precursors for both cell types. We developed a method to express any UAS-linked reporter gene in clonal progenies of neuroblasts that express GAL4 under a constitutive promoter. Using this method we performed cell lineage analysis in the adult *Drosophila* brain, to analyze the development and organization of the mushroom body (MB), which is an important center for higher-order sensory integration and learning. We showed that the four mushroom body neuroblasts (MBNbs) give

birth exclusively to the neurons and glial cells of the MB, and that each of the four MBNb clones contributes to the entire MB structure. The expression patterns of 19 GAL4 enhancer-trap strains that mark various subsets of MB cells revealed overlapping cell types in all four of the MBNb lineages. Partial ablation of MBNbs using hydroxyurea showed that each of the four neuroblasts autonomously generates the entire repertoire of the known MB substructures. See ref. 3.

(5) Hydra Peptide Project I. LWamides as Induces of Metamorphosis of Planula Larvae of Cnidarians

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Using the approach described previously (Ann. Report No. 46, 1995), we isolated 338 peptides from *Hydra magnipapillata*, determined the structure of 222 peptides and chemically synthesized 30 peptides. We selected two groups of peptides which are involved in morphogenesis of hydra as well as other cnidarians for further analyses.

We isolated 7 LWamides which have a consensus sequence of GLWamide in their C-termini. A similar peptide, metamorphosin A (pEQPGLWamide) was isolated from sea-anemone and shown to induce metamorphosis of planula larvae of *Hydractinia echinata*. We also showed that the *Hydra* peptides induced metamophosis of planula larvae of *Hydractinia serrata* and that the effective domain of the peptides for metamorphosis is GLWamide at their C-termini (Takahashi *et al.*, 1997). We further examined whether LWamides are general inducers of metamorphosis of cnidarian planulae. The phylum cnidaria is composed of 3 classes of animals: hydrozoa, anthozoa and schyphozoa. We chose one or two representatives in each class. *Hydractinia* is the representative of hydrozoa, reef-building coral, *Acropora* of anthozoa and *Cassiopea* and *Aurelia of* schyphozoa. Although preliminary, one of the LWamides, Hym-54 (GPMTGLWamide) was shown to be effective in inducing metamorphosis of *Acropora* planulae. Metamorphoses of schyphozoans are currently under examination. These results show that LWamides

are general inducers of metamorphosis in at least two of the 3 classes of cnidarians.

(6) Hydra Peptide Project II. Two Peptides Involved in Regulation of Foot Formation in Hydra

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We screened 30 synthetic peptides for their ability to enhance foot regeneration of hydra. Animals were treated with a peptide for 24 h and the upper foot region was isolated and allowed to regenerate for another 24 h in the absence of the peptide. At the end of regeneration, the pieces from treated and untreated control animals, as well, were fixed for immunostaining of foot specific monoclonal antibody (AE-03, a gift of Dr. Kobayakawa of Kyushu Univ.). Two peptides, Hym-323 and Hym-346 ,were identified as giving a positive signal in about 20% of regenerating tissues, while control tissues gave essentially no positive signal. Hym-323, composed of 16 amino acids, has some homology to head activator (Schaller and Bodenmueller, 1989) at its C-terminal half. Head activator was originally identified as a head specific morphogen but later it was shown to be a mitogen and inducer of neurons. Hym-323 has no effect on head formation. Hym-346, composed of 20 amino acids, is identical to a recetnly reported foot specific morphogenetic factor, pedibin purified from *H. vulgaris*. (Hoffmeister, 1996) except that Hym-323 lacks the C-terminal residue, glutamic acid, of pedibin.

Since the pretreatment of peptides resulted in the enhancement of foot regeneration, we assumed that the peptides were involved in chaging positional information along the body axis rather than in speeding up the regeneration process. To confirm this, lateral tissue transplantation experiments were carried out. A small tissue was excised from the mid-position between the head and the budding region of hydra which had been treated for several days and was transplanted to the same position of untreated host hydra. The frequencies of ectopic foot formation for Hym-323 and Hym-346 were 15% and 17%, respectively. In control transplants, where no peptide treatment was involved, the frequency of ectopic foot formation was 5%. These differences were statistically significant. The results strongly suggest

that these peptides are involved in determining axial positional values involved in foot formation.

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C–b. Division of Gene Expression

(1) Regulation of the *EDG84A* Gene by FTZ-F1 during Metamorphosis in *Drosophila melanogaster*

Takehide MURATA, Yuji KAGEYAMA, Susumu HIROSE, and Hitoshi UEDA

Transcription factor FTZ-F1 is a member of the nuclear hormone receptor superfamily and is transiently expressed during the mid- and late prepupal periods in *Drosophila melanogaster*. A putative pupal cuticle gene, *EDG84A*, is expressed slightly following FTZ-F1 expression during the prepupal period and carries a strong FTZ-F1 binding site between bases 100 and 92 upstream

of its transcription start site. In this study, *EDG84A* mRNA was found to be prematurely expressed upon heat induction of FTZ-F1 in prepupae carrying the heat shock promoter-FTZ-F1 cDNA fusion gene construct. Transgenic fly lines which had the 0.8-kb region of the *EDG84A* promoter fused to *lacZ* expressed the reporter gene in a tissue- and stage-specific manner. Base substitutions in the FTZ-F1 binding site within the 0.8-kb promoter abolished expression of *lacZ*. These results strongly suggest that the *EDG84A* gene is a direct target of FTZ-F1. Deletion studies of the cis-regulatory region of the *EDG84A* gene revealed that space-specific expression in imaginal disc-derived epidermis is controlled by the region -408 and -104 bp from the transcription start site. The region between bp-408 and -194 is necessary to repress expression in a posterior part of the body, while the region between bp-193 and -104 carries a positive element for activation in an anterior part of the body. These results suggest that FTZ-F1 governs expression of the *EDG84A* gene in conjunction with putative tissue-specific regulators. For details, see Ref. 1.

(2) Transcriptional Activation through Interaction of MBF2 with TFIIA

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Transcriptional activation of the *Drosophila melanogaster fushi tarazu* gene by FTZ-F1 or its silkworm counterpart BmFTZ-F1 requires two cofactors, MBF1 and MBF2, which do not directly bind to DNA. MBF1 is a bridging molecule that connects FTZ-F1 (or BmFTZ- F1), MBF2 and the TATA binding protein TBP. MBF2 is a positive cofactor that activates transcription (Li, F.-Q., Ueda, H., and Hirose, S., Mol. Cell. Biol., **14**, 3014–3021, 1994). To elucidate the mechanism of transcriptional activation by MBF2, we isolated the cDNA coding for the factor. Northern blot analyses showed temporally restricted expression of MBF2 mRNA similar to that of BmFTZ-F1 mRNA. The cDNA sequence predicts a polypeptide of 10 kDa whereas natural MBF2 is a glycoprotein of 22 kDa. The deduced amino acid sequence of the factor showed no homology with proteins in the databases. Farwestern analyses and glutathione *S*-transferase interaction assays demon-

strated that MBF2 makes direct contact with the β-subunit of TFIIA. In a HeLa cell nuclear extract, bacterially expressed recombinant MBF2 activated transcription from various promoters as natural MBF2 did. This activation requires the MBF2-TFIIA interaction. When recombinant MBF2 was added to the HeLa cell nuclear extract in the presence of MBF1 and FTZ622 bearing the DNA-binding region of FTZ-F1, it selectively activated transcription of the *fushi tarazu* gene. This selective activation also requires the MBF2-TFIIA interaction. From these results, we propose a model in which FTZ-F1 (or BmFTZ-F1) and MBF1 recruit MBF2 to a promoter carrying the FTZ-F1 site and MBF2 activates transcription through its interaction with TFIIA to allow selective activation in a FTZ-F1 site dependent manner. For details, see Ref. 2.

(3) Determination of Wing Cell Fate by the *escargot* and *snail* Genes in *Drosophila*

Naoyuki FUSE[1], Susumu HIROSE, and Shigeo HAYASHI[1] ([1] Laboratory of Invertebrate Genetics)

Insect appendages such as the wing and the leg are formed in response to inductive signals in the embryonic field. In *Drosophila*, cells receiving such signals initiate developmental programs which allow them to become imaginal discs. Subsequently, these discs autonomously organize patterns specific for each appendage. We here report that two related transcription factors, Escargot and Snail, expressed in the embryonic wing disc, function as intrinsic determinants of the wing cell fate. In *escargot* or *snail* mutant embryos, wing-specific expression of Snail, Vestigial and β-galactosidase regulated by the *escargot* enhancer were found as well as in wild-type embryos. However, in *escargot snail* double mutant embryos, wing development proceeded until stage 13, but the marker expression was not maintained in later stages, and the invagination of the primordium was absent. From such analyses, it was concluded that Escargot and Snail expression in the wing disc are maintained by auto- and crossactivation. Ubiquitous *escargot* or *snail* expression induced from the *hsp70* promoter rescued the *escargot snail* double mutant phenotype with the effects confined to the prospective wing cells. The similar DNA binding specificities of Escargot and Snail suggest that they control the same set of genes required for wing develop-

ment. We thus propose the following scenario for early wing disc development. Prospective wing cells respond to induction by turning on *escargot* and *snail* transcription, and become competent for regulation by Escargot and Snail. Such cells initiate auto- and crossregulatory circuits of *escargot* and *snail*. The sustained Escargot and Snail expression then activates *vestigial* and other target genes that are essential for wing development. This maintains commitment to wing cell fate and induces wing-specific cell shape change. For details, see Ref. 3.

(4) On a Critically Sensitive Period for the Methoprene-induced Supernumeral Larval Ecdyses in the Mulberry Silkworm, *Bombyx mori* (L.)

Akio MURAKAMI and Jun SHIMADA[1] ([1] Fac. of Agric., Tokyo Univ. of Agric. and Technology, Tokyo)

The process of metamorphosis from larval to pupal forms is principally controlled by developmental mechanisms, but this is to some extent influenced by certain endocrine mechanisms. The mechanisms are also concerned in the areas of the central nervous system and brain, involved with perception, recognition, and integration of environmental information. There is an accumulation of data on effects of juvenile hormone (JH) on metamorphosis in this insect. The action of JH has effects on several insect species in terms of larval maintainance or *status co*. However, the interaction between JH and brain function has not been analyzed with the silkworm, so far. In addition, the relationship between the supernumeral larval ecdysis events and regenerate of the mid-gut has not been elucidated in the insect. Therefore, we attempted to analyze the developmental processes of the JH analogue, methoprene-induced supernumeral larval ecdyses in the silkworm.

In this series of experiments, the biological materials used in this study were F_1 hybrid silkworms obtained from a cross between J106 females and Daizo males. The larvae were fed on mulberry leaves at 25℃ under standard conditions. Five different developmental groups, 48, 60, 72, 84 and 96 hr-old larvae after the 4th ecdysis, were prepared and 50 larvae were assigned to each group. The insects used in this experiment started spinning about 120 hr after the 4th ecdysis. Methoprene (GL Science, Tokyo) was used as a JH analogue, JH(a), which has a powerful function similar to JH. The chemical,

which was dissolved in acetone solutions at the rate of 10 μg per 10 ml acetone was applied to the larval body surface at 10 μg per body weight. Acetone alone was spread on larvae used as controls.

The results of the experiment clearly indicated that larvae at 48 hrs are indifferent to JH (a), but almost 50% of the treated larvae at 60 hr showed repeated larval molting. 84 hr larvae showed the most sensitive activity. A large number of supernumeral ecdysed larvae was detected among larvae ranging from 60 to 84 hrs. Most larvae at 96 hrs were normally developed into pupae, but less then 10% of the larvae were super molted again suggesting that older larvae are irreversibly directed to the next form. These observations showed that larvae ranging from 60 to 84 hrs are in a critical period for JH(a)-induced supernumeral ecdytsial events.

JH(a) is highly toxic to larvae at 48 and 60 hrs but all surviveing larvae were transformed to pupal forms, indicating that even the young last instar larva have the potential to start to some extent for the next form. The developmental process of the last larval stage before the critical period is a complicated situation: the larvae have not yet perfectly attained the state of metamorphosis into pupae but have the potential to do so. In any case, the last instar larvae are classified into three developmental steps: before and after the critical period, which ranges from 72 to 84 hrs. The critical period is the turning point for developmental direction, either normal pupal molting or repeated larval molting in the silkworm. During this period larval molting can occur again in contrast to the normal developmental process in this species. This suggests that even certain genetically determined developmental processes can be reversed by JH(a). This phenomenon seems to be exceptional, but the repeat of a small period of developmental growth in this insect may provide a clue to rejuvenescence. The larvae at 96 hr-old did not respond to the chemical, indicating that they had already irreversibly entered the metamorphosic process to the pupal form. The same view has been described by Sakurai (1985).

It is a well-known fact that tetramolting larvae at 48 hr-old have a significant amount of the JH titre, but not the ecdysone. No supernumeral ecdysial event has occurred in such a hormonal situation. There is a possibility that a JH receptor cell is involved in this developmentally stage specific phenomenon. It can be assumed that the receptor cell in young larvae just after the 4th ecdysis is inactivated by certain regulators under the control of the developmental mechanism. As will be made clear in the next

communication, the brain is a regulator of the JH receptor cell.

(5) Effects of Methoprene, a Juvenile Hormone Analogue, on the Induction of Supernumeral Molting under the Influence of Brain in the *Bombyx* Silkworm

June SHIMADA[1] and Akio MURAKAMI ([1] Fac. of Agric., Tokyo Univ. of Agric. and Technol. Tokyo)

This study was carried out to clarify the brain function on the methoprene induced in supernumerally molting larvae. The experimental materials and methods used in the research were the same as in the previous report except for certain kinds of operations, the extirpation and implantation of the brain. These operations were involved the ether anesthesia method at 20℃ (Shimada, 1989). Furthermore, certain larvae, which their brains removed were implanted with two brains per larva, obtained from two donors in the same developmental stage. The larvae were further kept for 12 hrs at 20℃ after anesthesia until complete recuperation. The larvae, which had brains removed, could not feed on mulberry leaves and they showed a fasting condition. Accordingly, the larvae on total abstinence from the leaves for several days were prepared as corresponding controls. Methoprene was also spread to the larvae starved as occasion demands. An ointment of methoprene dissolved in acetone solution was spread on the body surface of brainless larvae.

The results of the experiments under fasting conditions clearly showed that the supernumerary ecdysed larvae ranging from 72 to 84 hr-old were highly detected from 40 to 82% in the methoprene treated insects, but no supernumerary ecdysed larvae were observed in the sham operation as the control. The event was induced in 8% of the subject at 96 hr-old larvae. This feature was the same as the experimental group after treatment of larvae with methoprene under feeding condition as described in the previous communication.

In the brain extirpation group, all surviving larvae developed and/or metamorphosed to pupae, indicating that an effect of methoprene on the induction of the supernumerally ecdysed larva was not observed without the existence of the brain regardless of methoprene treatment.

In the next experiment, brain implantations were conducted to confirm the

experimental results. The experimental plane was limited to 20 larvae at 84 hr-old, because the operation is complicated and/or required much time. In this experiment, following extirpation, implantation of brain and application of methoprene, no dead larvae were detected and only two larvae among 20 insects developed to pupae, but the remaining 18 larvae exhibited supernumeral molting, and once again larval ecdysed. Thus, it can be concluded that in larvae treated with methoprene, the brain is absolutely required for induction of supernumerally ecdysed larvae.

Supernumeral events were not detected in brainless larvae regardless of treatment with methoprene. No supernumerally molt larvae at any stage were detected, in brainless larvae, irrespective of the post-spreading of methoprene in addition to a high frequency of death in larvae ranging from 48 to 84 hr-old. In other words, the brain has a critical function in the exertion of the original function of JH(a) or the JH function is under the control of the brain.

As is well-known, in the silkworm, JH titres of larvae during an early developmental stage of the last instar are significant, but this event did not take place. This contradiction can be explained on the basis of the hypothesis that an additional step, activation of the JH(a) receptor cells, as has been reported in numerous hormones, takes part in this biological event. At present, it appears that some substance secreted from secretary cells in the brain activates the receptor cell to receive JH(a) and consequently, the hormone becomes functional.

(6) On a Biological Clock in the Mulberry Silkworm, *Bombyx mori* (L.)

Akio MURAKAMI

In the silkworm, the daily rhythm of either hatching or eclosion is not detected in the strict sense of the word, because the egg-hatching never occurs again and/or the adult eclosion never occurs again. Nevertheless, some sericulturists have reported that hatching and/or eclosion in this insect is controlled by a circadian oscillator or biological clock system. Considering such a situation, we have analyzed biological rhythms on the level of the population.

We have been preserving several chronology mutants relative to both the

behaviors in the insect species. In the silkworm, normal hatching (h^+) and eclosion (e^+) behaviors begin 15 to 30 min after sun-rise and reach the maximum *ca.* 45 to 60 min after the start of these behaviors every day throughout the year. After that the behaviors usually decrease and are suspended about 1 hr later. The maximum frequency of these behaviors in normal stocks correctly changes with the seasons, indicating that both events are under the control of the movement of two heavenly bodies, the sun and the earth. For reference, the hatching event appears to some extent earlier than the eclosion. Accordingly, it seems unlikely to stand between certain circadian pacemaker systems and these biological phenomena.

Some larvae of a mutant stock showed a serious delay in hatching times for 4 to 5 hrs as compared with the normal stock, and in addition the stock is also *ca.* 15 min delayed for maximum time. A similar event is also observed in an eclosion mutant e^-. These phenomena seem to show a defect in time regulation mechanism in the brain rather than what is called the biological clock. A stock of h^+ (and e^+) showed earlier hatching (and eclosion) event than the normal stock (h^+ and/or e^+) and these stocks began their behavior before sunrise, suggesting that the unique behaviors are controlled by their own biological clock rather than the photoperiodic rhythm.

Accordingly, it is likely that these normal behaviors are under the control of both the biological clock and the movement of the heavenly bodies: the behaviors seemed to originally arise near sunrise.

When the embryos, at hatching time and/or near sunrise were kept in dark conditions, hatching was significantly prolonged and the remainder hatched the next day. This feature suggests the existence of a circadian oscillator. The embryos at hatching time were lit up for a very short time in the early morning around 2–3 o'clock, most of them hatched. The behavior is thought to be a reflex reaction to the flash light, since the behavior occurred a short time after illumination with the flash light, suggesting that these behaviors are mainly dependent on certain nervous systems. The same response seemed to be operating on the eclosion. Therefore, these biological behaviors in the silkworm are difficult to separate in term of the neurosecretory system and hormonal systems. Accordingly, it is unlikely that there is exclusive participation of the hormonal system in the behaviors. The instantaneous response seems to be involved in certain neurotransmittants.

Furthermore, adult exclusion consists of two steps: the first emerged from pupal cuticles and from cocoons in the next step. The primary behavior

occurs during the developmental process or their metamorphosis which slowly takes place during the latter half of the pupal stage within their cocoons. It is logical to assume that certain endocinological substances are concerned in the process to some exent. The moths survive on certain protein enzymes before emergence from their cocoon shells. This terminal event in the pupae seems to be regulated by either the strength (or weakness) of the sunlight conditions or their own circadian pacemaker or by both. Accordingly, it is possible to say that the behavior involves enzymes rather than hormonal substances. For reference, we are preserving a recessive mutant stock which shows an incomplete pupal ecdysis for adults.

(7) Biological Meanings of Silk Production in the Mulberry Silkworm, *Bombyx Mori* (L.)

Akio MURAKAMI

All living things ingest food to maintain life as well as to preserve their species. The silkworms use mulberry leaves to maintain their life, but why do they produce silk and/or cocoons. The cocoon may function to protect pupa from a various kinds of external hazardous factors. In fact, the silkworm spins silk fibers. In this report, the author considers the biological meaning of silk production in this insect.

It is axiomatic that the silkworm produces the proteinous fiber for their life, not for human beings. This statement is logical, even though, people are apt to think that the insect makes the silk for humans. This view is not acceptable from a biological viewpoint. The silkworm is regarded as an oligophagous insect feeds on mulberry leaves as its natural food. The leaves are complete in various kind of amino acids. The leaves are nutritional food stuffs for the silkworm larvae, but at the same time they contain many more non-essential amino acids. It is clear that the essential amino acids have a half of all kinds of fibroin and/or sericine, and contain large amounts of alanine (Ala), glycine (Gly), serine (Ser) and tryptophane (Try), indicating that these amino acids are of a non-essential type for the *Bombyx* larvae. As the larvae ingest a large quantity of both essential and non-essential amino acids from the mulberry leaves, the latter amino acids need to be excreted outsides of the body as polymeric silk proteins in neutralized forms. The more the insect feeds on mulberry leaves, the more nitrogenous substances

accumulate within the body. In general, an excess of nitrogenous substances in the body is toxic to the insect as in other organisms. This point requires attention in rearing the *Bombyx* silkworm as one step in detoxification of the unnecessary amino acids. Accordingly, the insect has to protect itself from the toxic substances. As is well known, the silk proteins are the product of polymerization of amino acids in silkglands. Polymerization of the amino acids during neutralization seems to be a sophisticated detoxification mechanism. It is clear that the silkworm excretes an excess of the non-essential amino acids for the larvae as the form of silk proteins rather than an objective product, suggesting that the silk production is necessary for maintaining life. In the silkworm, high productive lines often show physiological weakness, sensitivity to infection and so on. A mutant stock, *Nd*, is remarkably weak because this spineless line lacks the ability to spin the silk matter to the out side of the body, so the matter remains in the pupal-body. In this insect, an overfed larva is apt to have a shortened adult lifetime.

In domesticated animals, beef cattle, for example, the use of the entire or partial body is the object of breeding them. As learned from the mechanism of silk making, the strategy of *Bombyx* breeding is essentially different from that for mammals. However, the current breeding methods for silkworms is much the same as that for animals. It seems to be to force the larva to grow fat or to overfed it. In other words, it appears to force the insect to excrete the disused amino acids. But the silkworm appears to defend itself against from such rearing conditions. Consequently, it is necessary to improve the methods of rearing and/or breeding the domesticated silkworm in terms of better balanced dietetic control.

(8) Are Cultured Cells Situated between Uni- and Multicellular Organisms?

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Living organisms can be classified into unicellular and multicellular types according to the nature of their cell organization. Both types live as individual organisms. The latter type is composed of multiple cells, each of which does not act individually. In this type of organism, the central nervous system or brain maintains body function. Any cell of this class of organisms

has identical genetic information. However, each cell has a tissue specific character as a result of cell differentiation. Any cultured cell line, which is derived from a multiple organism, has a large genomic size compared to unicellulars.

Cultured cells, which are derived from various kinds of tissues in multicellular organisms, are free from the brain-nerve system and its related organs. Although cultured cells possess the same genome set as their original tissues, but only express the characteristics for living singly in culture media. Accordingly, nature may allow the cells to live a life of independence from the control of the brain-nerve system. It is likely that the primitive phase in the course of the establishment of cell lines takes place in three steps: the first step it becomes independent of cells from the original tissues, and the second one is adaptation of the cells to the culture medium, which is a novel environment for the cell line. In the adaptation of the cells, it should be necessary as a result of changes of gene expression in the new environmental condition. Thus, the cell line can be established an unicellular organism in the culture media independent of a subordinate state of the brain-nerve system.

The body structure and its activity in insects are regulated under the control of the brain-nerve and/or endocrine systems. In addition, it should be noted that a certain cultured cell line in insects is sensitive to ecdysone, as communicated in the previous report (Iwabuchi and Murakami, 1996). Certain ecdysone treated culture cells are transformed to the nerve cell type (Courgeon, 1972). Beermann and Clever (1964) reported a certain puffing on salivary gland chromosomes after the treatment of *Chironomus tentans* with ecdysone, suggesting a direct binding of the hormone to DNA. The response is considered to be the result of gene activation by ecdysone. Ecdysone binds to the hormone receptor (Turberg *et al.*, 1988) and finally to DNA (Turberg *et al.*, 1992) in the *C. tentans* cultured cell. From this evidence, it is thought that ecdysone directly activates gene transcription in cultured cells. It is also possible to say that certain cultured cell lines of the insect possess the ability to accept endocrinal regulation as does the original *in vivo* multicellular organism, suggesting that the culture cell may have an intracellular regulator. Consequently, it can be said that the cultured cell lines are generally more closely related to the multicellular organisms than to unicellular organisms.

(9) Origin of Holometabolous Metamorphosis in Insects

Kiyoshi MINATO

The evolutional origin of the holometabolous metamorphosis in endopterigote, which is characteristic to the specific developmental stage of pupa and the unique larval forms which are relatively simple and dissimilar to adult, which is one of the reasons insects have evolved so successfully, was investigated and analyzed through available literature and observations of the life styles of various insects in the fields.

Though the meaning of the pupal stage and how it originated in evolutionaly remain unclear, it seems now most reasonable that it corresponds to the last larval instar stage in the exopterigote, and that it has evolved as intermediate in the large morphological gaps between the larval and adult stages (Hinton, H. E., 1963b). However, considering the hormonal conditions during these stages in which the ecdysis into pupa occurs without any juvenile hormone, the pupal phase is likely to belong to the adult rather than the larval stage. The possibility that "the pupal stage is another one of the two adult stages (Poyarkeff, 1914; Hinton, 1948)" may remain to be reconsidered

The unique larval forms most different from those of adults in endopterygotes proved to have probably evolved in order to get actively the new styles of habitation and feeding, climbed and sticked to the labile, complicated, and sometimes large structure of grasses and trees, and bored or dipped into some three-dimensional structures such as wood, fruits, pasty-liquids, and soils, the both circumstances of which must have appeared newly in the process of evolution, rather than in order to get the advantages of the unnecessary of the competition for the same habitat and food between the larval and adult stages.

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snail genes in *Drosophila*. Development, **122**, 1059–1067, 1996.

C–c. Division of Physiological Genetics

(1) A New Class of Transcription Factor DSIF, Which Regulates RNA Polymerase II Processivity before Transcription Initiation

Tadashi WADA, Toshiyuki TAKAGI, Anwarul FERDOUS, Yuki YAMAGUCHI, and Hiroshi HANDA (Faculty of Bioscience and Biotechnology, Tokyo Institute of Technology)

The purine nucleotide analog 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) has been used to investigate the mechanism of transcription regulation *in vivo* and *in vitro*. We investigated the mechanism of DRB by using an *in vitro* reconstituted transcription system. We purified a DRB sensitivity inducing factor (DSIF) from HeLa cell nuclear extracts. DSIF is composed of at least two subunits with molecular masses of 160 kDa (p160) and 14 kDa (p14) that confer DRB-mediated transcription inhibition (DMTI) of RNA polymerase II and act as DRB-mediated elongation inhibitors. Biochemical studies on DMTI with a kinetically synchronized transcription reaction demonstratet that DSIF activity can be blocked by an unidentified protein phosphorylation which is inhibited by DRB and this phosphorylation event takes place before transcription initiation, but affects the processivity of RNA polymerase II during elongation. Isolation of a corresponding cDNA identified DSIF p160 as a 1087 residue protein with an extremely acidic amino terminus and a novel six amino acid repeat at the carboxyl terminus (consensus=K/R-T-P-A/M-Y-G). In a search of the available sequence databases it was found that, p160 has strong homology to the yeast SPT5 and a *Caenorhabditis elegans* SPT5 homolog of unknown function. Recombinant Supt4h protein which is encoded by a human homolog of yeast SPT4 gene was functionally equivalent to DSIF p14, suggesting that DSIF comprises human homologs of SPT5 and SPT4. The convergence of biochemical studies on DSIF and genetic analysis of SPT5 and SPT4 suggest that DSIF works as an elongation factor and regulates RNA polymerase II processivity.

(2) Analysis of the Mammalian Genome by Fluorescence *in situ* Hybridization

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We analyzed the structures and DNA replication in the human and mouse genomes using fluorescence *in situ* hybridization (FISH). We assigned the chromosomal localization of various genes by using cDNA or genomic clones. For details, see Ref. 19–26, 28, 29.

On the other hand, FISH was used to analyze how DNA replication timing is related to genome structure and how the specific genome domains are organized in the nucleus.

First, we determined the replication timing patterns of DNA segments within imprinted gene regions, such as those for *Igf2*, *Igf2r*, and Prader-Willi/Angelman Syndrome (PW/AS). Asynchronous replication timing was observed in the large area of each domain. These regions may consist of long-range replication domains which cover larger regions than those subjected to genomic imprinting. In particulor, we found that the PW/AS region consists of smaller replication timing domains in which either the paternal or maternal allele replicates earlier than the other. To analyze the intranuclear organization of imprinted gene regions, mouse embryonic fibroblast cells were cultured on microscope slides, and fixed with paraformaldehyde to keep the three dimensional structure of the nuclei. The nuclei were subjected to multicolor FISH using the three probes in the *Igf2r* domain. The FISH signals of the early replicating allele were dispersed in the nuclei, whereas those of the late replicating allele occupied a compact area, before and after their DNA replication. The experiment using the probes in the PW/AS region showed the same results, strongly suggesting that the imprinted gene regions are regulated to form the allele specific genome organization in the nuclei. For details, see Ref. 31.

Other studies on DNA replication timing and the chromosome bands were done in the human MHC region, as descriped in Ref. 17, 32.

We also studied biosynthesis and metabolism in NAD and its related compounds. For the details, see Ref. 18, 27, 30.

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D. DEPARTMENT OF POPULATION GENETICS

D–a. Division of Population Genetics

(1) Variation in Synonymous Substitution Rates Among Mammalian Genes and the Correlation Between Synonymous and Nonsynonymous Divergences

Tomoko OHTA and Yasuo INA

Using mammalian gene sequences, the variances in the numbers of synonymous and nonsynonymous substitutions among genes were estimated together with the correlation coefficient between the two. The expected correlation coefficient can be obtained under the neutral theory using these estimated values of the variances. The expected coefficient is found to often be one-half to two-thirds of the observed value. Possible causes for the disagreement were discussed, such as correlated selective constraints on the two types of substitutions and excess doublet mutations. The variance of mutation rate and that of selective constraint were also estimated. The results show that the coefficient of variation of the former is 0.2–0.3, whereas that of the latter is 0.7–0.9. For details, see Ref. 1.

(2) Population Biology of Antigen Presentation by MHC Class I Molecules

Peter PARHAM and Tomoko OHTA

In principle, the function of major histocompatibility complex (MHC) molecules is simple: to bind a peptide and engage a T cell. In practice, placing this function within the context of the immune response begs questions of population biology: How does the immune response emerge from the interactions among populations of peptides, T cells, and MHC molecules? Within a population of vertebrates, how does MHC polymorphism stamp individuality on the response? Does polymorphism confer differential advantages in responding to parasites? How are the pressures on the MHC reflected in

turnover of alleles? Examination of MHC molecules of North and South American Indians showed that allelic turnover occurred only in South populations. The roles of mutation, recombination, selection, and drift in the generation and maintenance of MHC class I polymorphism were discussed. For details, see Ref. 2.

(3) The Current Significance and Standing of Neutral and Nearly Neutral Theories

Tomoko OHTA

Comparative studies of DNA sequences provide opportunities for testing the neutral and the selection theories of molecular evolution. In particular, the separate estimation of the numbers of synonymous and nonsynonymous substitutions is a powerful tool for detecting selection of the latter. The difference in the patterns of these two types of substitutions of mammalian genes turned out to be in accord with the slightly deleterious or nearly neutral mutation theory for nonsynonymous changes. Interaction systems at the amino acid level were suggested to be responsible for such nearly neutral, or very weak selection. Synonymous substitutions are not strictly neutral, but because of their minute effect, random drift predominates such that the rate of substitution is only slightly less than the completely neutral prediction. It was concluded that the strictly neutral theory has not held up as well as the nearly neutral theory, yet remains invaluable as a null hypothesis for detecting selection. On the other hand, the main difference between the nearly neutral and the traditional selection theories is that the former predicts rapid evolution in small populations, whereas the latter predicts rapid evolution in large populations. For details, see Ref. 4.

(4) Study of Gene Evolution as Observed as Interspecific Hybrid Anomaly in *Drosophila*

Toshiyuki S. TAKANO

Loss of macrochaetes of the notum is one of the anomalies seen in interspecific hybrids between *D. melanogaster* and its closely related species. The emergence and divisions of sensory mother cells was monitored by using

a transformant line, A101 and a rabbit anti-asense antibody. There was no clear anomaly in late third instar larvae nor in pupae up to 1 hr after puparium formation (APF). Immunostaining using the mouse antibody 22C10 detected no neurons at many sites in the hybrid pupae of 25 hr APF, which means no double neuron phenotype. It was also found that hybrid pupae of 15 hr APF had no or very reduced levels of staining with the anti-cut antibody at the significant number of sites. These results suggest that the defect does not lie in the cell fate decisions during the development of bristles, but in the maintenance of neural fate and/or differentiation of the descendants of SMCs. Interspecific hybrids between a line of *D. melanogaster* and *D. simulans* iso-female lines exhibited a wide range in the number of missing bristles on the thorax. On the contrary, *D. mauritiana* and *D. sechellia* lines showed almost no reduction in bristle number in hybrids with *D. melanogaster*. This suggests that at least one of mutations occurred recently along the *D. simulans* lineage. Genetic analysis clarified a significant and partially recessive effect of the X chromosome of *D. simulans*, although gene(s) on the autosome(s) should interact with the gene(s) on the X chromosome. In attempts to isolate genes responsible for the bristle anomaly, deficiency screening was performed. QTL mapping based on the within-species variation of *D. simulans* was done as well by using nine length and SSCP polymorphism on the X chromosome. These screenings resulted in the identification of three candidate regions.

(5) Lineage-dependent Protein-sequence Evolution of *Drosophila* Genes

Toshiyuki S. TAKANO

The *achaete-scute* complex consists of the *achaete* (ac), *scute* (sc) and *lethal of scute* (*l'sc*) proneural genes, and the neural precursor gene *asense* (ase). All of these genes encode basic helix-loop-helix (bHLH)-type transcription factors. The DNA sequence of these four genes from *D. yakuba* were determined in order to compare their evolutionary rate. While there was no significant variation in synonymous substitution rate among the four genes, the ac gene accumulated significantly larger number of replacement substitutions than the other three genes based on the expectation from the estimates of the number of synonymous substitutions. This is mostly due to

a high replacement substitution rate along the lineage leading to *D. yakuba*. When the sequences were compared between *D. melanogaster* and its close relative, *D. simulans*, the ratio of replacement to synonymous substitutions for the ase gene was found to be significantly higher along the lines leading to these two species than along the *D. yakuba* lineage. In sum, these data suggests a significant variation in protein sequence evolution among the four AS-C genes, depending on lineage.

(6) Variance and Covariance of the Number of Amino Acid Substitutions Estimated by Kimura's Method

Yasuo INA

Since the process of amino acid substitution is stochastic, it is of great importance to consider the variance and covariance of the number of amino acid substitutions. Formulae for the variance and covariance of the number of amino acid substitutions estimated by Kimura's method are presented. The bootstrap method shows that the formulae are accurate. The formulae are useful for studies of molecular evolution, e.g., reconstruction of a minimum-evolution tree and the relative-rate test. For details, see Ref. 6.

(7) Correlation between Synonymous and Nonsynonymous Substitutions and Variation in Synonymous Substitution Numbers

Yasuo INA

To study the correlation between synonymous and nonsynonymous substitutions, two models of nucleotide substitution were considered. It was assumed that mutation rate and functional constraints on nucleotide changes are mutually independent in the first model, whereas these parameters are correlated with each other in the second model. Using an analytical method and computer simulations, the correlation coefficient between the numbers of synonymous and nonsynonymous substitutions under these two models was investigated. A correlation between functional constraint on synonymous sites and that on nonsynonymous sites does not strongly affect the correlation coefficient between the estimated numbers of synonymous and nonsynony-

mous substitutions, unless estimation errors are small. On the other hand, variation in mutation rate has a strong effect on increasing the correlation coefficient. For details, see Ref. 7.

(8) Pattern of Synonymous and Nonsynonymous Substitutions: An Indicator of Mechanisms of Molecular Evolution

Yasuo INA

Comparison of numbers of synonymous and nonsynonymous substitutions is useful for understanding mechanisms of molecular evolution. In this paper, I examine the statistical properties of six methods for estimating numbers of synonymous and nonsynonymous substitutions. The six methods are Miyata and Yasunaga's (MY) method; Nei and Gojobori's (NG) method; Li, Wu, and Luo's (LWL) method; Pamilo, Bianchi, and Li's (PBL) method; and Ina's (Ina) two methods. When the transition/transversion bias at the mutation level is strong, the numbers of synonymous and nonsynonymous substitutions are estimated more accurately by the PBL and Ina methods than by the NG, MY, and LWL methods. When the nucleotide-frequency bias is strong and distantly related sequences are compared, all the six methods give underestimates of the number of synonymous substitutions. The concept of synonymous and nonsynonymous categories is also useful for analysis of DNA polymorphism data. For details, see Ref. 8.

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D–b. Division of Evolutionary Genetics

(1) Replication Timing for the GC Content Transition Area between Classes II and III in the Human MHC

Toyoaki TENZEN, Yoshihisa WATANABE, Asao FUJIYAMA, Katsuzumi OKUMURA, Asako ANDO[1], Hidetoshi INOKO[1], and Toshimichi IKEMURA ([1] School of Medicine, Tokai University)

The human genome is composed of long-range G+C% (GC%) mosaic structures thought to be related to chromosome bands. We previously reported an example of a boundary of megabase (Mb)-sized GC% mosaic domains at the junction area between major histocompatibility complex (MHC) classes II and III, proposing it as a possible chromosome band boundary. DNA replication timing during S phase is known to be correlated cytogenetically with chromosome band zones, and thus the band boundaries have been predicted to contain a switch point for DNA replication timing. In this study, to identify at the nucleotide sequence level the replication switch point during S phase, we determined the precise DNA replication timing for MHC classes II and III, focusing on the junction area. To do this we used PCR-based quantitation of nascent DNA obtained from synchronized human myeloid leukemia HL60 cells. The replication timing changed precisely in the boundary region with a 2-hour difference between the two sides, supporting the prediction that this region may be a chromosome band boundary. We supposed that replication fork movement terminates (pauses) or significantly slows down in the switch region, which contains dense *Alu* clusters, polypurine/polypyrimidine tracts, di-, tri- or tetranucleotide repeats,

and medium reiteration frequency sequences (MERs). As the nascent DNA in the switch region was recovered at low efficiency, we investigated whether this region is associated with the nuclear scaffold and found three scaffold-associated regions (SARs) in and around the switch region. We are also determining the replication timing for the region containing pseudoautosomal boundary (PAB) of human sex chromosomes in order to assign the boundary of the X inactivation domain. For details, see Mol. Cell. Biol., **17**, 4043–4050.

(2) Human Pseudoautosomal Boundary-Like Sequences: Their Expression and Involvement in Evolutionary Formation of the Present-Day Pseudoautosomal Boundary of Human Sex Chromosomes

Tatsuo FUKAGAWA, Yasukazu NAKAMURA, Katsuzumi OKUMURA, Masahiro NOGAMI, Asako ANDO[1], Hidetoshi INOKO[1], Naruya SAITOU, and Toshimichi IKEMURA ([1] School of Medicine, Tokai University)

The human genome is composed of long-range mosaic structures of G+C% (GC%), which are thought to be related to chromosome bands. We previously identified a boundary of Mb-level domains of GC% mosaic structures in the human major histocompatibility complex (MHC) and found in the domain boundary, a sequence very similar to pseudoautosomal boundary (PAB) sequences of human sex chromosomes. We designated it "PABL" and found many PABLs in the human genome. By analyzing six genomic and six transcribed PABLs, a core and consensus sequence of about 650 nt were defined; the 3′- and 5′-edges of the PABLs were strictly conserved. Northern blot analysis showed sizes of PABL transcripts to be 5–10 kb in length. Divergence time of PABLs was estimated to be 60–120 million years ago from an analysis of human PABLs and PABXY1 of seven primates, and the evolutionary rates deduced showed PABLs to have been under selective constraints. A model for evolutionary formation of the present pseudoautosomal boundary was proposed by postulation of illegitimate recombination between two PABLs. For details, see Hum. Mol. Genet., **5**, 23–32, 1996.

(3) Chromosomal Localization of the Proteasome Z Subunit Gene Reveals an Ancient Chromosomal Duplication Involving the Major Histocompatibility Complex

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Proteasomes are the multi-subunit protease thought to play a key role in the generation of peptides presented by major histocompatibility complex (MHC) class I molecules. When cells are stimulated with interferon γ, two MHC-encoded subunits, low molecular mass polypeptide LMP2 and LMP7, and the MECL1 subunit encoded outside the MHC are incorporated into the proteasomal complex, presumably by displacing the housekeeping subunits designated Y, X, and Z, respectively. These changes in the subunit composition appear to facilitate class I-mediated antigen presentation, presumably by altering the cleavage specificities of the proteasome.

Here we show that the mouse gene encoding the Z subunit (*Psmb7*) maps to the paracentromeric region of chromosome 2. Inspection of the mouse loci adjacent to the *Psmb7* locus provides evidence that the paracentromeric region of chromosome 2 and the MHC region on chromosome 17 most likely arose as a result of a duplication that took place at an early stage of vertebrate evolution. Traces of this duplication are also evident in the homologous human chromosome regions (6p21.3 and 9q33–q34). These observations have implications for understanding the genomic organization of the present-day MHC and offer insight into the origin of the MHC. For details, see Proc. Natl. Acad. Sci. (USA), **93**, 9096–9101, 1996.

(4) Multivariate Diversity in Codon Usage in *Escherichia coli* Sequences Determined by Genome Projects

Shigehiko KANAYA[1], Yoshihiro KUDO[1], Yasukazu NAKAMURA, and Toshimichi IKEMURA ([1] Faculty of Engineering, Yamagata University)

We developed measures (called Z-parameters in this study) which reflect the diversity of codon usage in *Escherichia coli* genes using principal compo-

nent analysis. Protein-production levels for 1500 CDSs (protein-coding sequences) proposed by genome projects in Japan and USA were estimated by a correlation equation between Z_1 and cellular protein content, which was obtained through analysis of the genes which were experimentally characterized. Through a profile analysis of Z_1 for *E. coli* sequences determined in the Japan genome project, we predicted additional 36 CDSs that had not yet been annotated in the International DNA Databases. Thirty-two out of the thirty-six CDSs could be assigned to presumed protein genes also through a BLASTX search of recent protein databases in the Genome Net in Japan. Detailed examination of the Z_1-parameter profile also led us to assess sequencing errors which cause frame-shift. For details, see CABIOS, **12**, 213–225, 1996.

(5) cDNA Cloning of the Human Homologues of the Mouse Ke4 and Ke6 Genes at the Centromeric End of the Human MHC Region

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cDNA clones corresponding to the HKE4 and HKE6 genes at the centromeric end of the HLA region on human chromosome 6p21.3 were isolated and characterized. The predicted amino acid sequences of HKE4 and HKE 6 exhibited 81.5 and 85.6% identity to the mouse homologues, Ke4 and Ke 6, respectively. HKE4 may encode a membrane protein with histidine-rich charge clusters. HKE6 possesses remarkable amino acid sequence conservation with several bacterial proteins with oxidoreductase function and also shows significant homology with the two unique functional domains containing the nucleotide cofactor binding site and the consensus motif characteristic of the members of the superfamily of short-chain alcohol dehydrogenases such as human and rat steroid and prostaglandin dehydrogenases. For details, see Genomics, **35**, 600–602, 1996.

(6) Tenascin-X Is Essential for the Development of Epicardium and Coronary Vessels

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Tenascin-X (TN-X), a new member of the tenascin family of extracellular matrix proteins, is ubiquitously expressed, with the highest levels detected in the heart and muscle. However almost nothing is known about the functions of TN-X during embryo development. To investigate roles of this extracellular matrix in vivo, we attempted to generate TN-X-deficient mice by gene targeting in embryonic stem (ES) cells. But TN-X (+/−) chimeric mice with a high level of ES cell contribution were not obtained. To evaluate embryonic viability in TN-X (+/−) chimeras, embryos at various developmental stages were examined. The TN-X (+/−) chimeric fetuses expressed a reduced amount of TN-X and showed two conspicuous defects, both of which led to generalized edema and embryonic death. One defect occurred during the development of the epicardium and coronary blood vessels in the heart. Congestion of coronary vessels in the epicardium and thickened epicardium were observed. The other defect involved massive necrosis of the hepatocytes in the liver. The epicardium and coronary vessels are tissues where TN-X is most highly expressed, and thus are assumed to be where its important functions lie. Our results suggest possible roles of TN-X in epicardial cell migration and the development of coronary blood vessels in a tight dose-dependent manner.

(7) Gene Organization of Human NOTCH4 and $(CTG)_n$ Polymorphism in This Human Counterpart Gene of Mouse Proto-oncogene Int3

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The cDNA and genomic clones for the human counterpart of the mouse mammary tumor gene Int3 were isolated and sequenced. We designated this human major histocompatibility complex (MHC) class III gene as NOTCH 4, since very recently, by sequencing cDNA clones, the complete form of the mouse proto-oncogene Int3 was clarified and named Notch4. The present human NOTCH4 sequence is the first example of the genomic sequence for the extracellular portion of mammalian Notch4, and by comparing it with the mouse Notch4 cDNA sequence, the exon/intron organization was clarified. Comparison of the predicted amino acid sequence of human NOTCH4 with those of other Notch homologues of a wide range of species revealed four subfamilies for mammalian Notch. In the protein coding region of human NOTCH4, we found $(CTG)_n$ repeats showing variable number tandem repeat (VNTR) polymorphisms for different human leukocyte antigen (HLA) haplotypes.

Here we show also that ten genes mapped on 6p21.3, including NOTCH4, have counterparts structurally and functionally similar to those mostly mapped on 9q33–q34, indicating segmental chromosome duplication during the course of evolution. Similarity of genes on chromosome 1, 6, 9 and 19 was also discussed. For details, see Gene, **189**, 235–244, 1997.

(8) Reconstruction of Gene Trees from Sequence Data

Naruya SAITOU

Reconstruction of the phylogeny of genes is essential not only for study of evolution but also for biology in general. This is because replication of nucleotide sequences automatically produce bifurcating tree of genes. It should be emphasized that the phylogenetic relationship of genes is different from the mutation process. The former always exists, while mutations may

or may not happen within a certain time period and DNA region. Therefore, even if several nucleotide sequences happen to be identical, there must be a genealogical relationship for those sequences.

However, it is impossible to reconstruct the genealogical relationship without occurrence of mutational events. In this respect, the extraction of mutations from genes and their products is also important for reconstructing phylogenetic trees of genes. The advancement of molecular biotechnology has made it possible to routinely produce nucleotide sequences. We will therefore focus on the analysis of nucleotide sequences. However, a substantial part of this chapter also applies to other molecular data. For details, see Ref. 25.

(9) The Presence/Absence Polymorphism and Evolution of the p53 Pseudogene in the Genus Mus

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Distribution of the p53 pseudogene within the house mouse species (genus Mus) was studied with polymerase chain reaction for thirty seven individuals that were caught at different localities. Pseudogene-specific fragments were detected in some, but not all, individuals of Mus musculus subspecies regardless of locality and type of subspecies. Besides, three of seven individuals belonging to different Mus species carried the pseudogene in their genomes. These results show an existence of an interspecific presence/absence polymorphism of the p53 pseudogene in mice. Sequence analysis of eleven amplified 0.3 kb fragments suggested that the pseudogene originated in an ancestral mouse about 10 million years ago. Thus alleles with and without the p53 pseudogene have persisted through the mice speciation. The evolutionary rate for the p53 functional gene was also estimated to be about 2.5 10–9 per nucleotide site per year. For details, see Ref. 26.

(10) Contrasting Gene Trees and Population Trees on the Evolution of Modern Humans

Naruya SAITOU

A gene tree is an essential descriptor of any evolutionary process, for the semiconservative replication of DNA double helix automatically produces a bifurcating gene tree. It should be emphasized that the genealogical relationship of genes is independent from the mutation process, especially when the neutral evolution is considered. The former is a direct product of DNA replication, while the latter may or may not happen within a certain time period and DNA region. Therefore, even if many nucleotide sequences happened to be identical, there must be a genealogical relationship for those sequences. However, it is impossible to reconstruct the genealogical relationship without mutational events. In this respect, extraction of mutations from genes and their products is critical for reconstructing phylogenetic trees. We first reviewed gene trees for HTLV-I DNA, then compared gene trees and population trees for extant and ancient mitochondrial DNA. Our recent studies on gene and population trees for nuclear DNAs were also reviewd. Lastly, Importance of finiteness for evolutionary studies was discussed. For details, see Ref. 28.

(11) Extensive Polymorphism of ABO Blood Group Gene: Three Major Lineages of the Alleles for the Common ABO Phenotypes

Kenichi OGASAWARA[1], Naruya SAITOU, and Katsushi TOKUNAGA[2] ([1] Japanese Red Cross Tokyo Metropolitan Blood Center, [2] University of Tokyo Medical School)

Polymorphism of the ABO blood group gene was investigated in 262 healthy Japanese donors by a polymerase chain reactions-single-strand conformation polymorphism (PCR-SSCP) method, and 13 different alleles were identified. The number of alleles identified in each group was 4 for A1 (provisionally called ABO*A101, *A102, *A103 and *A104 according to the guidelines for human gene nomenclature), 3 for B (ABO*B101, *B102 and *B103), and 6 for O (ABO*O101, *O102, *O103, *O201, *O202 and *O203). Nucleotide sequences of the amplified fragments with different

SSCP patterns were determined by direct sequencing. Phylogenetic network analysis revealed that these alleles could be classified into three major lineages, *A/*O1, *B and *O2. In Japanese, *A102 and *B101 were the predominant alleles with frequencies of 83% and 97% in each group, respectively, whereas in group O, two common alleles, *O101 (43%) and *O201 (53%), were observed. These results may be useful for the establishment of ABO genotyping, and these newly described ABO alleles would be advantageous indicators for population studies. For details, see Ref. 27.

(12) Molecular Genetic Analysis of Variant Phenotypes of the ABO Blood Group System

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ABO is clinically the most important blood group system in transfusion medicine and includes many variant phenotypes. To understand the molecular genetic basis of this polymorphic system, we have analyzed genomic DNAs obtained from Japanese individuals possessing variant ABO phenotypes including A2, Ax, Ael, cis-AB, Bx, and Bel. By polymerase chain reaction-single-strand conformation polymorphism (SSCP) and nucleotide sequence analyses, we identified 11 different alleles. These alleles had nucleotide sequences different from those of the previously described 13 different alleles responsible for the common ABO phenotypes. Analysis of the nucleotide sequences of the alleles responsible for those variant phenotypes showed that the amino acid residues at positions 266 and 268 may be crucial for transferase specificity, whereas those at positions 214, 216, 223, 291, and 352 may be critical for the activity level. Nine of the 11 alleles, responsible for the A2, Ax, Ael, cis-AB, Bx, and Bel phenotypes, were presumed to be generated from common ABO alleles by single nucleotide mutations such as nonsynonymous substitution, deletion, or insertion. Two other alleles, responsible for the A2, and Ael phenotypes, may have originated by recombination, gene conversionlike events or accumulation of nucleotide substitutions. Our data indicate that different alleles could cause the same ABO variant phenotypes, and that these alleles do not necessarily belong to a single evolutionary lineage. For details, see Ref. 30.

(13) Evolution of Class III POU Genes: Amplification of Characteristic Amino Acid Residues by G+C Pressure in Mammals

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The class III POU transcription factor genes play an important role in nervous system. Comparison of their entire amino acid sequences disclosed a remarkable feature of particular mammalian class III POU genes. Alanine-, glycine-, and proline-repeats were present in the mammalian Brain-1 gene, whereas most part of these repeats were absent in the non-mammalian homologue. The mammalian Brain-2 gene had alanine-, glycine-, proline-, and glutamine-repeats, which were missing in the non-mammalian homologue. The mammalian Scip gene had alanine-, glycine-, proline-, and histidine-repeats, but the non-mammalian homologue completely lacked these repeats. In contrast, the mammalian Brain-4 gene had no amino acid repeats like its non-mammalian homologue. The mammalian genes containing the characteristic amino acid repeats had another feature, the higher GC content. We found a positive correlation between the GC content and the amino acid repeats ratio. Those amino acids were encoded by triplet codons with relatively high GC content. These results suggest that the GC pressure has facilitated generation of the homopolymeric amino acid repeats. For details, see Ref. 29.

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D–c. Division of Theoretical Genetics

(1) Current Topics on Molecular Evolution

Masatoshi NEI[1] and Naoyuki TAKAHATA[2] ([1] The Pennsylvania State University, [2] The Graduate University for Advanced Studies)

This volume is the outcome of the US-Japan Binational Workshop on Molecular Evolution held at the Graduate University for Advanced Studies, Hayama, Japan, August 25–27, 1995, in conjunction with the Third International Meeting of the Society for Molecular Biology and Evolution. The purpose of the workshop was to promote collaborative research between young investigators from the United States and Japan in the area of molecular evolutionary biology. Since studies on molecular evolutionary biology are so dispersed, emphasis was given to the study of molecular phylogeny and evolution of adaptive characters. In each of these areas, seven representatives each from the United States and Japan presented papers. The papers were then exposed to critical comments by other participants. There was ample time for the representatives from both countries to get acquainted and discuss each subject matter in detail. This volume includes short versions of the twenty-eight papers presented at the workshop. They are divided into two parts: (1) Molecular phylogeny and (2) Evolution of adaptive characters. However, the subject matters discussed are quite broad, and the distinction between the two parts is not always clear-cut. We hope this volume will give some flavor of the enthusiastic workshop attended by young investigators. For details, see refs. 9–11.

(2) Neutral Theory of Molecular Evolution

Naoyuki TAKAHATA (The Graduate University for Advanced Studies)

DNA sequence data are generally interpreted as favouring Kimura's

neutral theory but not without dissent and often with a great deal of controversy. Here I review the current status of the neutral theory with special reference to molecular clocks, DNA polymorphism, adaptive evolution, and gene genealogy. While the theory serves as a guiding principle, many issues concerning mutation, recombination, and selection remain unsettled. Of particular importance is the need for more knowledge about the function and structure of molecules. For details, see Ref. 12.

(3) Multiregional or Uniregional Origin of Modern Humans: What Do Genetic Data Indicate?

Naoyuki TAKAHATA[1] and Jan KLEIN[2] ([1] The Graduate University for Advanced Studies, [2] Max-Planck-Institut für Biologie, Abteilung Immungenetik and Department of Microbiology and Immunology, University of Miami School of Medicine)

We examined hypotheses on the origin of *Homo sapiens* in terms of gene genealogy and compared the theoretical results with available genetic data. All data suggest (1) frequent gene exchange and/or extinction and recolonization of local populations; (2) a relatively short coalescence time of neutral genes or a relatively small effective population size for humans throughout the late Pleistocene; and (3) characteristic intra- and inter-populational genetic diversity among Africans. Any hypothesis that regards human evolution as static cannot explain these observations. The most important factor, but one that has been overlooked in the evolution of *H. sapiens*, appears to be the frequent extinction and recolonization of local populations. For details, see Ref. 13.

(4) Evolution of the Primate Lineage Leading to Modern Humans: Phylogenetic and Demographic Inferences from DNA Sequences

Naoyuki TAKAHATA and Yoko SATTA (The Graduate University for Advanced Studies)

For the dating of major divergences that occurred in the primate lineage leading to modern humans, and to infer a demographic parameter (effective

population size) of the ancestral lineage that existed prior to each divergence, a maximum likelihood (ML) method was applied to autosomal DNA sequence data currently available for pairs of orthologous genes between human and chimpanzee, gorilla, Old World monkey (OWM) and New World monkey (NWM). A statistical test was carried out to support the assumption that silent substitutions have accumulated in a clock-like fashion over loci between primate taxa or even among sites within a locus. It was shown that the human ancestral lineage became distinct from the NWM 57.5 Myr ago, the OWM 31 Myr ago, the gorilla 8.0 Myr ago, and the chimpanzee 4.5 Myr ago, and that the effective population size at these divergences was generally much greater than that of modern humans. It was argued that the human ancestral lineage branched off from the NWM and OWM earlier than once thought and that significant demographic changes might have occurred at different evolutionary stages, particularly at the hominid stage. For details, see Ref. 16.

(5) Studies on the Structure of Visual Pigments Responsible for the Function of Photoreceptor Cells

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In spite of the high similarity in amino acid sequence between rod visual pigment rhodopsin and gecko blue-sensitive pigment (gecko blue), not only the spectral sensitivities but also the thermal decay rates of the meta II- and III-intermediates are noticeably different from one another. In order to identify the protein region(s) that contain(s) key residues being responsible for the functional difference, we constructed six chimerical mutants derived from gecko blue and bovine rhodopsin, with the aid of protein production in a human embryonic kidney cell line (293S). While the absorption maximum of every mutant was located in between gecko blue (466 nm) and bovine rhodopsin (500 nm), a large blue-shift (18 nm) was observed when helices I–III of rhodopsin were replaced with those of gecko blue. A time-resolved

spectroscopic study demonstrated that this replacement also accelerated the decay rate of the meta II-intermediate. The decay of the meta III-intermediate of the mutants became faster as the compartment of gecko blue was increased. Thus, the faster decay of the meta II-intermediate of gecko blue is largely attributed to residues within helices I–III, while the decay of the meta III-intermediate apparently depends on the overall structure of the protein. For details, see Ref. 1.

(6) Functional Analysis of Lipid Modifications on Photoreceptor G-Protein

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Photoreceptor G protein transducin [α- and $\beta\gamma$-subunits (Tα/T$\beta\gamma$)] play a central role in the visual transduction process. The amino-terminus of bovine Tα is modified by one of four distinct fatty acids—laurate (C12 : 0), myristate (C14 : 0), C14 : 1 (5-*cis*), and C14 : 2 (5-*cis*, 8-*cis*)—but the biological significance and the localization of the four isoforms of Tα are poorly understood. To investigate the cellular distribution of each isoform, we prepared monoclonal antibodies against a synthetic C12 : 0-, C14 : 0-, C14 : 1-, or C14 : 2-nonapeptide corresponding to the N-terminal region of Tα. Among several types of antibodies isolated, only one type, represented by LA4, reacted specifically with the C12 : 0-peptide as well as purified Tα but not with the other proteins in bovine retinal homogenate, including recoverin, indicating that the epitope comprises both C12 : 0 and the N-terminal amino acids of Tα. Immunohistochemical analyses of bovine retinal sections by LA4 showed the uniform distribution of C12 : 0-Tα in almost all the rod outer segments. Hence, it seemed unlikely that each isoform of Tα was localized in specific cells. This observation, together with evidence for a possible functional diversity among the isoforms, suggests that the four isoforms of Tα in a single rod cell may contribute simultaneously to a fine

tuning of the photon-signal transduction process. For details, see Ref. 3.

On the other hand, hydrophobic modifications of Tγ, such as farnesyl- and carboxyl-methylation, are essential for the association of T$\beta\gamma$ with the photoreceptor disc membrane, and MEKA/phosducin is known to inhibit the association. We examined the effect of MEKA on the hydrophobicity of T$\beta\gamma$. MEKA could bind to T$\beta\gamma$ without farnesyl/carboxyl-methyl moieties as well as native T$\beta\gamma$. In the triton X-114 phase separation assay, T$\beta\gamma$-MEKA complex was recovered in the aqueous phase, whereas T$\beta\gamma$ was recovered in the detergent phase. The N-terminal portion of MEKA which includes T$\beta\gamma$-binding domain was not sufficient to reduce the hydrophobicity of T$\beta\gamma$ or to dissociate T$\beta\gamma$ from the membrane. The data suggest that MEKA attenuates the hydrophobicity of T$\beta\gamma$ for dissociating T$\beta\gamma$ from the membrane without directly binding to farnesyl/carboxyl-methyl moieties. For details, see Ref. 5.

(7) Functional Studies on the Ca^{2+}-Binding Protein in Photoreceptor Cells

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Recoverin, a new member of the EF-hand superfamily, plays a critical role in the light/dark adaptation of retinal rods by regulating rhodopsin phosphorylation in a Ca^{2+}-dependent manner. In rods, Ca^{2+}-bound recoverin associates with disk membranes and inhibits light-dependent phosphorylation of rhodopsin. However, the functional significance of Ca^{2+}-induced membrane association of recoverin has not been fully evaluated. We found that Ca^{2+}-bound recoverin forms a complex with rhodopsin kinase preferentially at the membrane surface. The addition of increasing amounts of membranes promoted the membrane association of recoverin, and remarkably suppressed rhodopsin kinase activity. It was concluded that the Ca^{2+}-recoverin-rhodopsin kinase complex is stabilized by membrane association, leading to effective suppression of kinase activity. For details, see Ref. 4.

(8) Studies on Photoreception in the Brain.

Yoshitaka FUKADA, Toshiyuki OKANO[1], Kamon SANADA[1], Tomoko YOSHIKAWA[1], and Yoko TAKANAKA[1] ([1] Department of Biophysics and Biochemistry, Graduate School of Science, The University of Tokyo)

We are studying the molecular mechanism of the light-dependent phase-shift of the chicken pineal circadian clock system. Investigation of the photon-signalling pathway mediated by pinopsin will be one fruitful approach to the circadian oscillator.

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E. DEPARTMENT OF INTEGRATED GENETICS

E–a. Division of Human Genetics

(1) Isolation of Transcribed Sequences in Cloned Genomic DNA with Known Location on the Human Chromosome 18

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Identification and recovery of transcribed sequences from cloned human genomic DNA remain an important problem in isolating genes on the basis of their chromosomal location. Tetrasomy 18p and trisomy 18 syndromes are caused by an excessive number of genes on chromosome 18. To identify genes involved in these disorders, construction of high-resolution expression map of this chromosome is a rational strategy. We have developed a strategy that facilitates the recovery of transcribed sequences from random pieces of cloned genomic DNA. Thus, we isolated 600 cosmid clones of which 60 new cosmids are mapped on the short and long arms of chromosome 18 either by R or by DAPI banding and simultaneous fluorescence *in situ* hybridization. Our approach to generate region-specific cDNA libraries used hybridization of cDNA library inserts to genomic DNA from cosmids, and subsequent cloning of annealed cDNAs. In one application of the scheme, DNA fragments of a digested cosmid were ligated to an oligonucleotide linker/adaptor, which were hybridized with human fetal brain cDNA library bound to the surface of streptavidin-coated magnetic beads, and cDNAs thus selected were amplified by PCR before cloning.

While considerable progress has been made in genetic and physical mapping of the human genome, approaches are now needed to identify the protein coding sequences contained therein. Although most genes are composed of multiple exons, the identification of a gene requires the recovery of only a single exon. These candidate exons are ideally suited for establishing the presence of a gene in a cosmid insert and facilitating the subsequent isolation of this gene. Our current experience suggests that every one or two cosmids contain at least one transcribed sequence. Further screening of unchar-

acterized cosmids will determine the utility of transcribed sequence fragments of known chromosomal location, as identifiers of candidate coding sequences. For the details, see reference 1.

(2) Molecular Definition of Maternally Imprinted Genes on Chromosome 15 and the Development of Prader–Willi Syndrome

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The Prader–Willi syndrome (PWS) is characterized by generalized muscular hypotonia and severe feeding problems in early infancy, followed by hyperphagia and subsequent central obesity in childhood. In addition to short statue and characteristic dysfunction causing hypogonadism, dysmorphic signs such as almond-shaped palpebral fissures, narrow bifrontal diameter, down-turned mouth and acromicria become evident. In time, patients may manifest intellectual deficiency, behavioral abnormalities, and poor articulations. Originally defined as a clinical entity, PWS was later found to be associated with abnormalities of chromosome 15, in particular a small interstitial deletion of bands q11–q13 of the paternally derived chromosome 15. Non-deletion PWS patients exihits maternal disomy for chromosome 15, further demonstrating that loss of the expressed paternal alleles of maternally imprinted genes is responsible for the PWS phenotype. Thus, PWS is a distinct neurogenic disorder, caused by the loss of function of a gene or closely linked genes on chromosome 15. Paternal deletion and maternal disomy in PWS suggest that the PWS genes are transcribed from the paternal chromosome only. A system was developed in which subtraction and kinetic enrichment was used to purify restriction endonuclease fragments present in a population of cDNA fragments from the normal individual but not in the other from the patient. Application of this method to cDNA library of reduced complexity resulted in isolation of more than 20 probes present as single copies that are down-regulated for their expression in the PWS library. These probes could detect difference of levels between patient's cells and those of normal individuals.

A mechanism of imprinting may have evolved in mammals because of conflicting interests of maternal and paternal genes in relation to the transfer

of nutrients from the mother to her offspring. This hypothesis predicts most imprinted genes will affect how much nourishment an offspring receives from its mother at the expense of its siblings. Imprinted genes will include loci that influence placental growth, neonatal behavior, suckling and appetite control, nutrient metabolism, and postnatal neuronal growth. The mapping of genes identified here to within the PWS critical region, together with the indications that they function in the brain and may play a role in regulating neuronal growth and feeding behavior as well, should prompt us further to predict these genes as a candidate imprinting loci responsible for syndrome.

(3) Interleukin-4 Induces Association of the *c-fes* Proto-oncogene Product with Phosphatidylinositol-3-kinase and Activates Two Distinct Signal Transduction Pathways

Kenji IZUHARA

Interleukin-4 (IL-4) induces tyrosine phosphorylation of a protein closely related to the *c-fes* proto-oncogene product (FES), and association of this protein with IL-4 receptor α chain (IL-4Rα). IL-4 is known to induce association of phosphatidylinositol-3 (PI3) kinase with IL-4Rα. Since FES contains the consensus motifs for binding to PI3, FES may associate with PI 3 kinase upon IL-4 stimulation. We demonstrated that IL-4 stimulation induced in mouse T cell lines rapid association of FES (or a related protein) with PI3 kinase, and that human FES (hFES) associated with *src* homology 2 (SH2) domain of PI3 kinase in a COS7 cell expression system. The *in vitro* PI3 kinase assay using COS7 cells suggestes that hFES partly contributes to an association between hIL-4Rα and PI3 kinase. We further identified the important region in the cytoplasmic domain of hIL-4Rα for the association of tyrosine-phosphorylated hFES with hIL-4Rα and SH2 domain of PI3 kinase using a COS7 cell expression system. These results suggest that the FES (or a related protein)-PI3 kinase pathway may play a role in the pleiotropic effects of IL-4.

c-Fes protooncogene product (or a FES-related protein) associates with IL-4Rα and PI3 kinase in mouse T cell lines. PI3 kinase has been reported to associate with IL-4Rα through tyrosine-phosphorylated-insulin receptor substrate (IRS-2) in other cell types. We analyzed association of PI3 kinase with IRS-2, and tyrosine-phosphorylation of IRS-2 in a mouse pro-B cell line,

Ba/F3, and a mouse mast cell line, MC9. In both cell lines, IL-4 induced tyrosine phosphorylation of IRS-2, association of PI3 kinase with IRS-2, FES or a FES-related protein. These results indicate that IL-4 activates two distinct PI3 kinase pathways in the same cells. We further identified the critical region in the cytoplasmic domain of IL-4Rα for tyrosine phosphorylation of IRS-2. For the details, see Ref. 2 and 3.

(4) Mitochondrial DNA Polymorphism in East Asian Populations, with Special Reference to the Peopling of Japan

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Nucleotide sequences of the major noncoding (D-loop) region of human mitochondrial DNA from five East Asian populations including mainland Japanese, Ainu, Ryukyuans, Koreans and Chinese were analyzed. Based on a comparison of 482 base pair sequences in 293 East Asians, 207 different sequence types were observed. Of these, 189 were unique to their respective populations, whereas 18 were shared between two or three populations. Among the shared types, eight were found in common between the mainland Japanese and Koreans, which is the largest number in the comparison. The intergenic COII/tRNA^{Lys} 9 base pair deletion was observed in every East Asian population with varying frequencies. The D-loop sequence variation suggests that the deletion event occurred only once in the ancestry of East Asians. Phylogenetic analysis revealed that East Asian lineages were classified into at least 18 monophyletic clusters, though lineages from the five populations were completely intermingled in the phylogenetic tree. However, we assigned 14 out of the 18 clusters for their specificity based on the population from which the maximum number of individuals in each cluster was derived. Of note is the finding that 50% of the mainland Japanese had continental specificity in which Chinese or Koreans were dominant, while less than 20% of either Ryukyuans or Ainu possessed continental specificity. Phylogenetic analysis of the entire human population revealed the closest genetic affinity between the mainland Japanese and Koreans. Thus, the

results of this study are compatible with the hybridization model on the origin of modern Japanese. It is suggested that about 65% of the gene pool in mainland Japanese was derived from the continental gene flow after the Yayoi Age. For details, see Ref. 4.

(5) The Origin and Dispersal of Modern Humans as Viewed from Mitochondrial DNA

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Modern Homo sapiens originated in Africa somewhere between 125,000 and 161,000 years ago. Since then, a number of migrations out of Africa have taken place, one of the longest journeys being the peopling of the New World. To investigate the peopling all over the world, we examined the mtDNA sequence variations in the D-loop region. We have compared nucleotide sequences of the D-loop region for 271 individuals from six major geographic regions: the Americas, Africa, Europe, Southeast Asia, East Asia, and Papua New Guinea. The nucleotide diversity is highest in Africans, which is estimated at 2.11%, while the lowest value is observed in Europeans, 0.96%. Almost the same values are observed in four Mongoloid populations such as Asians from two regions, Native Americans and Papuans, although sampling size and nature of the populations are different; these range from 1.30 to 1.37%. On the basis of pairwise number of nucleotide substitutions, a phylogenetic tree was constructed for 271 humans. It is obvious that most Africans located near the root of the tree, while some appeared in various parts of the tree. Most Europeans fell into a final cluster, which is far from the root. This agrees with the lowest value of nucleotide diversity observed in Europeans. About 50,000 years ago, a sea level was over 80 meters below what it is today. The lower sea level connected the Australian continent and Papua New Guinea, and exposed Sahul land. The first appearance of humans in this region took place around this time. The Gidra people in Papua New Guinea are considered to be descendants of such early migrants, who speak a non-Austronesian language. Some Papuans appeared in early diverging clusters near the root, while the rest fell into four other clusters in different locations of the tree. East Asians such as Japanese, Koreans and mainland

Chinese are dispersed throughout the tree. The same applies to Southeast Asians such as Thais, Malays and Indonesians. Although 21 individuals were analyzed, they are dispersedly located in the tree. Contrary to the Asian populations, the majority of Native Americans fell into four distinct clusters. Assuming the deepest root of human mitochondrial divergence as 143,000 years ago, we estimated coalescence times of each human population; the longest coalescence time was observed in the African population, and the shortest in the European population. It is to be noted that Papuans also showed a deeper coalescence time, which was estimated around 124,000 years ago. This may indicate that ancestors of some Papuans were the earliest migrants of Homo sapiens who went out of Africa. For details, see Ref. 5.

(6) Molecular Phylogeny of Macaques: Implications of Nucleotide Sequences from an 896 Base Pair Region of Mitochondrial DNA

Kenji HAYASAKA, Kunihiko FUJII, and Satoshi HORAI

We determined the nucleotide sequences of an 896 base pair region of mitochondrial DNA (mtDNA) from 20 primates representing 13 species of macaques, a baboon, and a patas. We compared these sequences and the homologous sequences from four macaques and a human against each other and deduced the phylogenetic relationships of macaques. The results from the phylogenetic analyses revealed five groups among the macaques: (1) Barbary macaque, (2) two species of Sulawesi macaques, (3) Japanese, rhesus, Taiwanese, crab-eating, and stump-tailed macaques, (4) toque, pig-tailed, and lion-tailed macaques, and (5) Assamese and bonnet macaques. The phylogenetic position of Tibetan macaque remains ambiguous as to whether it belongs to the fourth or fifth group. Phylogenetic trees revealed that Barbary macaque diverged first from the other Asian macaques. Subsequently, the four groups of Asian macaques diverged from one another in a relatively short period of time. Within each group, most of the species diverged in a relatively short period of time following the divergence of the groups. Assuming that the Asian macaques diverged from the outgroup Barbary macaque three million years ago (MYA), the divergence times among groups of Asian macaques were estimated at 2.1–2.5 MYA, and within groups at 1.4–2.2 MYA. The intraspecific nucleotide diversity ob-

served among three rhesus macaques was so large that they did not form a monophyletic cluster in the phylogenetic trees. Instead, one of them formed a cluster with Japanese and Taiwanese macaques, while the other two formed a separate cluster. This implies that either polymorphisms of mtDNA sequences which existed before the divergence of these three species (ca. 700,000 years ago) have been retained in rhesus macaques or introgression has occurred among the three species. For details, see Ref. 6.

(7) Detection of DNA Fragments Encompassing the Deletion Junction of Mitochondrial Genome

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Rearrangements of human mtDNA are associated with not only mitochondrial diseases, including CPEO and Pearson's marrow-pancreas syndrome, but also with the aging process in various tissues such as the brain. Both Southern blot analysis and the PCR are usually applied to the detection of deletions in mtDNA because these methods have their own advantages. We here describe a combined method of a long PCR and digestion with three restriction enzymes that simplifies the detection of the deletion site and enables us to determine the adjacent junctional sequences. The long PCR method together with restriction enzyme digestion is also useful to map the deletion site on mtDNA, and it is simple to estimate the deletion site. One of the reasons why we chose Xba I, Hind III and Bam HI is that it is convenient to use the three enzymes in a single reaction tube because the optimal enzymatic conditions (e.g., salt concentration) are almost the same. Another reason is that polymorphisms associated with the three enzymes are uncommon. Among 120 Japanese, only one morph has been observed in XbaI and BamHI, and the most frequent morph in Hind III is seen in 97.5%. The above estimation immediately allows sequencing of the deletion junction. The determination of junctional sequences is essential for considering the probes within and without the deletion to detect a possible duplication on Southern blot. Because duplicated genomes are sometimes transmitted maternally, their presence offers an important information for genetic consultation in clinical practice. The disadvantages of the method described here

are the inability to detect duplications and to determine the proportion of mutants. These are the same disadvantages as those of the conventional PCR method, so they can be complemented by Southern-blot as before. We again emphasize that the combined use of Southern blot and PCR methods are necessary to determine the exact nature of mtDNA deletions. Nevertheless, the long PCR and three-enzyme digestion method is a very useful means for screening mtDNA rearrangements. For details, see Ref. 7.

(8) A Novel Mutation in the Mitochondrial tRNA^{Thr} Gene Associated with Mitochondrial Encephalomyopathy

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A novel G-to-A transition at nucleotide 15915 in mtDNA is described. The patient showed a combination of muscle weakness, hearing loss, mental retardation, and seizures. Muscle biopsy showed RRFs and focal COX deficiency. We sequenced all mtDNA, and found 5 novel nucleotide substitutions. Three of them were synonymous mutations, one was a missense mutation in cytochrome b gene (A→G at nt 15422), and the last one was the 15915 mutation in tRNA^{Thr} gene. We screened for the 15422 and the 15915 mutations with mismatch primers and found that one of 104 normal individuals carried the former one and none of 175 had the latter one. The 15422 mutation existed in homoplasmic states both in the patient and the normal individual, suggesting that this is a polymorphism. In contrast the 15915 mutation resided in heteroplasmic states in muscle, skin fibroblast and blood. The nucleotide substitution at nt 15915 disrupts a highly conserved base pair in anticodon stem of the tRNA^{Thr}. Our data suggest that the 15915 mutation is an additional mtDNA mutation responsible for mitochondrial encephalomyopathies. For details, see Ref. 8.

(9) The 3260 Mutation in Mitochondrial DNA Can Cause Mitochondrial Myopathy Encephalopathy Lactic Acidosis and Stroke-like Episodes (MELAS)

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Maternally inherited myopathy and cardiomyopathy (MIMyCa) is one of the phenotypic subgroups of mitochondrial diseases. There are two reports of European families with MIMyCa associated with an A-to-G transition at nucleotide (nt) 3260 in the mitochondrial $tRNA^{Leu(UUR)}$ gene. We have identified the 3260 mutation in a Japanese family with mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS) accompanied by myoclonic epilepsy. We directly sequenced a series of polymerase chain reaction (PCR)-amplified fragments encompassing all the mitochondrial tRNA genomes. Thirty sequence primers were set for both L and H strands. The data were compared with the reference sequence and only one substitution was found at nt 3260. Restriction fragment length polymorphism analysis using the previously reported mismatch primer that creates the Xmn I recognition site showed heteroplasmy not only in the proband but also in the mother and the siblings. To estimate the proportion of the mutant mitochondrial DNA, we performed a last cycle cold PCR/RFLP method which allowed us to avoid errors due to heteroduplex formation during PCR. The amplified fragments were digested with Xmn I over-night and analyzed in an automated sequencer. The proportions of the mutant mtDNA were estimated as follows: the mother 29.4% in blood; the proband 87.1% in muscle, 51.0%; the first brother 31.6%; and the second brother 44.1%. Is the MELAS phenotype of the 3260 mutation specific to the Japanese? The mitochondrial $tRNA^{Leu(UUR)}$ gene is a hot spot for the pathogenic mutations including the 3243, 3252, 3271, and 3291, all of which have been associated with MELAS. On the other hand, cardiomyopathy is a relatively frequent complication of mitochondrial encephalomyopathies including MELAS. Thus, we could consider both MELAS and MIMyCa as part of a spectrum of phenotypic expressions of the 3260 mutation. Although further studies are needed to elucidate the mechanism for the variable phenotypic expression, our data confirm that the 3260 mutation is a genetic

cause of MELAS as well as MIMyCa.

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E–b. Division of Agricultural Genetics

(1) Inbreeding Depression, Outbreeding Depression and Hybrid Vigor with Special Reference to the Mating System in Rice Species

Hiroko MORISHIMA

The level of inbreeding depression and outbreeding depression is fundamental to the evolution of the mating system in plants. This problem was investigated using wild-rice populations (*Oryza rufipogon*) having different outcrossing rates.

1) Estimation of the level of inbreeding depression in natural populations: Relative fitness of selfed progeny (inbreeding depression) was indirectly estimated from inbreeding coefficients observed before and after natural selection of the progeny generation (seed and adult), and selfing rate. These parameters were estimated based on isozyme polymorphism data obtained from four wild-rice populations collected in Thailand. Relative fitness was 0.28 and 0.09 for two predominantly selfing populations, and 0.42 and 0.68 for two partially outbreeding populations. The former values were significantly below the 0.5 threshhold thought to favor selfing.

2) Deleterious genes maintained in inbreeding and outbreeding populations: 25 first-generation individuals derived from the original seeds (collected in nature) of three wild-rice populations were selfed and crossed with each other (incomplete diallele cross). Seeds of their selfed progeny showed a high germination percentage in inbreeding annuals as well as in outbreeding perennials, though the germination percentage of original seeds was high in inbreeding annuals but low in partially outbreeding perennials. This indicates that outbreeding perennials harbor recessive deleterious alleles but they were purged through seed propagation. The germination percentages of F_1 seeds obtained from crosses between individuals belonging to an annual population was significantly lower than those from crosses between different populations. Histological observation of those ungerminated intra-populational F_1 seeds showed abnormal development of embryos. Outbreeding depression or hybrid invariability is usually considered a mechanism to separate species or

subspecies. The outbreeding depression within an inbreeding population found in this experiment could be a genetic mechanism to favor selfing.

3) Variation in hybrid vigor found within wild rice population: Hybrid vigor was quantified in terms of the F_1/midparent of plant biomass using F_1s obtained from the above-mentioned diallel cross and a number of previous crossing experiments. In hybrids between related species in rice, the values usually exceed 1.0, showing hybrid vigor. F_1s with wild rice generally showed lower values. Within a partially outbreeding wild-rice population, the values of hybrid vigor ranged from 0.6 to 1.8. The coexistence of hybrid vigor and hybrid weakness within a population might contribute to the maintenance of a mix-mating system in wild rice.

(2) Analysis of Quantitative Trait Loci Underlying Domestication in Rice

Hong-Wei CAI and Hiroko MORISHIMA

The essential differences between cultivated rice (*Oryza. sativa* L.) and wild rice (*O. rufipogon* Griff.) lie in self-propagating ability and seed productivity. The component traits underlying these characteristics are mostly quantitative. Recent developments in the rice genome project enabled us to map a number of loci controlling quantitative traits which could be analyzed only by quantitative genetic methods in the past. The objective of the present study is to map QTL responsible for rice domestication and elucidate their network, if any, on the rice genome. An F_7 recombinant inbred population consisting of 118 lines derived from a cross between cultivated rice (108; Indica from Taiwan) and wild rice (W1944: *O. rufipogon* from China) was used. Eighty-five RFLP and isozyme markers were examined. An RFLP assay was carried out using the ECL non-radioisotope method.

A total of 29 characters were observed. They included seed dormancy, seed shattering, regenerating ability and panicle exertion as self-propagating ability, with panicle number, panicle length, seed fertility, etc. as seed productivity. Cosegregation between markers and characters was analyzed using qGENE software. In all the characters studied, two to 44 QTLs were detected with single point analysis at a 5% significance level. The QTLs for respective characters generally had small gene effects and proved to be widely distributed on different chromosomes. This suggests that domestication of

rice was a gradual process accumulating a number of mutations with small effects. Further, we found that some developmentally correlated characters shared common QTLs indicating pleiotropy. On the other hand, several QTLs controlling coadapted characters tended to cluster on the particular regions of several chromosomes. This suggests that there may be an "adaptive gene block" promoting domestication.

(3) Geographical Differentiation Found in Annual Types of Oryza rufipogon

Hong-Wei CAI and Hiroko MORISHIMA

To examine whether or not differentiation exists in annual-type *O. rufipogon*, a total of 73 annual strains collected in tropical continental areas, such as Thailand, Laos, Cambodia, Burma, India, Bangladesh and Sri Lanka were examined. Thirty-two perennial strains collected in the same regions were used as controls. Polymorphisms at 20 polymorphic isozyme loci of 7 enzymes were analyzed.

The results showed that there are no clear differences in allele frequencies between annual and perennial types except for a few loci such as *Est1*. The alleles of *Est13-1*, *Est9-1* and *Amp1-3* frequently detected in Chinese strains in our previous study were rare in tropical strains. Strains collected on the west coast of India showed high frequencies of alleles *Est5-3*, *Sdh1-3*, *Pgi1-3* and *Pgi1-4* which were rarely detected in other areas. The results of factor analysis demonstrated that the strains examined can be separated into two major groups. One group consists of the strains collected mostly from the Indian west coast including both annual and perennial types, and another group contains the strains from other areas. These results indicated annual types of *O. rufipogon* showed geographical differentiation at the isozyme level in parallel with perennial types.

Judging from the results of our previous study (Cai *et al.*, 1995) and the present study, we concluded that Asian common wild rice can be largely divided into three geographical groups based on isozyme variation, i.e., 1) Chinese strains carrying some Japonica-specific isozyme alleles, 2) Indian west coast strains characterized by unique genotypes rarely found in other areas, 3) strains distributed in the tropics excluding the Indian west coast. The geographical differentiation thus revealed in Asian common wild-rice

seems to be independent of ecotypic differentiation into perennial and annual types. (Ref. 3)

(4) Basic Study of Genetic Erosion of Wild Rice (*Oryza rufipogon*) in Thailand

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In recent years, many natural populations of wild rice (*Oryza rufipogon*) have approached new extinction. The major factors of genetic erosion in wild plants are destruction of their habitats by human activity and genetic contamination by cultivated rice. To obtain basic information on the genetic erosion of wild rice, we are investigating the influence of environmental change on the genetic structure of wild-rice populations. The site we studied is a road-side swamp inhabited by perennial or an annual/perennial intermediate type of *O. rufipogon*, which is located in the suburb of Ayuthya city in the central plane of Thailand, and adjacent to a cultivated rice field on one side. We examined allozyme diversity at 15 loci of 10 enzymes by starch and polyacrylamide gel electrophoresis using two sets of seed samples collected from the same site in 1985 and 1994, respectively.

Based on allele frequencies, we estimated the gene diversity parameters. Gene diversity of the overall population decreased from 0.278 to 0.249 during the period between 1985 and 1994. Recently a gasoline station was constructed on the site and the wild-rice population was seriously damaged on account of destruction of their habitat and water pollution. Apparently a drastic decline in plant number forced surviving plants to inbreed and this caused a decrease in gene diversity.

An allele *Pgi1-1*, is known to be common among Indica rice varieties and so dominant in cultivars of this area, while wild rice in this area generally carry another allele, *Pgi1-2*. In this study-site, we found *Pgi1-1* in some wild-rice plants growing near cultivated rice. Most probably, introgression has occurred from cultivated to wild rice populations. The frequency of plants having the *Pgi1-1* allele was higher in 1994 than in 1985, indicating that the intrinsic features of wild rice have been continually replaced by those of cultivated rice.

We must recognize the present situation in which genetic erosion is rapidly

progressing and conservation efforts for natural environments are urgently needed. (Ref. 2)

(5) Inter- and Intra-Specific Differentiation of *Oryza glumaepatula* Found in Central and South America

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Oryza glumaepatula is a diploid wild rice species found in Central and South America. It shares the genome AA with Asian cultivated rice and is thought to be a useful genetic resource for rice breeding. However, we have little information on the life-history of this taxa and evolutionary relationships with other related species. We examined variation in ecological and morphological characters, isozymes and mtDNA polymorphisms among 40 accessions of *O. glumaepatula* collected in Central and South America. In addition, several accessions of Asian (*O. rufipogon*), and Australian rice (*O. meridionalis*), and two African (*O. longistaminata* and *O. barthii*) wild rice species were also examined for comparison. 1) From a survey of character variation, we could divide *O. glumaepatula* into three ecotypes. Type I; found in Central America and the northern part of South America. Regenerating ability and anther length (thought be related to outcrossing rate) were the same as those of perennial *O. rufipogon*. Type II; found in the Amazon flood basin. Regenerating ability and anther length showed an intermediate value between annual (predominantly selfing) and perennial (partially outbreeding) types of *O. rufipogon*. In the flooded period their culms are easily broken at the lower part and the upper parts of plant bodies become free floating on the water surface. Type III; found in southern Brazil (Pantanal swamp). Regenerating ability and anther length showed values similar to those of type II. Matured seeds (about 5 mm in length) of type III occupy only the lower half of the hull (11–12 mm length) leaving an empty space inside the hull.

2) Allozymes were not so variable within *O. glumaepatula* and no remarkable differentiation was recognized among the three ecotypes. It was found, however, that *O. glumaepatula* has developed unique isozyme genotypes by which it is separated from other related taxa.

3) Based on the mtDNA haplotype polymorphisms, *O. glumaepatula*

accessions were categorized into two groups. One group consisted of the accessions belonging to ecotype I and III, and their mtDNA haplotypes were similar to that of *O. longistaminata*. Another group consisted of the accessions belonging to ecotype II, and their mtDNA haplotypes were similar to that of *O. meridionalis*.

The phylogenetic relationships between *O. glumaepatula* and related taxa found on other continents remains unclear.

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E–c. Division of Applied Genetics

(1) Mitochondrial Genome Polymorphisms in *Lolium perenne*

Yoshiya SHIMAMOTO

The restriction fragment length polymorphisms (RFLPs) of mitochondrial DNA (mtDNA) of perennial ryegrass (*Lolium perenne* L.) were investigated to elucidate the genetic relatedness among 124 cultivars including diploid and tetraploid ones. Many patterns of RFLPs were observed and allowed to

assign the cultivars into the main eight haplotypes of mitochondrial genome. The American cultivars were classified to haplotype I and VIII which were remote at mitochondrial genome relatedness each other, the European ones were distributed thoroughly to all haplotypes and tetraploid ones were mostly assigned into the haplotype V. In addition the two genes, coxIII and nad9, on mitochondrial genome can be located closely each other. The assessment of RFLPs upon mtDNA may be a valuable method in analyzing a cytoplasmic differentiation among the perennial ryegrass cultivars. Further investigations remained to elucidate the mtDNA diversity in relation with the maternal effects on the agronomic traits of perennial ryegrass.

(2) The Extent of Natural Cross Pollination in Wild Soybean (*Glycine soja*)

Yoshiya SHIMAMOTO

To evaluate the extent of natural cross pollination in wild soybean (*Glycine soja*), the genetic structures of four populations along the Omono River in Akita Prefecture, Japan, were investigated by examining variations of several allozymes. In addition, observations of pollinators were made in the natural population. Although it was thought previously that *G. soja* is autogamous, as is cultivated soybean (*G. max*), within-population genetic variation (H_s= .315) were higher, and genetic divergence among population (G_{st}=.087) were lower than the values expected for selfing plant species. Based on allelic frequencies of isozyme loci, the means of mutilocus (tm) and single locus (ts) outcrossing rate estimates were 13% (9.3%–19%) and 8% (5%–14.7%) among the four populations, respectively. These values are much higher than the outcrossing rates previously reported for both the wild and cultivated soybeans. This higher outcrossing rate was supported by observations of the frequent visits of honey bees and hornets to the flowers. For details, see Ref. 1.

(3) Molecular Genetic Studies of Genomic Imprinting

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Genomic or parental imprinting denotes a biological process that modifies

the expression of the genome differently in male and female germline cells, leading to differential activity of the parental genome in offspring. In order to understand the molecular mechanisms involved in imprinting, we are studying the distal portion of mouse chromosome 7, which contains at least three imprinted genes, insulin 2 (*Ins2*), insulin-like growth factor 2 (*Igf2*), and *H19*. The results obtained so far are as follows:

(1) Based on findings obtained in human and mouse, we discussed the possibility of imprinted genomic regions serving as a model to study the long-range mechanisms that control gene expression (ref. 2). (2) We isolated YAC clones that cover the imprinted *Ins2/Igf2/H19* domain, and are making a physical map of the region. (3) We reported the complete nucleotide sequence of the 28-kb region containing the entire *Igf2* (ref. 3). (4) We cloned the *L23mrp* gene, which is located downstream of *H19* and is biallelically expressed. Furthermore, it was shown that *L23mrp* is functionally insulated from the *H19* enhancers, suggesting that there may be a domain boundary between *H19* and *L23mrp* (in preparation). (5) We have sequenced most of this putative boundary region, which is about 30 kb in size. (6) We showed that *Igf2*, which has three different promoters, is controlled mainly by a locus-wide mechanism (in preparation). (7) A convenient method for detection and mapping CpG islands has been devised (in preparation). This technique should be useful in identifying new imprinted genes. (8) We determined the timing of the erasure and establishment of the methylation imprint which is present in the 5′ flank of *H19* (in preparation). (9) In collaboration with Hayashizaki's group in RIKEN, we isolated a new paternally expressed mouse gene, RAS-specific guanine nucleotide exchange factor (*Grf1*), on chromosome 9 (ref. 4). (10) We found; in cultured embryonic fibroblast cells; an apparently new arrest-specific up-regulatory sequence common to many imprinted genes, which may give us a clue to the evolution of the genomic imprinting mechanisms (submitted).

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F. GENETIC STOCK RESEARCH CENTER

F–a. Mammalian Genetics Laboratory

(1) Geological Distribution of a Y chromosome Specific Sequence DYH1 in Wild Mice

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Polymorphic genes on the Y chromosome are unique markers for study of genetic differentiation in the wild mice population, because the Y chromosome is inherited from male and the genes on the Y chromosome have never been shuffled by recombinational event except for the pseudautosomal region.

DYH1 is a novel sequence specific to the Y chromosome, which has homology with the sequence for the human proto-oncogene, KRAS2. We investigated the geographical distribution of polymorphisms of the DYH1 in wild mice populations by analyzing the PCR amplified sequences. As a result, we found that DYH1 is absent in populations of *Mus musculus domesticus* in west-Europe and *Mus musculus castaneus* in southeast Asia. It was distributed in populations of *Mus musculus musculus*, from east Europe to east Asia including Japan. This distribution pattern is unique when compared with those obtained from analyses with two other markers specific to the Y chromosome, the Zinc-finger protein on Y-2 (*Zfy-2*) and the Sex determining region-Y (*Sry*). Based on the geographical distribution pattern of these three different markers specific to the Y chromosome, we intend to study the process of subspeciation of mouse.

(2) Molecular Characterization of *Pb* Hotspot in the ClassII of Mouse MHC

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The murine major histocompatibility complex (MHC) is an excellent system for studying the molecular mechanisms of meiotic recombination, where breakpoints of recombinations are systematically studied at the molecular level. It is well known that meiotic recombinations in this region do not occur at random but are clustered in limited regions known as hotspots. Thus far, four hotspots have been identified in this region. Three hotspots other than *Pb* have been well characterized at the molecular level. Namely, the fine locations of breakpoints of these three hotspots were analyzed and sequences around the *Eb* and *Lmp2* hotspots were determined. Comparison of the sequence between the *Eb* and *Lmp2* hotspots revealed several molecular motifs commonly shared by the two hotspots. In order to elucidate the roles of these motifs in recombination at the hotspots and to understand the mechanism by which recombinations are restricted to hotspots, we need to characterize other hotspots at the molecular level.

Meiotic recombination takes place at a high frequency at a hotspot in the vicinity of the *Pb* gene, when the wild mouse-derived cas4 haplotype is used in the genetic cross. Molecular characterization of this hotspot, has not yet been done. So far, we have obtained six independent recombinants at the *Pb* hotspot after screening six hundred mice generated from crosses between cas 4 and wm7 haplotypes. The breakpoints of these recombinants were localized to a 15 kbp of DNA fragment in the vicinity of the *Pb* gene. In this study, we attempted to make a more complete map of the breakpoints of the six cas4/wm7 recombinants. First, we constructed the restriction map of the 15 kbp of DNA fragment including the breakpoints. Subsequently, we determined the parental origins of several short DNA segments. At a result, at least five recombinants were found to be confined to a 2.4 kbp of DNA segment located proximal to the 3′ end of the *Pb* gene. Then we determined the sequence of this segment. The sequence of this *Pb* hotspot appeared to have neither MT-middle repetitive family, the TCTG repeat or the solitary type

LTR, indicating that these molecular motifs commonly shared by the *Eb* and *Lmp2* hotspots do not play key roles in determining the site specificity of recombinational breakpoints in the mouse MHC.

In addition to the cas4/wm7 recombinants, we obtained other recombinants generated from cas4/cas3 and cas4/b crosses. The breakpoints of these recombinants were analyzed in the same way used for the cas4/wm7 recombinants. The results indicated that the breakpoints are located in a comparatively larger fragment at least 15 kbp in length, including the same 2.4 kbp segment to which the breakpoints of the cas4/wm7 recombinants were confined.

(3) Genetic Analysis of Coat-Color Mutation, *rim2*. III

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As previously reported, one of the RIMs (Recombination Induced Mutants), *rim2*, is a new allele of an old coat color mutation, *pearl*, and has abnormalities similar to human hemorragic symptoms accompanied with albinism, platelet storage pool deficiency (SPD). Platelet aggregation and ATP release in platelets of the *rim2* mouse using several agonists were clearly reduced in comparison to those of wild type mouse. These results indicate the possibility that *rim2* could serve as a mouse model for human SPDs.

Prior to the positional cloning of the *rim2* or *pearl* gene, we tried to make the YAC contig covering the *rim2* region. We screened YAC libraries with microsatellite markers tightly linked to the *rim2* gene. First we obtained two YAC clones including two marker sequences which perfectly cosegregated with the *rim2* phenotype in 1570 back cross progeny. These clones were nonchimeric, but were discontinuous with each other. Then we isolated the STS end markers from the two clones and screened the YAC libraries again. Finally we assembled a contiguous array of YAC clones spanning of *rim2* region. The length of the entire contig was speculated to be a maximum of 1.5 Mb. Now we are ready to carry out a rescue experiment via the transgenesis of these YAC clones into the *rim2* homozygotes to identify the

YAC clone including the *rim2* gene.

(4) Mapping and Histological Analysis of the Mouse Mutant (*Rim3*) Affecting Skin and Eye

Hajime SATO, Tsuyoshi KOIDE, Hiroshi MASUYA, Shigeharu WAKANA[1], Tomoko SAGAI, Kazuo MORIWAKI[2], and Toshihiko SHIROISHI ([1] Central Institute for Experimental Animals, Kawasaki, [2] The Graduate University for Advanced Studies, Kanagawa)

Rim3 is a dominant mutant derived from an intra-MHC recombinant. It is characterized by hairlessness and corneal opacity. In order to determine the locus of the *Rim3* gene, we carried out two kinds of crosses, cross I: (C57BL/10SnJ-*Rim3*/+ × MSM)F_1 × C57BL/10Snslc; cross II: (C57BL/10SnJ-*Rim3*/+ × JF1)F_1 × C57BL/10Snslc. MSM is an inbred strain derived from Japanese wild mouse, while JF1 is an inbred strain derived from Japanese fancy mouse. These mice are suitable for linkage analysis because their genetic backgrounds are far from those of laboratory mice. We finally obtained 1545 progeny from cross I and 667 progeny from cross II. Linkage analysis using microsatellite markers revealed that the *Rim3* locus was tightly linked to *D11Mit145*, *14*, *124*, *197* on Chromosome 11. Gene and microsatellite markers orders obtained from the two crosses are as follows, cross I: centromere–*Mit145*–(0.26 cM)–*Rim3*–(0.06 cM)–*Mit14*, *124*, *197*; cross II: centromere–*Mit145*–(0.30 cM)–*Rim3*, *Mit14*, *197*–(0.15 cM)–*Mit124*.

The *Krt-1* (Keratin type 1) locus has been mapped near this position. Keratins are a group of water-insoluble proteins that form 10-nm intermediate filaments in epithelial cells. These can be divided into acidic and basic subfamilies according to their relative charge, immuno-reactivity, and sequence homologies. They are designated as type I and II keratins, respectively. Type I and II keratins contribute to cytoskeletal architecture as heterodimers. It is known that *Krt-1* consists of at least 11 genes and its expression patterns are tissue- and differentiation- specific. Therefore we intended to examine whether *Krt1-12* which is expressed in cornea and *Krt1-10*, *14*, *16*, *17* which are expressed in epidermis are possible candidates for the *Rim3* gene. At the present time, we have excluded *Krt1-10*, *12*, *14*, *17* as candidate genes for the *Rim3* gene, because linkage analysis has indicated segregation of *Rim 3* phenotype and these marker loci.

Several mouse mutants affecting the skin and cornea have also been mapped to the region near the *Rim3* locus. The bare skin (*Bsk*) mutation causes baldness and corneal opacity. The denuded allele at the *Rex* locus (Re^{den}) has a similar phenotype. To characterize the corneal opacity and hairlessness in detail, we carried out histological analysis in *Rim3* and Re^{den}. We observed remarkable changes in the center of the cornea of *Rim3* mutants. Keratin covered the surface of the cornea and the corneal epithelium were about three fold thicker than that of control mice. Keratinocytes in the stromal layer proliferated and neutrophils infiltrated into the stromal layer, which may have caused the thickness of the stromal layer. There were also some vessels in this lesion. In contrast, we could not find any changes in the periphery of the cornea of *Rim3* mutants. Additionally, morphological alterations in the epidermis of *Rim3* were observed. The epidermis of *Rim3* mutants was much thicker than that of control mice and contained two to three times the number of spinous layers. The number of the hair follicles of *Rim3* mutants were markedly reduced. Similar abnormalities could be seen in Re^{den} mutants.

We are making a physical map around the *Rim3* locus by using YAC and BAC sequences and searching for candidate genes which may play roles in growth and differentiation of epithelial cells.

(5) Organization of the Type1 Keratin Genes (*Krt1*) in the Mouse Genome

Hajime SATO, Tsuyoshi KOIDE, and Toshihiko SHIROISHI

Mapping studies of keratin genes in the mouse genome have revealed that most of the type 1 keratin genes (*krt1*) are located in the region 58.0 cM from the centromere on Chromosome 11. This is a syntenic region 17q12–q21 in the human Chromosome, where most of the human type 1 keratin genes are clustered. In the human type 1 keratin genes, *KRT10*, *13*, *14*, *15*, *16*, *17*, *18*, *19* have been already cloned, and the genes separated into two clusters. One contains *KRT15* and *KRT19*, and the other cluster contains *KRT14*, *KRT16* and *KRT17*. Recently, it was reported that the two clusters were very closely located and confined to at most a 55-kb DNA fragment in the human genome. The genes were organized in the following order: *5′-KRT19-KRT15-KRT17-KRT16-KRT14-3′* (Milisavljeic *et al.*, 1996).

In mouse, *Krt1-10, 12, 13, 15, 19, 14* (partial), *17* (partial) have been already cloned, but their relative positions and the physical distance between them have not been studied. In order to examine the organization of the mouse *Krt1* gene cluster, we carried out fine linkage analysis using the panels which were constructed for *Rim3* mapping. Our results suggested that there are at least two clusters in the mouse *Krt1* genes as well. One contains *Krt1-10* and *Krt1-12*, and the other cluster contains *Krt1-15,19, 14* and *17*. We are now making a physical map using YAC sequences containing these *Krt1* genes and intend to resolve the organization of *Krt1* genes in mouse genome.

(6) Gene Mapping of *Rim4* Modifier

Hiroshi MASUYA, Tomoko SAGAI, Shigeharu WAKANA[1], and Toshihiko SHIROISHI ([1] Central Institute for Experimental Animals, Kawasaki)

A network of interactions among the products of numerous genes is probably essential for morphogenesis in limb buds. An analysis of mutants in which such genes are affected is obviously critical if we are to identify the key roles of the various genes. Mating experiments between different mutants with similar phenotypes would provide information about the interactions among genes that are involved in morphogenesis. Although such genetic studies have been very successful in the fruitfly, fewer studies have been performed in house mouse, *Mus musculus*. There are many mutants with preaxial polydactyly in mice. We reported previously that disruption of the anteroposterior axis formation of limb bud induces the preaxial polydactyly in six preaxial polydactyly mutants, namely, *Recombination induced mutant 4* (*Rim4*), *Hemimelic extra toes* (*Hx*), *extra-toes-Jackson* (Xt^J), *Strong's luxoid* (*lst*), *luxate* (*lx*), and *X-linked polydactyly* (*Xpl*). In these mutants, region specific expression of *Sonic hedgehog* (*Shh*) which is mediator gene for the anteroposterior axis formation was affected in early limb development.

It has been well documented that a mutant phenotype depends not only on the genotype at the mutated locus but also on the genotypes at other loci unlinked to the mutant gene. This fact, known as the genetic background effect, indicates that there is a second gene, called a modifier, which interacts with the first mutant gene and that such modifier genes are functionally polymorphic among inbred strains. We have also reported that the appearance of the phenotype of the polydactyly mutants depended on the genetic

background of mouse strain. For example, polydactylous phenotype of *Rim4* heterozygotes is dominantly and completely suppressed by the MSM background, which is derived from Japanese wild mouse and is dominantly accelerated by NZB background.

We carried out the gene mapping of the modifier for the one of polydactyly mutants, *Rim4*. We crossed C57BL/10J-*Rim4*/+ with the F_1 progeny of MSM and NZB. We obtained 57 polydactylous progeny out of total 381 progeny generated from the above cross. Using the 57 polydactylous progeny, we carried out a systematic search for loci which show significantly biased frequency for one parental allele. As a result, two marker loci were detected as the candidates for the modifier genes of *Rim4*. At *D2Mit13*, *D2 Mit126* and *D2Mit130* in chromosome 2, the frequency of the MSM derived allele was 22.8% and the LOD score was 3.9. At *D15Mit2*, *D15Mit31* and *D15Mit6* in chromosome 15, the LOD score was 1.6. When randomly extracted progeny, irrespective of polydactylous or not, were used in the same analysis, the frequency of MSM allele at the *D2Mit13* and *D15Mit2* was almost 50%. Thus, it revealed that the biased frequency in the polydactylous progeny was not due to transmission ratio distortion (TRD).

Recently, it was reported that preaxial polydactyly mutant, *lst*, was tightly linked to *D2Mit130*. We previously reported that *lst* exhibits a phenotype similar to that of *Rim4*. It is possible that *lst* per se is a downstream modifier gene for *Rim4* in the genetic cascade regulating the anteroposterior axis formation in mouse limb development.

(7) Genetic Analysis of *Hemimelic extra-toe* (*Hx*) Mutant

Tomoko SAGAI, Hiroshi MASUYA, and Toshihiko SHIROISHI

Hemimelic extra-toe (*Hx*) is a limb morphological mutant which arose in the B10.D2 strain. Heterozygotes have preaxial polydactyly on all four feet. Typical expression includes shortening of the radius, tibia, and talus with supernumerary metacarpals, metatarsals, and digits. The supernumerary digits are always preaxial. The fibula and ulna are normal size but often bowed. The *Hx* gene is located on chromosome 5 and very closely linked to the *Hammer-toe* (*Hm*) mutation which shows interdigital webbing regression. This region is homologous to a segment of human chromosome 7q36, where congenital limb deformity (polysyndactyly) in a large family has been

located. These data suggest that the homologous region of human and mouse contain genes involved in limb pattern formation. Additionally ectopic expression of *sonic hedgehog* (*Shh*) has been detected in several preaxial polydactylous mutants including *Hx* (Masuya *et al.*, Genes & Dev., **9**, 1645–1653, 1995). Because *Hx* and *Hm* are mapped in a position close to the *shh* locus, *shh* is one possible candidate gene for *Hx* and *Hm*. For the elucidation of genes concerning limb patterning through *Hx* mutation, we are carrying out fine linkage analysis of the *Hx* locus.

(8) Positional Cloning of the Mouse Skeletal Mutant, *Tail short* (*Ts*)

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Tail short (*Ts*) was initially found as a mutant which produces a shortened kinked tail in heterozygotes. *Ts* gene was mapped to the teromeric region of chromosome 11. It has been proposed that *Ts* is a animal model for a human skeletal dysmorphology known as Meckel syndrome (MES1). MES has been mapped to 17q21–24 in human chromosome and this region is homologous and syntenic to the distal region of mouse chromosome 11.

To elucidate the function of the *Ts* gene in mouse embryogenesis, we are trying to clone the gene using positional cloning. The *Ts* gene was mapped within a region of 0.16 cM that is located between two microsatellite markers, *D11Mit128* and *D11Mit256*. The distance between these two markers is estimated to be approximately 320 kb from calculations based on the average recombination frequency in the mouse genome. We screened the mouse YAC library and obtained YAC clones which carry microsatellite markers tightly linked to the *Ts* locus. A new YAC library constructed by Whitehead Institute, MIT has been made available from Research Genetics Co. Ltd. We obtained six additive YACs by screening this new library. In the present study, we isolated terminal sequences of YAC clones. Further screening of the mouse YAC and Bacterial Artificial Chromosome (BAC) library and chromosome walking is underway to make a contig between these two markers.

(9) Morphological Characterization of the Mouse Mutant *Tail-short*: Skeletal Patterning Along the Anteroposterior Axis

Junko ISHIJIMA, Kikue UCHIDA, Akihiko MITA, and Toshihiko SHIROISHI

Mice heterozygous for *Tail-short* (*Ts*) exhibit shortened a kinked tail and numerous skeletal abnormalities throughout the whole body and severe defects in head structural development. These phenotypes differ depending upon the genetic background in which the mutant gene is introduced. In particular, *Ts* heterozygotes generated from the cross of (C57BL/6J- *Ts*/+ $\times$MSM)$F_1\times$C57BL/6J show several homeotic transformations that are highly reproducibe. Two most characteristic phenotypes of the mutant mice were transformation of the cervical vertebrae to thoracic vertebrae and an additional vertebrae in upper thorathic region. It is of interest to note that the cervical region transformation was observed asymmetrically, namely, left side of the vertebra was preferentially transformed to a posterior structure. Thus, it is possible that *Ts* gene plays some role in providing segmental identity along anteroposterior and left-right axes in mouse embryogenesis. The precise relationship between this mutant phenotype and a number of genes known to be involved in determination of segmental identity along two axes remains to be determined.

(10) Searching for Genes Regulating the Phenotype of *Tail-short*

Junko ISHIJIMA, Kikue UCHIDA, Akihiko MITA, and Toshihiko SHIROISHI

The phenotype of *Tail-short* (*Ts*) mutant is varies according to different genetic backgrounds as is seen in many other mouse mutants. This indicates that some unknown genes play critical roles in mouse embryogenesis in cooperation with *Ts* gene. The genes which interact with the mutant gene and change the mutant phenotype are called modifier gene. The large variation in *Ts* phenotype observed in different inbred strains indicates that *Ts* modifier genes are functionally polymorphic among inbred strains. We have carried out two experiments to reveal the nature of the modifier genes.

(1) Polymorphism of *Ts* modifier genes in mouse inbred strains. In order to examine the effect of genetic background of different inbred strains on *Ts* phenotype, *Ts* heterozygotes were crossed with several inbred strains, such as A/J, C3HSW/SnJ, CBA/J, AKR/J, BALB/cAnN, C57BL/6J, DBA/2J

and SJL/J. Microsatellite markers linked to *Ts* locus were used to determining the genotype of the resultant progeny. This mating experiment clearly indicated that crosses with A/J, C3HSW/SnJ, CBA/J and AKR/J mouse strains did not give rise to viable progeny which had *Ts* mutant allele. This led us to the conclusion that the above four strains possess polymorphic gene (s) which causes prenatal lethality in cooperation with *Ts* mutant gene. We intended to map the gene which causes the lethality. The above four inbred strains were crossed with C57BL/6J strain, then F_1 mice were subsequently crossed with *Ts* heterozygotes. The resultant progeny were subjected to genotyping through entire chromosomes. The result clearly demonstrated that the progeny carrying alleles of A/J, C3HSW/SnJ, CBA/J and AKR/J only at the microsatellite loci linked to *Ts* were lethal. This indicated that the objective gene is tightly linked to *Ts* locus and probably is *Ts* locus *per se*. It is possible that the allelic forms of the four inbred strains at *Ts* locus improperly interact with *Ts* allele which results in prenatal lethality. A/J, C3HSW/SnJ and CBA/J originated from a common stock and have diverged into three inbred strains about seventy years ago. Detailed analysis of early embryogenesis revealed that mutant embryos were mostly defective in their distal mesodermal region at early primitive streak stage, leading to lethality in embryos in utero around 12.5 dpc. Thus, the results indicate that *Ts* gene plays a crucial role in early embryonic stage and that there is polymorphism at *Ts* locus among inbred strains. The functional differences of the polymorphisms become evident when they interact with *Ts* allele.

2) Searching for Modifier Genes for the Phenotype of Mutation *Tail-short* When (TSJ/Le-Ts/+ × C57BL/6J)F_1 mice were backcrossed to C57BL/6J, the frequency of the resultant progeny carrying the *Ts* allele is almost 0%. Fetal death soon after birth is associated with a high incidence of cranial neural tube defects including exencephaly, spinal bifida and cleft palate. In turn, when the MSM inbred strain was used as a backcross partner, mice with the *Ts* allele were less defective and viable. The differences in expressivity of the mutant phenotype indicates that there are non-allelic genes which modify the severity of *Ts* phenotypes and are polymorphic among C57BL/6J and MSM strains. We intend to map these genes and investigate the mechanisms of the neural tube defects.

(11) A New Inbred Strain JF1 from Japanese Fancy Mouse Carrying the Classic Piebald Allele for Use in Biomedical Research

Tsuyoshi KOIDE, Kikue UCHIDA, Akihiko MITA, Tomoko SAGAI, Toshihiko SHIROISHI, and Kazuo MORIWAKI[1] ([1] The Graduate University for Advanced Studies, Kanagawa)

There is strong evidence that laboratory mice were established from the European or North American wild mouse, *M. m. domesticus*. Analysis of the mitochondrial genome, and many of the nuclear genes of the laboratory mouse have revealed that it shares common polymorphisms with those of the west European subspecies, *M. m. domesticus*. Most laboratory strains probably originated from a small number of fancy mice kept by mouse breeders in Europe and North America. It is known that Abbie E. C. Lathrop maintained a number of fancy mice on her farm in Granby, Massachusetts and also supplied her mice to scientists in the early 1900s. Old laboratory strains such as C57BL/6, and C58 were established from her mice.

On the other hand, there have been many mouse fanciers in Japan and China since the late 1700s. Varieties of mutant mice such as albino, pink-eye dilution, dwarf, waltzer and piebald already existed in Japan and those mice were described in the book titled "Chingan-sodategusa" published in 1787. One of those mice, Japanese Waltzing Mouse, famous for its characteristic behavior was introduced into Europe in the late 1800s by Brehm. Historical records indicate that Japanese fancy mice were crossed with European and North American mice and some genetic factors were introduced into them. An analysis of genes on the Y chromosome like *Zfy2* and *Sry* has shown that the Y chromosome of most strains of laboratory mouse was derived from Asian mice, probably the Japanese wild mouse, *M. m. molossinus*. Even though *M. m. molossinus* contributed to the gene pool of many laboratory mouse strains, morphological similarities are not apparent between *M. m. molossinus* and laboratory mice. Thus, most laboratory strains are genetically similar to *M. m. domesticus* and differences between laboratory strains are quite small, even though there has been substantial contributions from *M. m. molossinus* to the establishment of laboratory inbred strains. This implies that if we use only current laboratory strains, there is a risk of ignoring many unknown genetic factors which could be found only in strains genetically

remote from laboratory strains. Such polymorphisms are precious and useful for much scientific research. In fact, a new inbred strain which is genetically far different from old laboratory strains but easy for use in biomedical research work in terms of breeding, handling and manipulation has been required for a long time.

Although the original strains of the Japanese fancy mouse were believed to have become extinct by the middle of the 1900s, we found a strain of fancy mouse being maintained in Denmark with the name "Japanese Mouse" in 1987. We introduced this Japanese Mouse stock into the National Institute of Genetics, Japan and have bred the stock through full-sib mating. Now, the mouse has been established as an inbred strain and designated JF1. The mouse is phenotypically similar to one of the mice reported in "Chingan-sodategusa". Morphological analysis of JF1 showed that the mouse is highly similar to the Japanese mouse *M. m. molossinus*. In addition to this, many genetic features such as the polymorphism in *Zfy2* on the Y chromosome, the genotype for biochemical markers, and the haplotype of *H-2K*^{f} suggest that JF1 has the genetic profile peculiar to *M. m. molossinus*. Therefore we concluded that JF1 originated in Japanese wild mouse *M. m. molossinus*. Linkage analysis for the spotting coat color in JF1 showed that the mutation is located on the *piebald* locus. Characterization and comparison of the causative gene for piebald, endothelin receptor type B (*ednrb*), indicated that the allele of *ednrb* in JF1 is the same as that of the classic *piebald* allele suggesting that these two mutations have a common origin. Possibly, the classic *piebald* mutation was introduced from a Japanese tame mouse which was already reported at the end of the 1700s.

JF1 has the potential to provide a new source for genetic research. It appeared from SSLP analysis that the microsatellite markers are highly polymorphic between JF1 and old laboratory strains. We observed that 80% of the microsatellite markers are polymorphic between JF1 and laboratory strains. These polymorphisms facilitate a high density mapping of any objective genes. It is also noteworthy that both the males and females of the F_1 progeny between JF1 and either C57BL/6 and DBA/2 are completely fertile and have no difficulty when crossing to any of the parental strains. This makes mapping experiments extremely easy. We found that F_1 progeny from these crosses showed more increased activity than any of the parental strains. This can be explained by heterosis. These F_1 progeny are still easier to handle than any strains established from wild mouse.

Currently, we are investigating modifier effects on several morphological mutants and observed different phenotypes of mutations in the genetic background of JF1. These modifier effects must be caused by polymorphic genes in JF1, which functionally cooperate with the mutant genes. It is theoretically easier to detect modifier effects for a variety of mutations by introducing mutant genes into a genetically different strain such as JF1 rather than into closely related strains.

JF1 will also provide a new experimental system for studying mouse behavior, because JF1 has been highly domesticated and is obedient compared to the genetically related strain MSM which still maintains the character of wild mouse and is very active. The clarified genotypes of JF1 are "*aa BB CC DD ss*".

(12) Establishment of Intersubspecific Consomic Mouse Strains II

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By definition, two strains of mouse are referred to as consomic when they differ by one complete chromosome pair. Given that mouse has a karyotype with 21 different chromosomes (19 autosomes + X + Y), 21 consomic mouse strains are required for full coverage of mouse chromosomes by donor strains, from which one complete chromosome is introduced into the common genetic background referred to as the recipient. Chromosome substitution is achieved by six to seven times backcrossing of individuals identified as heterozygous for the selected chromosome to the recipient strain.

It has been well established that many pathological conditions and diseases like cancer and diabetes are under multigenic control. They are generally difficult to analyze when a great number of genes are involved and epistatic interactions are present among the genes. In these cases, if a phenotype of interest appears to deviate in a consomic strain from the recipient strain, its genetic control must be related to the chromosomal substitution concerned. Further genetic analysis will then be considerably simplified.

We have started to establish consomic strains, using the MSM strain, which is derived from Japanese wild mouse, *Mus musculus molossinus*, as the donor strain and, C57BL/6J laboratory strain as the recipient. These two strains are genetically very remote from each other so that they differ in a number of genetic traits. This genetic difference facilitates chromosomal assignment of any phenotype to be examined and is of great use in linkage analysis based on polymorphisms of DNA markers. We are using microsatellite DNA markers, at least five markers per chromosome, for selection of individuals in each step of the backcrossing. At present, we have obtained third to fourth generations of the backcross for most of the chromosomes.

(13) Genetic Analysis of Non-MHC Susceptibility Genes to IDDM in Mice

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The NOD mouse is a good animal model for insulin-dependent diabetes mellitus (IDDM). It is also used as a model for studying multigenic diseases, because at least sixteen different *Idd* loci responsible for susceptibility to IDDM have been reported. The first discovered locus *Idd*, is linked to the MHC, and essential to the development of diabetes. However, the contributions of other *Idd* loci not linked to MHC, to IDDM remain unclear. To investigate the functions of the *Idd3* and *Idd4* without the influence of other susceptibility genes, we have established two congenic strains for *Idd3* and *Idd4* by introducing the chromosomal segments of a Japanese wild mouse-derived MSM strain into the NOD mouse. The NOD-*Idd3*^{msm} congenic mice showed a reduced level of IDDM, but were not completely free from it. On the other hand, the NOD-*Idd4*^{msm} congenic mice developed insulitis, although the incidence of diabetes and insulitis in these congenic mice was not lower than that in NOD mice. To elucidate the function of the *Idd3* in detail, we constructed a fine map for *Idd3* using a cross between NOD-*Idd4*$^{msm/msm}$ and NOD-*Idd3*$^{nod/msm}$. A region responsible for severe insulitis could be localized in the *Idd3*-region between *D3MIT169* and *D3MIIT181* including the *IL2* gene. Moreover, the coding sequence of *IL2* from NOD was different from

that of MSM. The amount of *IL2* secreted from anti-CD3 stimulated T cells of NOD/shi and MSM was measured by ELISA. The level of *IL2* in the MSM was higher than that of NOD/shi. These results suggest that *IL2* is a plausible candidate for *Idd3*.

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F–b. Invertebrate Genetics Laboratory

(1) Specification of Embryonic Limb Primordium by Graded Activity of Decapentaplegic

Satoshi GOTO and Shigeo HAYASHI

Two thoracic limbs of *Drosophila*, the leg and the wing, originate from a common cluster of cells including the sources of two secreted signaling

molecules Decapentaplegic and Wingless. We showed that Wingless, but not Decapentaplegic, is responsible for the initial specification of limb primordia with a distal identity. Limb formation is restricted to the lateral position of the embryo by negative control of the early function of Decapentaplegic and the EGF receptor homolog that determines the global dorso-ventral pattern. Late function of Decapentaplegic locally determines two additional cell identities in a dosage dependent manner. Loss of Decapentaplegic activity resulted in a deletion of the proximal structures of the limb, which is in contrast to the consequence of *decapentaplegic* mutations in the imaginal disc that cause a deletion of distal structures. The results indicate that the limb pattern elements are added in a distal to proximal direction in the embryo, which is opposite to what is happens in the growing imaginal disc. We propose that Wingless and Decapentaplegic act sequentially to initiate the proximodistal axis. For details, see Ref. 7.

(2) Cadherin-mediated Cell Adhesion and Cell Motility in *Drosophila* Trachea Regulated by Transcription Factor Escargot

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Coordination of cell motility and adhesion is essential for concerted movement of tissues during animal morphogenesis. *The Drosophila* tracheal network is formed by branching, migration and fusion of tubular ectodermal epithelia. The tracheal tip cell is located at the end of each branch that is going to fuse. Tip cells extend filopodia to search for targets and later change their cell shape to that of a seamless ring to allow passage of lumen. The cell adhesion molecule *D*E-cadherin is required for tracheal formation (Ref. 2), and accumulates at the site of contact to form a ring that marks the site of lumen entry, and is essential for fusion. *D*E-cadherin expression in tip cells of a subset of branches is dependent on *escargot*, a zinc finger gene expressed in all tip cells. Such *escargot* mutant tip cells failed to adhere to each other and continued to search for alternative targets by extending long filopodia. We present evidence indicating *escargot* positively regulates transcription of the *D*E-cadherin gene, *shotgun*. Overexpression of *D*E-cadherin rescued the

defect in one of the fusion points in *escargot* mutants, demonstrating the essential role of *D*E-cadherin in target recognition and identifying *escargot* as a key regulator of cell adhesion and motility in tracheal morphogenesis. For details, see Ref. 6.

These studies have proven that the tracheal system is ideal for the study of cell motility and adhesion during morphogenesis. To facilitate the study of tracheal formation in live animals, we developed a gfp expression vector that enabled us to observe tracheal cell migration in living embryos. For details, see Ref. 4.

(3) Role of the *Drosophila* EGF Receptor in Determination of Dorsoventral Domains of *escargot* Expression during Primary Neurogenesis

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Primary neurogenesis in the central nervous system of insects and vertebrates occurs in three dorsoventral domains on each side of the neuroectoderm. Among the three dorsoventral domains of the *Drosophila* neuroectoderm, the medial and lateral columns express the zinc-finger gene *escargot* (*esg*), whereas the intermediate column does not. We studied *esg* expression as a probe to investigate the mechanism of neuroectoderm patterning. The effect of dorsoventral patterning genes on *esg* expression was studied. *decapentaplegic*, *snail* and *twist* were found to repress *esg* expression outside of the neuroectoderm. The expression of *escargot* in the intermediate column is normally repressed, but repression was eliminated when the EGF receptor homologue (DER) activity was either elevated or reduced. A neurogenic enhancer of *escargot* was identified, and was shown to be separable into a distal region that promotes ubiquitous expression in the neuroectoderm and a proximal region that represses the intermediate expression. It was concluded that *decapentaplegic*, *snail*, *twist* and an activator act through the distal region to initiate transcription of *escargot* in the neuroectoderm. We propose that the combination of opposing gradients of DER and its ligand creates a peak of DER activity in the intermediate column where DER represses *escargot* transcription through the proximal repressor region. These two kinds of regulation establish the early *escargot* expression that prefigures

the neuroectoderm patterning. For details, see Ref. 8.

(4) *plexus*, a Gene Required for Establishment of Adult Wing Vein Pattern

Hitoshi MATAKATSU[1], Sumiko GAMOU[1], and Shigeo HAYASHI ([1] Osaka Prefectural University)

Veins on the adult wing are good landmarks for anteroposterior and dorsoventral positional information. They also provide a stage where the EGF receptor, Decapentaplegic and Notch/Delta signaling pathways interact. The gene *plexus* is required for supression of extra veins in specific intervein positions (between vein two and three, and between vein four and five). The study of *plexus* may provide a clue to the mechanism that organizes the global pattern of the wing as well as vein differentiation. To this end, we are cloning the *plexus* gene.

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F–c. Plant Genetics Laboratory

(1) Structural and Functional Analysis of the Genes Expressed in Early Embryogenesis in Rice (*Oryza sativa*)

Yukihiro ITO, Mitsugu EIGUCHI, and Nori KURATA

To elucidate the gene expression network essential for early embryogenesis in rice, we cloned and conducted a functional analysis of transcription activating factor genes. We constructed cDNA libraries of embryos at 1, 2, 3, 4 and 5 days post anthesis (DPA) and selected 33 cDNA clones probed by homeo-domain-containing PCR fragments isolated originally from the 3 DPA embryo. These clones were categorized into 8 kinds of clones; HOS 9, 13, 16, 24, 58, 59 and 66, through sequence analysis. In addition, we cloned another cDNA coding zinc-finger protein probed by a maize homologue and designated it HAZ 1. To detect the expression specificity of these genes, RT-PCR analysis was carried out using fixed-cDNA libraries of 0, 1, 2, 3, 4, 5, 6 and 22 DPA embryos. Three HOS clones, HOS 13, 16 and 24, and the HAZ 1 clone showed stage specific expression patterns in the embryo. We next analysed the spacial expression patterns of these clones by in situ hybridization on sections of 3, 4 and 5 DPA embryos. The results showed that HOS 24 expresses on the shoot/radicle primordium at 3～4 DPA and HAZ 1 expresses on the surface cell layer of globular shape embryo at 3～4 DPA. These genes appear to have important roles in early rice embryogenesis. The analysis will be followed by searches for genes operating in up- and down-stream flows of gene expression in embryogenesis as well as through further functional analysis through generations of transgenic rice.

(2) Positional Cloning of a Segregation Distortion Gene Detected in the Progenies of Crosses with *Japonica* and *Indica* Rices

Yoshiaki HARUSHIMA and Nori KURATA

Site specific reduced gene transmission, which is called segregation distortion, has been detected in several chromosomal regions in F_2 progenies of

crosses between *japonica* and *indica* rices (ref.s 1, 2 and 6). To detect the fine positions and degree of causative genetic factors for the segregation distortion, each of 200 reciprocal backcrossed lines derived from either a backcross of the *japonica* rice, Nipponbare, or that of the *indica* rice, Kasalath, to an F_1 plant of Nipponbare crossed with Kasalath were planted and leaf samples were harvested. Fine mapping and positional cloning of these genetic factors will be continued in 1997.

(3) Molecular Cytogenetic Analysis of Nuclear Organization of Rice Chromosomes in Meiosis and Mitosis

Kenichi NONOMURA and Nori KURATA

This study focused on unravelling the mechanisms of spacial and functional organization of chromosomes in the nucleus. Before screening of mutants affecting both chromosome arrangement and function in the nucleus, we have begun with the following two approaches starting in October, 1996. We are now in the initial stage of the first important step of figuring out the basic and normal chromosome architecture in the nucleus by reconstructing three-dimensional images of *in situ* hybridization using position-known probes. Another approach is to investigate the effect of mismatch repair genes in relation to homologous and/or homoeologous chromosome interraction and recombination in rice. We have cloned a candidate partial fragment of a rice mismatch repair gene by PCR amplification using an adult leaf cDNA library.

(4) Generation of Enhancer and Gene Trap Lines in Rice

Yukihiro ITO, Mitsugu EIGUCHI, Kenichi NONOMURA, and Nori KURATA

To establish and to screen a lot of mutants affecting the steps of early embryogenesis and of chromosome organization in meiotic and mitotic stages in rice nuclei, we have started to produce gene and enhancer trap lines for most rice genes. For efficient generation of trap lines, we decided to use an Ac/Ds transposon system of maize, GUS or GFP genes as reporters and the T-DNA mediated gene transfer system of *Agrobacterium*. We chose useful constructs already used in the Arabidopsis gene/enhancer trapping system

and replaced some components with fragments suitable for rice transformation system. We are beginning to transform rice calli with either the vector carrying Ac-transposase or that carrying Ds+GUS gene and intend to cross these two Ac- and Ds-GUS transformed plants to obtain a lot of Ds-GUS transposed gene/enhancer trapped progenies.

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F–d. Microbial Genetics Laboratory

(1) Molecular Mechanism that Determines the Timing of Cell Division in the Cell Cycle of *Escherichia coli*

Akiko NISHIMURA

During the cell cycle of *E. coli*, several fundamental events take place through strictly periodic processes, though the synthetic pattern of DNA, cytoplasm, and membrane in the cell cycle are completely different from each other (Cooper, 1990), and two identical daughter cells are produced. The cell must have a mechanism coordinating each event. Cell division, for example, occurs after a constant period (20 min) and the coefficient of variation in cell size at the time of cell division is very small, about 10% (Schaechter *et al.*, 1962). These facts suggest that *E. coli* must have a mechanism that triggers the initiation of cell division at a specific cell length.

We isolated the novel mutants cfcA11 and cfcB1 which uncouple between cell division and DNA replication in the normal cell cycle. The cfc mutations reduced the period from nuclear division to cell division producing many small cells. The mutants grew exponentially, and the rates of macromolecular synthesis, DNA per mass ratio, elongation speed of DNA replication, and the mass at the initiation of DNA replication were not affected significantly by the mutations. Therefore, the cfc mutations affect only cell division, and the time shortened by the early division might be compensated for by the extension of the period for cells staying mononuclear.

We also found that Ap4A accumulates in the mutant strain of the *glyS* gene and overproduction of Ap4A causes early division in the cell cycle

uncoupling between cell division and DNA replication. It is known that Ap 4A affects many cellular processes (Zamecnik, 1983; Johnstone and Farr, 1991; Farr *et al.*, 1989). The Ap4A nucleotide is synthesized by certain aminoacyl-tRNA synthetases in a reaction in which an enzyme-bound aminoacyladenylate intermediate donates AMP to ATP (Zamecnik *et al.*, 1966). However, neither aminoacyl-tRNA synthetase mutants, inhibitory treatments of aminoacylation, nor structural mutants of tRNA are known to produce Ap4A *in vivo* (Bochner *et al.*, 1984). This is the first indication that Ap4A is involved in the regulation of timing of cell division in *E. coli*.

F–e. Genetic Resources Laboratory

Construction of Genetic Resources Database

Yukiko YAMAZAKI

The Genetic Resources Databank Project has entered its second trial phase. Information on wheat, rice, and drosophila genetic resources maintained by several laboratories in Japan are provided by individual databases via a WWW server (http://www.grs.nig.ac.jp) as well as local information on mouse stocks and cloning vector collections. A unified database of wheat and rice strains has also been constructed and has started to be used for experimental purposes.

(1) Wheat germplasm database—KOMUGI version 1.0—has been released. The database contains 13679 accessions of wheat stocks from 16 research laboratories in Japan. The data were classified into 8 groups according to their genetic properties. Each record consists of 9 categories and 63 items including genetic, biological and bibliographical information. The server offers both global and targeted keyword searching of the database. A data submission system, through which researchers can submit a new strain,and add or update their data using a Web interface, has been developed and is ready for test use.

An improved format with additional information is scheduled to appear in KOMUGI version 2.0. The construction of a barley germplasm database and wheat DNA clone databases have already been started with the collaboration of researchers.

(2) The first electronic database of rice genetic resources in Japan has

been constructed and distributed this year. In addition to information on 11020 strains maintained by 33 laboratories in Japan, basic biological knowledge, such as anatomical data, gene designations and a morphological linkage map, and about 900 citations are included. The data were divided into 10 groups and each group has individual items. Although the database is a trial version and still contains known inconsistencies, this is the most substantial collection among the currently accessible rice databases in the world. Release 1.0 will soon appear both on line and as a published catalog. Partnership with the scientific community is necessary to enrich the database and improve the quality of it.

(3) A unified database which integrated wheat and rice strain data was experimentally constructed under Sybase DBMS. A database schema was designed by which any syntactic inconsistencies between two plants were accepted as long as they were semantically identical, allowing cross-species search between them. The data were roughly divided into 9 semantic categories and 90 items which cover all data compiled. Although a more sophisticated interface would be desirable to read browsing data, the method allows quick integration of several different databases without changing any entities. The next plan is an application of the idea to other species databases.

F–f. Mammalian Development Laboratory

(1) Analysis of Cellular and Molecular Mechanisms in Development of Mouse Fetal Germ Cells

Norio NAKATSUJI and Yasuaki SHIRAYOSHI

Recent studies have shown that the stem cell factor (SCF), leukemia inhibitory factor (LIF), basic fibroblast growth factor (bFGF), and enhancement of the cAMP level increase proliferation and survival of mouse primordial germ cells (PGCs) in vitro. Even after the addition of these factors, however, it is still not possible to obtain proliferation of PGCs at a rapid rate similar to that in vivo, suggesting the presence of other growth factor(s) in vivo. We found that tumor necrosis factor-α (TNF-α) stimulates proliferation of PGCs at earlier migration stages. We also analyzed the functional role of gp130-mediated signaling in PGC growth in vitro (for details, see Ref. 1). A combination of IL-6 and soluble IL-6 binding subunit (sIL-6R), which

is known to activate intracellular signaling via gp130, fully reproduced the LIF action on PGCs. The addition of a neutralizing antibody against gp130 in culture markedly blocked PGC survival. These results suggest the pivotal role of gp130 in PGC development. We further demonstrated that a combination of LIF with forskolin or retinoic acid caused continuation of proliferation of PGCs, leading to propagation of embryonic stem cell-like EG cells.

Then, we showed that use of Sl/Sl4-m220 feeder cells and the addition of medium conditioned with Buffalo rat liver cells (BRL-CM) and forskolin to the culture medium stimulate PGCs obtained from 8.5 days post coitum (dpc) embryos to proliferate in culture at a rate comparable to that in vivo. In this condition, their proliferation continued several days past the timing of growth arrest, which we studied in detail by using the clonal culture method (for the details, see Ref. 2). Such proliferating PGCs continue to express c-kit and Oct-3 proteins. The characteristics of the medium and the requirement of feeder cells were different from those for embryonic stem (ES) cells. For details, see Ref. 3.

Also, we evaluated electroporation, liposome-mediated transfection, and the calcium phosphate (CaPO4) co-precipitation method for gene transfection of mouse primordial germ cells (PGCs) in culture as a prelude to the investigation of molecular mechanisms of germ cell development. We found that electroporation severely damaged PGCs, and the efficiency of liposome-mediated transfection was very low. In contrast, using the CaPO4 co-precipitation method, 18% of PGCs transfected with plasmid pSV-LT expressed simian virus 40 large tumor antigen (SV40 T-Ag) transiently. However, we did not detect any effects on the proliferation and survival of PGCs obtained from embryonic gonads at 11.5 days post coitum (dpc) during 2 days of culture after transfection. PGCs isolated from 11.5 dpc gonads change from spread- to round-shape and exhibit growth arrest during a few days of culture, and these rounded PGCs quickly disappear from the culture. We found that the transfection and expression of Bcl-X_L or adenovirus type 2 E1B 19,000-molecular-weight protein (E1B 19K) significantly promoted the survival of PGCs and retarded the disappearance of rounded PGCs from the culture system. These results suggest that Bcl-X_L or E1B 19K can prevent the apoptosis of PGCs and inhibit cell death of rounded PGCs in culture. For details, see Ref. 4.

(2) Establishment of Neuronal Stem Cell Lines for Analysis of Cell Lineages and Differentiation in Mouse Central Nervous System Development

Norio NAKATSUJI and Yasuaki SHIRAYOSHI

We established a large number of immortalized cell lines from early neuroepithelium through isolation of the forebrain region at E8.5 or E9.5 from mouse embryos harboring transgene tsA58, the temperature sensitive SV40 T-antigen gene (T-Ag) driven by the H2-Kb promoter. Expression of the oncogene product can be controlled by temperature and also by the presence or absence of interferon in cell culture. All of the NES8 lines obtained from such embryos at E8.5 expressed marker proteins of the neural precursor cells, but they could not be induced to differentiate into neurons or glia in various culture conditions. When injected into the ventricle of fetal brains at E14.5, however, NES8 cells showed engrafting into brain tissues and produced typical neurons in the cerebral cortical plate. Thus, NES8 cell lines seem to represent the undifferentiated neuroepithelial cells at the earliest stage. Half of the NES9 lines established from embryos at E9.5 were similar to the NES8 lines, but the remaining half expressed neuronal or glial differentiation marker proteins dependent on culture conditions. Thus, they seemed to contain cell lines at various stages in their determination and differentiation. These results suggest that the early neuroepithelium at E8.5 is made of homogeneous cell population of the early stem cells, and there is a beginning of cell determination and differentiation at E9.5 producing various types of multipotent stem cells and neuronal progenitor cells. In addition, the engrafting of some of NES lines into fetal brains through the ventricular zone indicates an important possibility that they may be used as a vehicle to investigate roles of various genes during CNS development by gene transfection of a NES line and examination of effects of the expressed gene on fates of the engrafted cells and also on the surrounding nervous system.

(3) Molecular Analysis of Cell Differentiation and Morphogenesis in Postimplantation Mouse Embryos

Yasuaki SHIRAYOSHI and Norio NAKATSUJI

We cloned one Notch related gene "int-3" from the 8.5 day cDNA library. The entire structure of the int-3 gene resembles that of Notch or vertebrate Notch homologues, and the int-3 gene showed 50–60% identity to the related Notch homologues in its amino acid sequence. We consider int-3 to be one of the mouse homologues of Notch. RT-PCR and in situ hybridization analysis revealed that int-3 was expressed in blood vessels in 9.5–10.5 day old embryos. The expression pattern of int-3 coincided with the expression of receptor tyrosine kinase Flk-1, which is regarded as a major regulator of vasculogenesis and angiogenesis. These results suggested that int-3 might be involved in the formation of blood vessels in early mouse embryos. For details, see Ref. 5.

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G. STRUCTURAL BIOLOGY CENTER

G–a. Molecular Biomechanism Laboratory

(1) Mechanism of Abortive Synthesis by *E. coli* RNA Polymerase

Tomoko KUBORI, Hiroki NAGAI, Ranjan SEN, and Nobuo SHIMAMOTO

Transcription is regulated at initiation, and there are both positive and negative controls. The regulation is afforded either by changing the rate of the rate-determining step (r. d. s.), or by changing the active fraction of the transcription complex or RNA polymerase. The latter mechanism works irrespective of the position of r. d. s., and seems to be more general from a kinetic viewpoint. The former mechanism, however, is conventionally supposed to be the case of most regulatory mechanism of transcription initiation.

Transcription of full-length products is always accompanied with abortive synthesis *in vitro*; iterative synthesis and release of 2–14 nucleotide transcripts. The physiological significance of abortive synthesis is still unknown, and its existence in a cell is technically difficult to prove. We speculated that abortive synthesis may relate to the regulatory mechanism classified in the latter mechanism.

In transcription from the λP_R promoter, we found a new transcription complex which produces only abortive transcripts (see Ref. 1). The complex, named moribund complex, cannot convert into the normal productive complex producing full-length transcript unless moribund complex is dissociated from DNA. The complex releases abortive transcripts with a time constant of 2.5 min, and iteratively synthesizes abortive transcripts (see Ref. 2). It also converts into a complex unable to elongate transcripts in 20 to 30 min. RNA polymerase prepared from the productive complex can convert into moribund complex, showing that productive and moribund complexes are generated from the same molecule of RNA polymerase.

Some mutations in the σ^{70} subunit decrease the synthesis of abortive transcripts. We compared transcription from several promoters by using RNA polymerases containing the wild-type and mutant σ^{70} subunit. Kinetic analysis showed that decrease of abortive synthesis is due to the destabiliza-

tion of binary complexes of polymerase and a promoter, rather than a decrease of moribund complex. A part of the dissociated binary moribund complex generates a binary productive complex upon rebinding to a promoter, resulting in indirect conversion from moribund complex into productive complex. We are accumulating evidence for difference between protein conformation of moribund complex and that of productive complex using protein and DNA footprinting techniques.

(2) Single-Molecule Dynamics of Transcription: Sliding of Proteins along DNA

Hiroyuki KABATA, Minoru TAKEUCHI, Takashi KINEBUCHI, Hisashi MIYASHITA, and Nobuo SHIMAMOTO

We previously showed the existence of a sliding motion of protein along DNA through direct visualization of single molecules of *E. coli* RNA polymerase. To check the generality of sliding, we applied similar single molecule dynamics to a bacterial repressor protein, *P. putida* CamR, which was observed to slide along DNA. In the absence of its inducer, d-camphor, CamR molecules were trapped at three positions on a λ DNA, one was its cognate operator cloned in λ DNA, and the other two are likely to be homologous to the operator. All trapping occurred at specific sites, and only sliding complexes were observed at non-specific sites. This observation indicates that the non-specific complex is the sliding complex itself.

Based on this observation, we developed a general theory of sliding. The theory predicts that sliding does not always cause an enhancement of specific binding. Sliding can accelerate not only association to a specific site but also dissociation from it. Under certain conditions sliding enhances specific binding, and the enhancement depends on the length of DNA. In this case non-specific sites close to a specific site, work as antenna to harvest protein molecules, but distant sites are competitors for specific sites. The critical distance distinguishing these two classes of non-specific sites is also derived from the theory. This theory solves many seemingly conflicting results previously obtained in kinetic assays of sliding.

We have been developing manipulating techniques for single molecules (see Ref. 3). As a fluorescent technique to detect single molecules, we have been improving the *A. victoria* Green Fluorescent Protein. The following two

awards were given for these lines of work.

James Melcher Prize Paper Award of IEEE/IA Trans, 1996

Saitou Award, 1996

(3) Crystallographic Analysis of *E. coli* Replication Termination Protein

Katsuhiko KAMADA, Kosuke MORIKAWA, and Nobuo SHIMAMOTO

Genome replication is terminated at specific sites, and a protein-DNA complex is reqired for this function. In *E. coli*, the Tus protein binds to one of *Tar* sites, and blocks replisome which is progressing in one direction, but the Tus-*Tar* complex does not block the one progressing in the other direction. This polar block is required for full replication of the genome. The mechanism of this polar block is studied by clarifying the structure of the Tus-*Tar* complex.

Among the 50 kinds of oligo DNA synthesized and tried, two DNA formed protein-DNA cocrystals suitable for X-ray diffraction study. The obtained structure with 2.7 angstrom resolution showed detailed features of Tus-*Ter* interactions (see Ref. 4). In the direction of blocking, replisome seems to collide with two alpha helices surrounding DNA, breaking no Tus-*Ter* interactions on the other side of the helices. In the other direction replisome collides with flat beta-sheets interacting specific bases of *Ter* DNA, and appears to break the interactions one by one, explaining the polar block by the complex.

This work was done under collaboration with Takashi Horiuchi, and Katsufumi Ohsumi (National Institute of Basic Research).

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G–b. Multicellular Organization Laboratory

(1) Fluoride-resistant Mutants of the Nematode *Caenorhabditis elegans*

Masaya TAKE-UCHI, Takeshi ISHIHARA, and Isao KATSURA

We are isolating and characterizing fluoride-resistant mutants of *C. elegans* to discover a new regulatory system. These mutants are recessive and map in five new genes, *flr-1* to *flr-5*. They are grouped into two categories: strongly resistant, slowly growing mutants (*flr-1*, *flr-3* and *flr-4*) and weakly resistant, normally growing mutants (*flr-2* and *flr-5*). Interestingly, the latter mutations suppress the slow growth of the former (Katsura, I. *et al.*, Genetics, **136**, 145–154, 1994). All those mutations seem to affect the nervous system, because the slowly growing mutants have short defecation-cycle periods (Dr. J. H. Thomas, University of Washington), because all except the *flr-2* mutations have synthetic dauer-constitutive phenotypes (See section (3)), and because some of them are defective in chemotaxis.

To learn what molecular mechanism is involved in the action of *flr* genes, we cloned the genes and cDNAs of *flr-1*, *flr-3*, and *flr-4*. The DNA sequences suggest that *flr-1* encodes an ion channel related to amiloride-sensitive ion channels of mammals. *flr-3* and *flr-4* code for a kinase-like molecule and a novel protein kinase, respectively, both of which have a hydrophobic domain on the carboxyl-terminal side of the kinase domain. This year we made several advances in our research on *flr-1* and *flr-4*. We obtained their full-length cDNA and determined the genomic DNA fragments that rescue the mutant phenotypes by microinjection. Two mutations each in *flr-1* and *flr-4* were identified by DNA sequencing. Both of the *flr-1* mutations were missense mutations near membrane-spanning regions, while the *flr-4* mutations were a splice-acceptor mutation in the kinase domain and a missense mutation in the C-terminal hydrophobic domain. This shows that the hydrophobic domain, in addition to the kinase domain, is essential for the function of the FLR-4 protein. We noticed a strange phenomenon on *flr-3*. Although we detected a single band of 2 kb in the Northern analysis, an 11 kb genomic fragment containing all of the 2 kb cDNA sequence did not

rescue any of the *flr-3* mutant phenotypes. However, a fragment 3.1 kb longer on the 3′ side did rescue all of the mutant phenotypes. We think there is an essential mRNA in *flr-3* that was not detected by the Northern analysis and that extends to this downstream region. We actually detected a candidate for such an mRNA by RT-PCR.

It is known from our previous study that a *flr-3-lacZ* fusion gene is expressed in most intestinal cells, beginning just before body elongation in the mid-embryonic stage and ending at the end of the L1 or the beginning of the L2 larval stage. This year we investigated the expression of GFP fusion genes of *flr-1* and *flr-4*. Both of them were expressed in the intestinal cells from middle-stage embryos to adults. Furthermore, the *flr-4*-GFP fusion was expressed also in a pair of head neurons from L1 larvae to adults. Thus *flr-1* and *flr-3*, and possibly also *flr-4*, may act in the intestine and affect neural functions from outside the neurons.

We plan to continue the analysis of the *flr* genes to elucidate the molecular mechanisms of their action regulating various neural functions as well as fluoride-sensitivity and growth-rate.

(2) A Gene That Controls Both Hatching and Cell Migration in *C. elegans*

Ryuichi HISHIDA, Takeshi ISHIHARA, and Isao KATSURA

Mutants in the *hch-1* gene have abnormalities both in hatching and in cell migration. They cannot digest protein components of the eggshell, and a neuroblast called QL and its descendants move anteriorly instead of posteriorly during larval development (Hedgecock, E. M. *et al.*, Development, **100**, 365–382, 1987). Having isolated a transposon-insertion mutant in *hch-1* (K. Kondo (Soka University) and I. Katsura, unpublished results), we cloned the gene by the transposon-tagging method and the cDNA by using a genomic fragment as the probe. The cloned cDNA encoded a protein that consisted of a signal peptide, a Zn protease domain, an EGF domain and a CUB domain, and that resembled the *Drosophila* TOLLOID and the mammalian BPM-1, both of which act in cell-differentiation. By whole-mount *in situ* hybridization of embryos, we found that expression starts just before body-elongation, continues through the comma stage and ends at the 1.5-fold or 2-fold stage, while no expression was detected at later embryonic stages. The

hybridization signal was found on the dorsal and lateral sides of the middle and posterior part of embryos before elongation, but was limited to the lateral sides (probably seam cells) at the 1.5-fold stage.

This year we investigated five *hch-1* alleles for their DNA sequence, the hatching defects, and the cell-migration abnormality. These alleles consisted of one nonsense and two missense mutations in the protease domain, a transposon-insertion mutation in the CUB domain, and a missense mutation in the Cys-rich region near the carboxyl terminal. While the former four mutations were recessive and had low penetrance in the cell-migration abnormality, the last allele was semi-dominant and had high penetrance, which shows the importance of the C-terminal part of the molecule for the function. Although most mutants showed an average delay of several hours in hatching and hatched completely in the end, the transposon-insertion mutation had a delay of more than 10 hours, and about half of its embryos did not ever hatch. Thus there was almost no correlation between the abnormality of hatching and that of the cell-migration.

We found that *hch-1* mutants show abnormality, though at much lower penetrance, in the direction (normally anterior) of migration of the QR neuroblast and its descendants, as well as in the direction (normally posterior) of migration of the QL neuroblast and its descendants. It is known that both of them migrate anteriorly in a loss-of-function mutant of the homeotic gene *mab-5*, and posteriorly in a gain-of-function mutant. We constructed double mutants between each of the five *hch-1* alleles and both types of *mab-5* mutants. None of the double-mutants showed the same cell-migration phenotype as either of the single-mutants, i.e., *hch-1* was neither epistatic nor hypostatic to *mab-5*. Thus it is unlikely that *hch-1* gene acts simply in the upstream or downstream of *mab-5* in the regulatory cascade. Protease activity in the hatching fluid was assayed *in vitro* with the soft eggshells of *hch-1* embryos as the substrate. Activity in the hatching fluid of a *hch-1* null mutant was not detectable, whereas a transformant embryo having many copies of the wild-type *hch-1* gene exhibited about 10 times greater protease activity than wild-type embryos. Thus, *hch-1* gene seems to encode the hatching enzyme itself or another factor that determines the level of hatching enzyme activity.

(3) Analysis of the Head Neural Circuit of *C. elegans* as Studied by Formation of Dauer Larvae

Norio SUZUKI, Takeshi ISHIHARA, and Isao KATSURA

If newly hatched larvae of *C. elegans* are in a crowded state and given a limited food supply, they deviate from the normal life cycle and develop to enduring, non-feeding larvae called dauer larvae. The developmental decision to become dauer larvae is regulated by the neural circuit in a pair of sensory organs called amphids, with the dauer pheromone and food as the input. Since the assay of dauer formation is much less time-consuming than behavioral assays such as chemotaxis assays, we are analyzing the head neural circuit by detecting dauer formation as the output. We found that more than 50 genes are involved in the synthetic dauer-constitutive (syn Daf-c) phenotype, i.e., the phenotype in which combinations of two mutations cause worms to develop to dauer larvae irrespective of environmental conditions. The synthetic nature of this phenotype, we think, is based on the structure of the neural circuit, in which many neurons are positioned in parallel or in a network, only a part of which can be blocked by a single mutation. If this is the case, the specific combinations of mutations for the phenotype should contain information on the structure of the functional neural network on which the dauer regulatory signal is transmitted and processed.

We found that the pattern of combinations for the syn Daf-c phenotype is very specific and can be explained by a model assuming three parallel pathways, if we exclude *flr* mutations. However, a more complicated model is necessary to explain the suppression of the syn Daf-c phenotype of various double-mutants by known dauer-defective mutations.

We isolated and mapped 44 new mutations that show the syn Daf-c phenotype in combination with the *unc-31* (*e169*) mutation. Eight of them were alleles of 4 known genes (*tax-2*, *osm-6*, *che-11*, and *aex-3*), but most of the remaining 36 mutations, which map in at least 18 genes, seem to be alleles of novel genes. We also examined the penetration of the fluorescent dye diO into amphids and phasmids (sensory organs in the tail) to investigate possible structural defects in those organs. Six mutants, of which four were alleles of *osm-6* and *che-11*, showed clear abnormalities in dye-filling. We also found that one of the mutants, which is normal in dye-filling, escapes from benzaldehyde and isoamyl alcohol, which attract wild-type worms. These results

show that the syn Daf-c phenotype is useful for the isolation of new neural mutations. It is known that wild-type worms become dauer-constitutive if three types of amphid sensory neurons (ADF, ASI, ASG) are killed, while the killing of only the ASI neurons is necessary to make the *unc-31* mutant dauer-constitutive. This suggests that many, if not all, of the above mutants have defects in ASI. We plan to characterize them further to elucidate their roles in sensory reception, transduction, or processing.

(4) Reverse Genetics of Neural Genes of *C. elegans* and the Analysis of Their Expression Using Jellyfish Green Fluorescent Protein

Takeshi ISHIHARA, Manabi FUJIWARA, and Isao KATSURA

To analyze the formation and function of the neural circuit in *C. elegans*, we made about 20 types of worms in which specific sets of neurons emit fluorescence. They were prepared by integrative transformation of worms by DNA constructs in which the cDNA of GFP (jellyfish green fluorescent protein; Chalfie, M. *et al.*, Science, **263**, 802–805, 1994) is fused with various *C. elegans* neural promoters. Three of the promoters were selected by the promoter trapping method (Hope, I., Development, **113**, 399–408, 1991), and others by looking at the genome DNA sequences. Most of the cells that express GFP were identified for each promoter.

Of the promoters obtained by promoter trapping, H20 gave expression in all neurons, H13 only in AFD neurons, and I85 in the post-embryonic blast cell G2 and its descendants, including RMF neurons. The gene for H13 encoded a putative transcriptional factor possessinging Zn fingers.

Of the genes selected by looking at DNA sequences, we investigated three glycine/$GABA_A$ receptor homologs and two metabotropic glutamate receptor homologs in more detail. We made their cDNA by RT-PCR, 5′-RACE, and 3′-RACE. We also isolated their deletion mutants by first selecting transposon-insertion mutants and then selecting deletion mutants made by imprecise excision of the inserted transposon. The behavioral defects of those mutants are now under study. We hope to elucidate the roles of interneurons by studying the functions of those receptors, which are expressed in some interneurons as well as other cells. More specifically, we are interested in metabotropic glutamate receptors because they play a role in learning in

mammals. Glycine/$GABA_A$ receptors are ligand-gated chloride channels that repress the activity of neurons. Hence, the disruption of their genes may be a useful means of producing the effects opposite to those from the killing of the neurons expressing them.

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G–c. Biomolecular Structure Laboratory

(1) Crystallographic Study of $\alpha_3\beta_3$ Sub-complex of F1-ATPase: Structural Analysis of Supramolecule

Yasuo SHIRAKIHARA

F1-ATPase is a catalytic sector of the membrane bound ATP synthase which plays a central role in energy conversion in mitochondria, chloroplasts and bacteria, generating ATP from ADP and inorganic phosphate using energy derived from a trans-membrane electro-chemical potential. The $\alpha_3\beta_3$ sub-assembly of F1-ATPase is an active complex with 20–25% of the ATPase activity of intact F1, and has similar catalytic properties to those of F1. Both F1-ATPase and the $\alpha_3\beta_3$ sub-assembly have been challenging targets for crystallographic study because of their large sizes e.g. 380 kDa for F1 and 320 kDa for the $\alpha_3\beta_3$ sub-assembly.

The nucleotide-free form of $\alpha_3\beta_3$ sub-assembly from Bacillus PS3 F1 formed the crystals which diffracted to 3.2Å resolution. We solved the structure of the sub complex by the molecular replacement technique using bovine F1 structure as a search model, and have finished refinement of the structure this year (manuscript in preparation). The final model has R factor of 20.5% (free R factor 29.5%) and has a reasonable stereochemistry e.g. the root mean square deviations from ideal values of 0.012Å for bond lengths, and 1.6° for bond angles. The structure solution was done in collaboration with Andrew Leslie, Jan Pieter Abrahams and John Walker at MRC Laboratory of Molecular Biology, Cambridge, UK.

The following structural features have been established after finishing the refinement. In the absence of nucleotides and the single copy subunits, the $\alpha_3\beta_3$ sub-assembly adopts a strictly symmetrical structure: the three α and three β subunits have identical conformations. This is in contrast to the asymmetrical crystal structure of nucleotide-bound bovine mitochondrial F1. The bacterial β subunits adopt a conformation essentially identical to that of the nucleotide-free β subunit in mitochondrial F1. In contrast, the a subunits have a similar conformation in both structures. When compared to the mitochondrial enzyme, the major differences in structure are localised in membrane-proximal half of the enzyme, while the remainder of the structure is highly conserved.

(2) Crystallographic Study of the Transcription Activator, PhoB

Toshihiko AKIBA and Yasuo SHIRAKIHARA

PhoB Protein is a positive transcriptional activator for the genes in the phosphate (*pho*) regulon of *E. coli*, such as *phoA* and *pstS*, that are induced by phosphate starvation. PhoB acts by binding to the *pho* box in the promoter region, which is the consensus sequence shared by the regulatory regions of *phoA*, *phoB*, *phoE* and *PstS*. The activity of PhoB is regulated by phosphorylation by PhoR. The N-terminal domain of PhoB is responsible for this regulatory role, whereas the C-terminal domain has a DNA binding ability.

The DNA-binding C-terminal domain, spanning from a residue 125 to the C-terminus, of the protein has been crystallized in phosphate solutions by the microdialysis method. Diffraction data were collected using synchrotron radiation and the Weissenberg camera installed at the beam line BL18B of the Photon Factory in KEK, Tsukuba. Crystals diffracted to 2.0Å resolution and showed good stability against X-ray irradiation at an ambient temperature. The crystals belong to the space group $P2_1$ with unit cell dimensions a = 30.74 Å, b = 105.91Å, and c = 30.90Å, $\beta = 110.27°$. Using the collected data set, a structural analysis has been attempted employing the molecular replacement method with the structural model of OmpR, which has become available recently. This work has been done in collaboration with Kozo Makino, Research Institute for Microbial Diseases, Osaka University.

(3) Crystallographic Study of the Transcription Repressor, CamR

Yasuo SHIRAKIHARA

CamR protein is a repressor that regulates transcription of the cytochrome P-450cam hydroxylase operon of *Pseudomonas putida*. Expression of the *camDCAB* operon and the *camR* gene is regulated through interaction of the CamR protein with the single operator located in the overlapping promoter region between the *camDCAB* operon and the *camR* gene. D-camphor is an inducer which allows those genes to be transcribed. CamR is a homodimer with a molecular mass of 40 kDa. Crystals of CamR with satisfactory size was previously obtained from polyethyleneglycol solution in the presence or absence of D-camphor. However, recent crystallization experiments have failed to grow such crystals, probably due to variability of protein preparations. While trying to improve the quality of the CamR protein preparation, we searched for new crystallization conditions and found that Na-K-phosphate gave crystals with the similar size in the presence of D-camphor. These crystals diffract to at least 2.8Å resolution, but are temperature sensitive: the crystals grown at a low temperature got cracked at room temperature where a diffraction study was made. Effort is being made to overcome the problem. This work has been done in collaboration with Hironori Aramaki, Daiichi College of Pharmaceutical Sciences.

G–d. Gene Network Laboratory

(1) Expression Pattern Map of the *C. elegans* Genome

Yuji KOHARA, Tomoko MOTOHASHI, Hiroaki TABARA, Hisako WATANABE, Masako SANO, Akiko MIYATA, Tokie OHBA, Yuko MITANI, Kouichi IIDA, and Hiroko UESUGI

Aiming to ultimately understand the network of gene expression in the development of the nematode *C. elegans*, we are constructing an expression pattern map of the 100 Mb genome by identifying and characterizing its cDNA species, whose total number is estimated to be around 15,000.

More than 100,000 cDNA clones were picked up randomly from different cDNA libraries, stored and gridded. cDNA clones derived from already

analyzed abundant cDNA species were identified by probing the high density clone grids and removed to avoid unnecessary redundancy of analysis. The remaining clones were subjected to analyses of (1) tag-sequencing from both 5′-ends (with vector primers) and 3′-ends (with anchored oligo-dT primers), (2) mapping onto the genome, and (3) analysis of expression patterns. Thus far, 32,000 clones have been processed, and 21,500 clean 3′-tag sequences were obtained, which were classified into >6,000 unique cDNA species (nearly half of the total genes) by comparing the 3′-tags. About 70% of them were located on the genomic sequence determined by the Sanger Ctr/WASH-U sequencing consortium. We are analyzing the expression patterns during development of the classified cDNA species using the newly developed method of *in situ* hybridization on whole mount specimens of embryos, larvae and adults in the standard multi-well format. Thus far, about 30% of them have shown specific expression patterns. The cDNA sequence and mapping information has been made available at our WWW site (http://www.ddbj.nig.ac.jp/htmls/c-elegans/html/CE_INDEX.html) in collaboration with DDBJ staff and we are preparing to make the expression patterns open at the WWW site.

(2) *Pos-1*, a Gene Which Shows Localization of Its mRNA to the P Lineage during Early Cleavage of *C. elegans* Embryos

Hiroaki TABARA, Craig MELLO[1], Russell HILL[2], Jim PRIESS[1], and Yuji KOHARA ([1] Cancer Center, University of Massachusetts, Worcester, MA, USA, [2] Fred Hutchinson Cancer Research Center, Seattle, WA, USA)

Taking advantage of our cDNA project, we have identified a lot of maternal genes which showed interesting patterns of expression. One of them, the cDNA group CELK01662 (=YK1662 in ACEDB), showed an asymmetric distribution of its mRNA in 2-cell stage embryos. Closer examinations of the behavior of the mRNA in gonads and embryos showed that (1) the mRNA started to appear at the turn of gonad, (2) it was distributed evenly in oocytes, (3) it was segregated posteriorly as the first cleavage proceeded, (4) the mRNA remained in P4 but disappeared in other somatic blastomeres (perhaps through a mechanism of degradation). Thus, we named this gene *pos-1* (posterior segregation).

Sequencing analysis showed that pos-1 had strong homology to the zinc-

finger region of the mammalian Tis11 growth factor inducible genes. This zinc-finger motif is also found in a maternal gene *pie-1*. Interestingly, the physical map position of *pos-1* turned out to be very close to the genetic map position of another maternal gene, *skn-2*. The *skn-2* gene like *pie-1*, is required for proper development of the P lineage and *skn-2* and *pie-1* mutants exhibit dominant genetic interactions. Based on the following evidence we concluded that *skn-2* is a mutation of the *pos-1* gene; (1) injection experiments using *pos-1* antisense RNA caused phenocopies of *skn-2*, and (2) missense or deletion mutations were found in the zinc finger region of *pos-1* in five *skn-2* mutant alleles.

The overall phenotype of *skn-2* mutants is the lack of germ line development. Although *skn-2* embryos are lethal, some somatic lineages seem to differentiate properly; we found the differentiation of hypodermis, gut and pharynx, but not body-wall muscle, in *skn-2* embryos. It was shown that the *pie-1* gene, a genetically related gene to *skn-2*, repressed the transcription of somatic genes in the germ line. *In situ* hybridization experiments, however, showed that this was not the case in the *skn-2* mutants. Antibodies against the N-terminal part of the *pos-1* protein were raised and the distribution of the *pos-1* protein was examined. The expression of the protein was coincident with the pattern of the localized mRNA. Essentially no expression of the protein was detected in 1-cell embryos or AB blastomeres in 2-cell embryos. The expression of the protein was detected in P1, P2, P3 and P4. Interestingly, the expression of the POS-1 protein was cytoplasmic in contrast to other genes containing the Tis11 zinc finger motif.

Based on these facts and preliminary observations of other genes containing the Tis11 zinc finger motif, we hypothesize that the function of the POS-1 protein is to stabilize or activate the translation of mRNA of other factors which are necessary for establishing the fate of the germ line.

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H. CENTER FOR INFORMATION BIOLOGY

H–a. Laboratory for DNA Data Analysis

(1) Large-Scale Search for Genes on Which Positive Selection May Operate and Analysis of Their Biological Meanings

Toshinori ENDO[1, 2] and Takashi GOJOBORI[1] ([1] National Institute of Genetics, [2] New Energy and Industrial Technology Development Organization)

With the aim of revealing the role of positive selection in gene evolution, we have developed a method for searching and identifying the genes on which positive selection may operate by comparing the numbers (dS and dN) of synonymous and nonsynonymous substitutions of homologous DNA sequences. By searching through 3,595 homologous gene groups representing all the coding sequences in the DDBJ database, we obtained 17 gene groups as the candidates. We published the result in Mol. Biol. Evol., **13**, 685–690, 1996. This method, however, failed to detect the MHC and Abalone sperm lysin genes on which positive selection was shown to have operated previously. We found that the problem came from the comparison between dS and dN for whole coding regions. To resolve this problem, we developed a new method for detecting positive selection within a small region of coding genes. We named this method as Window Search. As a result, we could identify 192 candidate gene groups on which positive selection may operate. They included genes that are reported to be under positive selection. We examined genes for Abalone sperm lysin and Rhodobacteria photosynthetic reaction center H subunit whose tertiary structure had been resolved, to study the relationships between the positive selection and tertiary structure of the gene product. In the former case, the region under positive selection has a shape similar to scissors. This region is considered to have interaction to egg surface molecules and it may be important for the egg to identify the sperms of the same species among many sperms in the ocean. Thus, positive selection might help develop distinguishable genes for lysin molecules. In the latter case, the gene product is a component of multisubunit protein. It is suggested that the gene in question corresponds to the protein that interact with

photoharvesting protein, which might vary depending on the wavelength of absorbing light. We reported these results at the 19th Annual Meeting of the Japan Society of Molecular Biology in Sapporo.

(2) Phylogenetic Analysis of Duplicated Regions within Human Chromosomes

Toshinori ENDO[1, 2], Tadashi IMANISHI[1], Takashi GOJOBORI[1], and Hidetoshi INOKO[3] ([1] National Institute of Genetics, [2] New Energy and Industrial Technology Development Organization, [3] Tokai University School of Medicine)

We examined the evolution of duplicated band regions in human chromosomes on 6p21.3 and 9q33-34. The band 6p21.3 is well known as the major histocompatibility complex (MHC) region and is well characterized. The two regions contained more than 11 pairs of paralogous genes. We examined the phylogenetic relationships between each pair of genes in the two regions, together with the homologous genes from other organisms including mice and Drosophila. We concluded that those genes in the two regions diverged around the time when vertebrates emerged, with some exceptions. The gene order is conserved as well, though we need to consider some duplication and rearrangement events of some chromosomal segments. It is possible that the duplication event might have triggered the emergence of vertebrates. Inspired by the finding above, we attempted to identify such duplicated regions by conducting homology searches among genes whose chromosomal locations are already known. As a result, we found that at least 200 chromosomal bands carry genes that have homologous genes on different chromosomal bands. However, we could not identify a large-scale duplicated tract extending over several bands except for the chromosome-long homology found between chromosomes 22 and X. Thus we concluded that duplication and rearrangement of chromosomes may be common events. We reported the result above at International Symposium on Molecular Evolution at Guana Caste in Costa Rica 1997.

(3) The Origin of Domestic Fowls

Akishinonomiya FUMIHITO[1], Tetsuo MIYAKE[2], Masaru TAKADA[3], Ryosuke SHINGU[4], Toshinori ENDO[5,6], Takashi GOJOBORI[5], Norio KONDO[3], and Susumu OHNO[6] ([1] Yamashina Institute for Ornithology, [2] Wakunaga Pharmaceutical Company Central Laboratories, [3] The Research Institute of Evolutionary Biology, [4] Osaka University Medical School, [5] National Institute of Genetics, [6] New Energy and Industrial Technology Development Organization, [6] Beckman Research Institute of the City of Hope)

We attempted to identify the origin of domestic fowls by molecular phylogenetic analysis of mitochondrial DNA. We concluded that all the domestic fowls have a single origin and their most closely related species might be the red junglefowl in the Indochinese Peninsula. We published the result in Proc. Natl. Acad. Sci., **93**, 6792–6795, 1996.

(4) The Molecular Mechanisms of Choosing Infected Clones in Mother-To-Child Transmission of HIV-1

Kazunari TAKAHASHI and Takashi GOJOBORI

Mother-to-child transmission of human immunodeficiency virus (HIV-1) is one of the major paths of AIDS infection. With the aim of understanding which virus clones are transmitted from mother to child, we extracted nucleotide sequences of viruses in mothers and their children from DDBJ (DNA Data Bank of Japan) or journals and investigated the patterns of mutations in the V3 amino acid sequences of the env gene product soon after the birth of an infected child. By comparing the sequences between mothers and children, we obtained the characteristic features of sequence differences between transmitted and nontransmitted viruses. We also estimated the amino acid sites of the V3 region which may play an important role during mother-to-child transmission.

(5) HLA Class II Alleles in Ainu Living in Hidaka District, Hokkaido, Northern Japan

Makoto BANNAI[1], Katsushi TOKUNAGA[1], Tadashi IMANISHI, Shinji HARIHARA[3], Kiyoshi FUJISAWA[4], Takeo JUJI[4] and Keiichi OMOTO[5] ([1] Department of Research, Japanese Red Cross Tokyo Metropolitan Blood Center, [2] Department of Human Genetics, School of International Health, The University of Tokyo, [3] Department of Biological Sciences, Graduate School of Science, The University of Tokyo, [4] Japanese Red Cross Central Blood Center, [5] Division of Research, International Research Center for Japanese Studies)

The Ainu people are considered to be the descendants of preagricultural native populations of northern Japan, while the majority of the population of contemporary Japan (Wajin) is descended mainly from postneolithic migrants. Polymorphisms of the HLA-DRB1, DRB3, and DQB1 alleles were investigated in DNA samples of 50 Ainu living in Hidaka district, Hokkaido. Unique features of the Ainu in this study were high incidences of DRB1* 1401, DRB1*1406, and a newly described allele, DRB1*1106 (20%, 17%, and 5%, respectively). On the other hand, several common alleles in Wajin (DRB1*1502, 1302, 0803, and 1502) were found at relatively low frequencies (1–2%) in Ainu. Previously DRB1*1406 was described as a characteristic allele of some Native American or northeast Asian ethnic groups, and DRB1*1106 had been found in only two Singapore Chinese and one Korean. Principal component analysis of various populations based on HLA class II allele frequencies places the Ainu population midway between other east Asian populations, including Wajin, and Native Americans. These observations support the hypothesis that the Ainu people are the descendants of some Upper Paleolithic populations of northeast Asia from which Native Americans are also descended. See Ref. 5 for details.

(6) Patterns of Amino Acid Substitutions in the V3 Region of HIV Within Single Hosts

Yumi YAMAGUCHI and Takashi GOJOBORI

The third variable envelope region (V3) is one of the major epitopes of HIV. We intended to elucidate the evolutionary mechanism of molecular

evolution of the V3 region within a human body. We collected the nucleotide sequence data of HIV clones which were isolated from single hosts at several time points after infection. From the comparisons between the rates of synonymous and nonsynonymous substitutions, we previously showed that there were the particular periods when positive selection was operating in the V3 region of HIV within single hosts. In this study, we attempted to characterize patterns of amino acid substitutions. For this purpose, we estimated frequencies and types of amino acid substitutions that occurred in the V3 region. We also compared the substitution pattern obtained with that expected by the assumption of the JTT model (Jones *et al.*, 1992). We then found that the proportion of amino acid substitutions altering their electric charge was significantly high in the V3 region compared with the expectation from the JTT model. Our results indicate that the V3 region may have characteristic patterns of amino acid substitution due to positive selection which favors amino acid substitutions causing antigenic or phenotypic variations.

(7) An Analysis of Amino Acid Diversity at One Site in the Envelope Glycoprotein of HIV-1

Yumi YAMAGUCHI and Takashi GOJOBORI

In protein evolution, the variability of amino acids at each site in proteins may be affected by the level of functional constraints or selective advantages for amino acid changes. We intended to elucidate the evolutionary mechanism of the protein at one amino acid site level by examining the correspondence of the variability of amino acids at one site to its biological function.

We developed a new method to estimate variability at each amino acid site by extending the idea of the "nucleotide diversity." Our method uses aligned amino acid sequence data. The amino acid diversity at a given site is defined as the proportion of pairs of two sequences whose amino acids on the site are different. This quantity is estimated from data of frequencies of amino acids or gaps at each amino acid site. In this method, the amino acid diversity can be divided into several components of interest. For example, the whole diversity including gaps can be divided into diversity by gaps and that by different amino acids. We applied our new method to the envelope glycoprotein of HIV-1 to elucidate the relationships between the diversity of amino

acids generating selective advantages and the functional constraints of the protein that are important for the viral replication.

We analyzed 67 nucleotide sequences of gp120 (surface glycoprotein) of HIV-1 (subtype B). Our findings are as follows: 1) insertions or deletions are concentrated on six regions, 2) the V3 region (one of the major epitopes of HIV-1) is one of the most variable regions in gp120, 3) there are several highly variable regions with variability on electric charge of amino acids as much as the V3 region in gp120, 4) the amino acid sites that are responsible for binding to CD4 locate on relatively conservative region.

(8) Exceptionally Conserved Regions Found in Extensively Rearranged Microbe Genomes

Hidemi WATANABE, Takeshi ITOH[1], Hirotada MORI[1], and Takashi GOJOBORI ([1] Nara Institute of Science and Technology, Japan)

To investigate the evolutionary process of genome structures in microbes, completely or nearly completely sequenced genomes of *Haemophilus influenzae* Rd, *Mycoplasma genitalium*, *Mycoplasma pneumoniae*, *Synechocystis* sp. strain PCC6803, *Methanococcus jannaschii*, *Saccharomyces cerevisiae*, *Escherichia coli* and *Bacillus subtilis* were compared to each other using their DNA sequences. The comparisons were carried out by examining the locations of orthologous genes in different genomes. As a result, we found that the genomes of these microbes must have undergone frequent rearrangements except for a few extremely conserved gene clusters. Here, orthologous genes are defined as those which originated from a single gene in the last common ancestral genome. The orthologous gene pairs were identified according to the following four criteria: (1) Each of an orthologous pair of genes should be more similar to each other than to any other genes it can be paired with; (2) Each orthologous gene pair should show similarity of statistical significance; (3) All the possible pairs of the genes comprising an orthologous gene group should satisfy the above-mentioned two criteria; (4) Gene phylogeny based on similarities of genes should be consistent with the phylogenetic relationships among those species. Through these analyses, we revealed that the order of orthologous genes identified between different genomes is so unstable that almost all operons were broken, although operons have been believed to be very conservative structural units. This indicates

that dynamic rearrangements have frequently occurred in those microbe genomes during their evolution. In the extensively rearranged microbe genomes, however, we found several exceptionally conserved regions such as ribosomal protein gene clusters, the RNA polymerase operon, the ATPase operon, and a cluster composed of genes for cell wall synthesis and cell division. This suggests that the gene order in these conserved regions must be essential for survival of those organisms, implying the existence of special structural constraints on the regions. Thus, this kind of study will provide significant insights into the ancestral genome structures and their relationships to gene functions. This study was presented in the International Symposium on Network and Evolution of Molecular Information, The 1996 Cold Spring Harbor Meeting on Molecular Genetics of Bacteria and Phages, and J. Mol Evol., **44**(Suppl. 1), S57–S64.

(9) Characterization of the *Methanococcus jannaschii* Genome in Terms of Its ORF Composition and the Structures within and around ORFs

Hidemi WATANABE, Takashi GOJOBORI, and Kinichiro MIURA[1] ([1] Institute for Biomolecular Science, Gakushuin University)

As a result of genome projects, the sequence of the whole genome of an archaeon, *Methanococcus jannaschii*, was recently presented as well as other complete sequences of bacterial and eucaryal genomes. Although previous investigations mostly using translation-related genes showed that Archaea were closely related to *Eucarya* rather than *Bacteria*, a classification of the entire genes of *M. jannaschii* on the basis of the sequence similarity clearly shows that this archaeon had chimeric sets of bacterial-type (70%) and eucaryal-type ($<30\%$) genes. Most of the minor eucaryal genes are involved in the translation and transcription pathway. However, a few genes such as the 16S ribosomal RNA and an EF-Tu-like gene, are bacterial. To understand the discrepancy between the phylogenetic place and the gene composition of this archaeon, we investigated whether the genome structure of *M. jannaschii* is eucaryal or bacterial in terms of the base compositional pattern around and within ORFs. In this investigation, a statistical method, G-test, was applied to evaluate the base biases around the boundaries of ORFs. Then, we found that *M. jannaschii* possesses some bacterial features in the

base biases around and even within ORFs. This indicates that the exceptional bacterial genes for translation play crucial roles in the translation pathway. We dare to hypothesize that this archaeon is more closely related to *Bacteria* than *Eucarya* on the whole. This study was presented in the International Workshop on Recent Advance in Genome Biology of Micro-organisms and the Meeting of the International Society of Molecular Evolution "JUNK DNA: The Role and the Evolution of Non-coding Sequences," and also is going to be published in a special issue of GENE.

(10) Implication about the Evolutionary Process of the Generation of Regulator-Regulatee Relationships after Speciation between *Escherichia coli* and *Haemophilus influenzae*

Hidemi WATANABE, Kousuke GOTO[1], and Takashi GOJOBORI ([1] Teijin systems technology Co., Ltd.)

The transcriptional regulation relationships among genes in *Escherichia coli* have been extensively studied. However, little about the generation process of the relationships has been investigated. To get insight into the evolutionary processes of the generation of regulatory relationships, we examined which regulators and regulatees in *Escherichia coli* were conserved in *Haemophilus influenzae*. For this examination, we first compiled the data on transcriptional regulation relationships among the *E. coli* genes from literal sources: ninety-seven regulons in which 262 regulated operons (455 genes) are regulated. By identifying the orthologues of these *E. coli* genes in the *H. influenzae* genome, it was revealed that only twenty-seven orthologous regulators (28%) and 151 orthologous regulated genes (33%) had been retained in *E. coli* and *H. influenzae* since the beginning of speciation, and that among the ninety-seven operons each of which were composed of two or more genes in *E. coli*, only fifteen operons were completely conserved in *H. influenzae*. These findings strongly suggest that regulation systems and regulation relationships among genes in these organisms have been frequently reconstituted after speciation. By searching for the origins of regulatory regions, we are currently investigating the evolutionary events for the neo-genesis of the regulatory regions of the genes and operons in *H. influenzae* whose orthologous genes or operons together constitute a single operon in *E. coli*.

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H–b. Laboratory for Gene-Product Informatics

(1) Improvement of Protein Sequence-Structure Compatibility Method by the Best-Five Test

Motonori OTA and Ken NISHIKAWA

We proposed a new assessment, called the best-five test, for the pseudo-energy potential empirically derived from the protein structural database. The object of the test is the three-dimensional (3D) profiles of proteins, which are directly connected to pseudo-energy potentials. In the 3D profile, the fitness of each amino acid type is ranked at each residue site of a protein. A site whose native residue type is ranked within the best-five out of 20 amino acids is regarded as satisfactory and the ratio of satisfactory sites over all the sites of all proteins examined is indicative of the efficiency of the pseudo-energy potential employed. We applied the test to our potential functions, by setting various kinds of definitions to constitute energy functions. Through this test, the validity of the minus average operation was confirmed, where the energy level of potential functions is adjusted by referring to the random-environmental state of proteins. Failure without the operation was ascribed to bulky hydrophobic residues, which almost always occupy higher ranking positions in the 3D profile table. A maximum success ratio of 55.6% was attained with the final set of potentials. The efficiency of the final set was further checked in the fold-recognition test for distantly related proteins. The best-five test is a new use of the 3D profile table for assessing the ability

of pseudo-energy potentials. See Ref. 1 for details.

(2) A Distant Evolutionary Relationship between Bacterial Sphingomyelinase and Mammalian DNase I

Yo MATSUO[1] and Ken NISHIKAWA ([1] Institute for Social Information Science, Fujitsu Laboratories Ltd.)

The three-dimensional structure of bacterial sphingomyelinase (SMase) was predicted using a protein fold recognition method; the search of a library of known structures showed that the SMase sequence is highly compatible with the mammalian DNase I structure, which suggested that SMase adopts a structure similar to that of DNase I. The amino acid sequence alignment based on the prediction revealed that, despite the lack of overall sequence similarity (less than 10% identity), those residues of DNase I that are involved in the hydrolysis of the phosphodiester bond, including two histidine residues (His 134 and His 252) of the active center, are conserved in SMase. The functional roles of SMase residues identified by sequence comparison were consistent with results from mutant studies. Two *Bacillus cereus* SMase mutants (H134A and H252A) were constructed by site-directed mutagenesis. They completely abolished their catalytic activity (collaboration studies with Prof. Hiroh Ikezawa of Nagoya City University). A model for the SMase-sphingomyelin complex structure was built to investigate how SMase specifically recognizes its substrate. The model suggested that a set of residues conserved among bacterial SMases, including Trp 28 and Phe 55, might be important in substrate recognition. The predicted structural similarity and the conservation of functionally important residues strongly suggest a distant evolutionary relationship between bacterial SMase and mammalian DNase I. See Ref. 2 for details.

(3) Comparison of Protein Structures Using 3D Profile Alignment

Mikita SUYAMA[1] and Ken NISHIKAWA ([1] Biomolecular Engineering Research Institute)

A novel method for protein structure comparison using 3D profile alignment was presented. The 3D profile is a position-dependent scoring matrix

derived from three-dimensional structures, and is basically used to estimate sequence-structure compatibility for prediction of protein structure. Our idea was to compare two 3D profiles using a dynamic programming algorithm to obtain optimal alignment and a similarity score between them. When the 3D profile of hemoglobin was compared with each of the profiles in the structural library, all the profiles of other globins were detected with relatively high scores, and proteins of the same structural class followed the globins. Exhaustive comparison of 3D profiles in the library was also performed to depict protein relatedness in the structure space. Using multidimensional scaling, a planar projection of points in the protein structure space revealed an overall grouping in terms of structural classes, *i.e.*, all-α, all-β, α/β, and $\alpha+\beta$. These results differ in implication from those obtained by the conventional structure-structure comparison method. Differences are discussed with respect to the structural divergence of proteins in the course of molecular evolution. See Ref. 3 for details.

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H–c. Laboratory of Gene Function Research

(1) DNA Database and Its Applications to the Study of Molecular Evolution

Takashi GOJOBORI and Yoshio TATENO

With the recent advancement of molecular biology and biotechnology, the DNA sequence data are accumulating with an enormous rate. In particular, the rapid development of genome projects for various kinds of species including humans spurs the increase rate of the DNA sequence data in

collaboration with EBI (European Bioinformatics Institute) at EMBL (European Molecular Biology Laboratories) and NCBI (National Center for Biotechnology Information) at NLM (National Library of Medicine)/NIH (National Institutes of Health). Moreover, these international DNA data banks also play roles in providing the data to researchers worldwide. In Ref. 1, we briefly overviewed the present activities of the international DNA data banks with special reference to DDBJ. Then, we presented how the DNA sequence data are important for various research areas of life sciences. We focused on the study of molecular evolution and showed some examples of the study of evolution of genes and genomes by use of the DNA sequence database.

(2) A Biological Taxonomy Database System on World-wide Web

Hajime KITAKAMI[1], Yasuma MORI, Yoshio TATENO, and Takashi GOJOBORI ([1] Hiroshima City University)

We newly developed an intelligent system which is needed to integrate and search across taxonomy databases constructed by the international DNA data banks over international computer networks. Integration across any two taxonomy databases raises complex problems. We tried to resolve the problems by introducing the intelligent mechanisms to integrate across the taxonomy databases with semantic heterogeneity. We also described intelligent tree search methods implemented in stored procedure and loosely coupled interface between WWW and database servers. Existing database systems do not support such devices and easy interface to access the taxonomy databases over computer networks. For details, see Ref. 4.

(3) Development of New DDBJ DNA Sequence Database with Data Annotation Tool Yamato II

Tomohiro KOIKE[1], Toshitsugu OKAYAMA[1], Jun ISHII[1], Tadashi MIZUNUMA[1], Takurou TAMURA[2], Yoshio TATENO, Hideaki SUGAWARA, Ken NISHIKAWA, Tadashi IMANISHI, Kaoru FUKAMI-KOBAYASHI, Kazuho IKEO, and Takashi GOJOBORI ([1] Hitachi Software Engineering Co., Ltd., [2] Association for Propagation of the Knowledge of Genetics)

As the molecular biology has made a rapid progress, there has been a great number of changes required for the methodology for maintaining and utilizing DNA sequence data. For example, annotation to sequences has become complex and extensive. DDBJ which recognized the impending requirements decided to develop a new DNA sequence database system in 1995. To cope with frequent changes of the data structures and significant increment of the data in terms of quality and quantity, we designed a completely new database schema. In the new system, physical changes of the data structure no longer affect such applications as a tool for annotation. We also designed a new annotation tool with object oriented concept that allows us to handle DNA sequence data in computers as intuitively as in the real world. The annotation tool is named as YAMATO II. We also take care of needs from DDBJ itself in the new system. Data traffics and security in the database access are especially analyzed and outside reviewers of data for DDBJ are now able to process the data safely and comfortably in the new system. The new system also realizes more robust and effective data exchange with partners in the international nucleotide sequence banks, EBI and GenBank. For details, see Ref. 5.

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H–d. Molecular Classification Laboratory

(1) Improvement of the Data Processing System for DDBJ

Hideaki SUGAWARA, Yoshio TATENO, Kazuho IKEO, Naruya SAITOU, Tadashi IMANISHI, Kaoru FUKAMI-KOBAYASHI, Satoru MIYAZAKI, Motonori OTA, Takashi GOJOBORI, and Katumi ISONO[1] ([1] Kobe University)

We improved the data processing system for DDBJ which faces the explosion of sequence data. The core system is the database management system named YAMATOII that was designed based on object-oriented concept and has three layers of application, access and client. The improved system is flexible and expandable enough to accommodate massive and complicated sequence data in the future. We also implemented a new data submission system named SAKURA. It takes advantage of INTERNET and World Wide Web technology and easy to use. Therefore more than half of the submission has been already done through SAKURA and quality of the data is higher than those submitted by other methods. For details, see Ref. 6 and Ref. 15.

(2) Databases and Network Agents on Biological Data

INTERNET and distributed data management systems will be an indispensable information environment for biology. We surveyed, studied and

developed systems which are not model systems in laboratory but process actual data in the real world. They will be the elements or modules of the integrated system for biology in the future. For details, see Ref. 8, 9, 11, 13 and 14.

a. Information retrieval from distributed data sources in INTERNET

Satoru MIYAZAKI, Junko SHIMURA[1], Takashi NAKASE[1], Hideaki SUGAWARA ([1] The Institute of Physical and Chemical Research)

We developed an agent that helps researchers retrieve reliable data from servers which are distributed in INTERNET. The subject of the prototype is microbes such as bacteria, fungi and cell lines. For details, see Ref. 4 and Ref. 12.

b. Utilization of Broad Band Network for Science and Technology

Satoru MIYAZAKI and Hideaki SUGAWARA

We participated G7 GIBN projects, in which we carried out on-line demonstration of virtual laboratory, tele-education, video-on-demand and multi-media database between Japan and US. We could show the value and feasibility of ATM switch network, especially, the usefulness of the virtual laboratory composed of a large database, a high performance computer and an experimental group which are physically distributed in Japan and US.

c. Database on EPITOPE

L. LING[1], Junko SHIMURA[2], Hideaki SUGAWARA and Akira TSUGITA[1] ([1] Science University of Tokyo, [2] The Institute of Physical and Chemical Research)

We developed a database on epitopes which are located in antigens and whose sequences are determined. The database has links to PIR-International, DDBJ/EMBL/Genbank and Hybridoma Data Bank and is a valuable tool for the analysis of the nature of molecular interaction. For details, see Ref. 3.

d. Statistical Analysis and Database on Human Leukocyte Differentiation Antigens (HLDA)

Hitoshi KIKUTANI[1], Hideaki SUGAWARA, Satoru MIYAZAKI, Tadao OHNO[2], Kaoru SIAJO[2], Junko SHIMURA[2] ([1] Osaka University, [2] The Institute of Physical and Chemical Research)

We developed and provided to the public the database on HLDA. It includes the reaction of 333 antibodies which were tested in the occasion of the 6th International Workshop of HLDA in 1996. Based on the result of cluster analysis, the new CD numbers are assigned to the new antibodies.

(3) Research and Development of Classification and Identification of Biological Objects

Biological diversity will be one of major subjects of biology in 21st century and systematics forms the common base of the relevant researches. The systematics in the stage of the integration of conventional taxonomy based on phenotypic data and the phylogenetic analysis based on molecular data. Therefore, we have developed tools for poly-phasic analysis of biological data.

a. Information-base for Systematics

Hideaki SUGAWARA, Satoru MIYAZAKI, Junko SHIMURA[1], Yohihiro MASUDA[2], and Yasuhiro ISHITOBI[2] ([1] The Institute of Physical and Chemical Research, [2] Xerox Co.)

The information-base aims to provide an integrated information environment for taxonomists. The user is able to apply many kinds of analyses to the same subset from the database and enjoy sophisticated graphical user interface (GUI). Thus he/she is able to compare the results to get a consistent view of the relationship among species and strains. It is proved by the explosive increase of users of Web browsers that a good GUI is powerful, valuable and important. For details, see Ref. 2

b. Homology modeling of 3D structure of imunogloblin

Satoru MIYAZAKI, Junko SHIMURA[1], Hideaki SUGAWARA, Sachiko HIROSE[2], Shunichi SHIRAI[2] ([1] The Institute of Physical and Chemical Research, [2] Juntendo University)

We investigated effects of somatic mutations in immunoglobulin variable region genes on the affinity maturation of autoantibodies using single precursor B cell-derived anti-DNA monoclonal antibodies generated from an autoimmune disease-prone NZB×NZW F1 mouse. Analyses of DNA sequences, homology modeling on a graphic computer and molecular dynamics simulation of antigen-binding sites showed that any single site of mutation

and changes in the electrostatic or hydrogen-bonding potential of the residues and in the three dimensional structure could not solely explain the difference in DNA-binding activities. However, a significant increase in the flexibility of antigen-binding Fv loops was associated with affinity-maturated anti-DNA antibodies. For details, see Ref. 1

c. A New Measure for the Study of Molecular Evolution

Satoru MIYAZAKI, Hideaki SUGAWARA, and Masanori OHYA[1] ([1] Science Univeristy of Tokyo)

We examined the efficiency of the entropy evolution rate for the construction of phylogenetic trees with DNA sequences. Taking model trees with branches of given evolutionary rates, we generated sequences with 1,000 nucleotides along the branches of each model. We repeated computer simulation 100 times for each model and found that the entropy evolution rate gives the correct topology of trees more often than the other methods when the evolutionary rate of a lineage (branch) was higher than 0.5 per site. For details, see Ref. 7.

d. The Construction of Phylogenetic Tree from the Large Scale Data of Gene Sequences

Satoru MIYAZAKI and Hideaki SUGAWARA

The maximum likelihood method has several advantages to other methods to construct phylogenetic trees. However, it is not practical in the case of a large data sets because it requires a lot of computer resources. We implemented fastDNAml (a parallelized program of the maximum likelihood algorithm) to VPP500 and evaluated the program. We constructed the tree for full sequences of 16SrRNA and checked computational time changing the number of PEs (processors) from 1 to 40. The increase of the number of PEs decrease the computation time linearly and we find a possibility to draw a large phylogenetic tree by use of fastDNAml on VPP500. For details, see Ref. 5 and Ref. 10

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I. RADIOISOTOPE CENTER

(1) Transcriptional Analysis of the *Bacillus subtilis* Sporulation Gene: Promoter Selectivity of RNA Polymerase

Masaya FUJITA and Yoshito SADAIE

Nutrient deprivation in *Bacillus subtilis* leads to a process of differentiation that results in endospore formation. The first critical event of sporulation is an asymmetric cell division that produces two cell types of different size: a larger mother cell and a smaller forespore. More than fifty sporulation genes are expressed sequentially during sporulation and their expressions are controlled differently in the mother cell and in the forespore. Differential gene expression during sporulation is largely due to promoter selectivity by the five sequentially activated RNA polymerase sigma factors. To understand the molecular mechanism of their promoter selectivity, we constructed an *in vitro* transcription system with σ^A- and σ^H-RNA polymerases. The first sporulation specific σ^H factor directs transcription of a large number of early sporulation genes whereas the principal σ factor, σ^A, is essential for the transcription of the genes for vegetative growth and early sporulation. The genes encoding σ^A or σ^H were cloned into an expression vector under the control of the T7 promoter. Both proteins were overproduced in *Escherichia coli* BL21 (DE3) and purified from inclusion bodies after solubilization with guanidine hydrochloride. Antigenicities and N-terminal amino acid sequences of the overproduced proteins were used to identify both proteins. Unlike the σ^A protein, the σ^H protein showed DNA-binding ability. To compare the promoter selectivity of the σ^A protein with that of the σ^H protein, transcription *in vitro* of 16 promoters was performed using RNA polymerase holoenzymes reconstituted from the purified core enzyme with either σ^H or σ^A. These holoenzymes correctly recognized each of the cognate promoters; σ^H-RNA polymerase recognized σ^H promoters but not σ^A promoters, and *vice versa*. We also determined the promoter strength, i.e., the rate of open complex formation between RNA polymerase and the promoter, and the saturation level of the open complex formation at equilibrium. σ^H bound to core RNA polymerase could be replaced effectively by σ^A *in vitro*; this was unexpected as the

replacement of σ^A in the σ^A-RNA polymerase holoenzyme with σ^H in the late logarithmic growth phase is essential for entering the developmental process of sporulation. We infer from these results that growth phase-coupled replacement of the σ subunit in the holoenzyme from σ^A to σ^H may require an additional factor, or the modification of the core enzyme or σ factor.

(2) Nucleotide Sequence of the *phoB-rrnE-groESL* Region of the *Bacillus subtilis* Chromosome

Yoshito SADAIE, Katsunori YATA, Masaya FUJITA, Hitoshi SAGAI[1], Mitsuhiro ITAYA[2], Yasuhiro KASAHARA[3], and Naotake OGASAWARA[3] ([1] Laboratory for Pharmacology, Asahi Chemical Industry Co., Ltd., [2] Mitsubishi-kasei Institute of Life Sciences, [3] Nara Advanced Institute of Science and Technology)

Large-scale nucleotide sequencing eventually will reveal the structure and organization of genes on the chromosomes of bacteria that have been estimated by classical genetic methods in a limited number of organisms. A cooperative effort, by many laboratories of different countries, to sequence the whole genome of *Bacillus subtilis* is providing fundamental knowledge of the gene organization of the spore-forming bacterial cell.

We determined a 36 kb sequence of the *phoB-rrnE-groESL* region of the *Bacillus subtilis* chromosome at around 55°. The sequenced region contained thirty-six open reading frames including the *phoB* and *groESL* genes, and the whole *rrnE* operon. The *phoB* gene is transcribed in the direction opposite to that of chromosome replication while most ORFs, including *groESL* and the *rrnE* operon, are transcribed in the same direction. Two newly identified tRNA genes upstream of the *rrnE* operon were those for Arg-tRNA and Gly-tRNA. The sequenced region contains an operon consisting of genes for degradation and the uptake of mannan. The *rrnE* operon and its downstream ORFs are well conserved among *Mycoplasma genitalium*, *Haemophilus influenzae*, *Synechocystis* sp, and *Methanococcus jannaschii*. Sigma H consensus sequences are present in the promoter regions of three ORFs, including *groESL*. (Microbiology, **143**, 1861–1866, 1997).

Publications

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J. EXPERIMENTAL FARM

(1) Development and Reevaluation of the Genetic Stocks of Rice

Ken-Ichi NONOMURA, Mitsugu EIGUCHI, Toshie MIYABAYASHI, and Nori KURATA

We have conducted the reproduction and distribution of genetic stocks of wild and cultivated rice. From October 1, 1997, we try to include a new system of rice genetic stock generation and application. Additional resources we will produce, utilize and distribute are the enhancer trap and gene trap rice lines. Another trial program to develop and reevaluate interspecific hybrids of rice stocks in terms of the mechanism of reproductive isolation by chromosomal unpairing is also in progress. These projects are being conducted in cooperation with the plant genetics laboratory. For details, see the reports of the plant genetics lab.

ABSTRACTS OF DIARY FOR 1996

Biological Symposium

464th–Jan. 24	Cloning of Genes for Rifampicin Inactivation from Mycobacteria and other Organisms (Eric Dabbs)
465th–Feb. 26	Cloning and Expression of the Ecdysone Receptor and Ultrasperacle Homologs in Manduca sexta (Marek Jindra)
466th–Mar. 14	Molecular analysis of pattern formation in Hydra (Thomas C. G. Bosch)
467th–Mar. 28	Promoter Activation by *E. coli* RNA Polymerase: Alternative Programs (Olga N. Ozoline)
468th–Apr. 4	Regulation of rRNA Transcription (Richard L. Gourse)
469th–Apr. 4	The Role of Antisense RNA and a Pseudoknot in Controlling Replication of B Group Plasmids (J. Pittard)
470th–Apr. 11	Transcription Activation by the Cyclic AMP Receptor Protein (Susan Garges)
471st–Apr. 11	Structure and Function of *E. coli* RNA Polymerase: The Translocation Process (Hermann Heumann)
472nd–May 13	ATP-sensitive K^+ Channels and Insulin Secretion (Joe Bryan)
473rd–June 7	Of mice and Meisl: the identification of a novel homeobox gene and its role in myeloid leukemia (Arthur Buchberg)
474th–June 6	Mapping the Surface of RNA Polymerase with Metal Ions (Claude Meares)
475th–June 24	Proteome Research: Complementarity and limitations with respect to the DNA and RNA worlds (Ian Humphery-Smith)
476th–Sept. 12	Genetic and Immunological Dissection of the Beta-Subunit of RNA Polymerase (Robert E. Glass)
477th–Sept. 19	The Role of Pax Genes in Organogenesis (Rudolf Balling)
478th–Oct. 17	Wrestling with *E. coli* Sigma: Results of Kon-Basho

(Richard S. Hayward)

479th–Oct. 17 Proteomics, towards a full description of the cell readout (Andrew Gooley)

480th–Oct. 21 IMAGE: Integrated Molecular Analysis of the Human Genome and its Expression (Charles Auffray)

481st–Oct. 24 Function and Molecular Structure of Nematocytes. A one vesicle system of exocytosis (Thomas W. Holstein)

482nd–Nov. 11 Helical Filaments and Hexameric Rings in DNA Replication and Recombination: A Single Conserved Structure (Edward H. Egleman)

483rd–Dec. 3 Site-specific recombinases: enzymes or building blocks in morphogenesis? (Kiyoshi Mizuuchi)

484th–Dec. 9 LINEAGE COMMITMENT AND FATE OF NEURAL CREST-DERIVED SUBPOPULATIONS (James A. Weston)

485th–Dec. 5 TRANSFORMING MEN INTO MICE (Pavel Pevzner)

486th–Dec. 20 Evolution of Genome Composition: Effect of Mutation Pressure and Selection (Noboru Sueoka)

487th–Dec. 25 The Evolution of Self-incompatibility in Natural Populations of Flowering Plants (Marcy Uyenoyama)

Mishima Geneticists' Club

482nd–Jan. 18 Information theory and its application to DNA sequence analysis (Satoru Miyazaki)

483rd–Feb. 22 Molecular evolution of aldolase: how tissue specific functions of isozymes were acquired in vertebrate (Katsuji Hori)

484th–Feb. 26 Molecular biology of retroposons and its applications (Norihiro Okada)

485th–Feb. 23 Molecular evolution: approach from informatics (Hiroshi Tanaka)

486th–Feb. 23 Genetic variation and differentiation of wild rice in Asia (Cai Hong-Wei)

487th–Mar. 18 Molecular mechanism of neuronal determination in the Drosophila eye (Yasushi Hiromi)

488th–Mar. 21 Micronuclei formation and DM (double minute chro-

mosome) capture (Noriaki Shimizu)

489th–Apr. 1 Regulatory mechanism of asymmetric cell division in the nematode *C. elegans*—Role of *Wnt* and a receptor-like molecule (Hitoshi Sawa)

490th–Apr. 18 Protein Structure(3D)-Sequence(1D) compatibility search (Motonori Ohta)

491st–Apr. 24 Genetics of Atopy (Taro Shirakawa)

492nd–May 15 How left and right of body axis is determined? (Naohiko Yokoyama)

493rd–May 29 Three-dimentional structure of bovine cytochrome bc1 complex by electron cryomicroscopy and helical image reconstruction (Toshihiko Akiba)

494th–June 20 Imaging of intermolecular interactions of biological molecules—development of an atomic-force microscope—

495th–Aug. 1 FtsH, a membrane-bound, ATP-dependent metalloprotease in *Escherichia coli*: structure and function of the AAA family proteins (Teru Ogura)

496th–Sept. 17 Making a gene by combining microgenes (Kiyotaka Shiba)

497th–Oct. 30 Mechanisms of substrate recognition by eIF2 α kinase (Makiko Kobayashi-kawagishi)

498th–Dec. 5 Protein crystallography and structural biology (Kunio Miki)

499th–Dec. 18 Color pattern formation on the wing of the butterfly: Color determination and scale development (Eriko Takayama)

500th–Dec. 19 The functional analysis of *unc-51* and *unc-14* genes required for the formation of a neural network of a nematode *C. elegans* (Kenichi Ogura)

FOREIGN VISITORS IN 1996

June 9, 1994–March 31, 1996	H. W. Cai, Beijing Agriculture University, China
Jan. 24, 1996	Eric Dabbs, University of the Witwatersrand, South Africa
Feb. 26	Marek Jindra, University of Washington, U.S.A.
Mar. 14	Thomas C. G. Bosch, University of Munich, Germany
Mar. 28	Olga N. Ozoline, Institute of Cell Biophysics, Russia
Apr. 4	Richard L. Gourse, University of Wisconsin, U.S.A.
Apr. 4	J. Pittard, University of Melbourne, Australia
Apr. 11	Susan Garges, NCI, National Institutes of Health, U.S.A.
Apr. 11	Hermann Heumann, Max-Planck-Institute fur Biochemie, Germany
May 13	Joe Bryan, Baylor College of Medicine, U.S.A.
May 16	Claire Stuart, Mayor of New Primouth, New Zealand
May 22–23	Marcy K. Uyenoyama, Duke University, U.S.A.
June 6	Claude Meares, University of California at Davis, U.S.A.
June 7	Authur Buchberg, Jefferson Medical College, U.S.A.
June 24	Ian Humphery-Smith, University of Sydney, Australia
Sept. 12	Robert E. Glass, Nottingham University, U.K.
Sept. 12	Enrique Alberto Fugueroa, National Institute of Agriculture Technology, Argentina
Sept. 12	Adel Mohamed Abd El Razek Ghoneim, Rice Research and Training Center, Egypt
Sept. 12	Rapolu Mahender Kumar, Directorate of Rice Research, India
Sept. 12	Daniel Atula Masatia, National Irrigation Board, Ahero Irrigation Research Station, Kenya
Sept. 12	Zulkifli Bin Romli, Muda Agricultural Development Authority, MADA Headquarters, Malaysia
Sept. 12	Muhammad Imtiaz Ali Asad, Rice Research Institute, Pakistan
Sept. 12	Maria Chona E. Maleza, Bohol Agricultural Promotion

	Center, Department of Agricuture, Philippines
Sept. 12	Julmanee Pithuncharunlap, Rice and Field Crops Promotion Division, Department of Agricultural Extension, Thailand
Sept. 19	Rudolf Balling, Institut fur Saugetiergenetik, Germany
Oct. 1–31	John H. Gillespie, University of California at Davis, U.S.A.
Oct. 17	Richard S. Hayward, University of Edinburgh, U.K.
Oct. 17	Andrew Gooley, Macquarie University, Australia
Oct. 21	Charles Auffray, CNRS Research Unit on Molecular Genetics and Developmental Biology, France
Oct. 23	Hidesaburo Hanafusa, Rockefeller Univesity, U.S.A.
Oct. 24	Thomas W. Holstein, JW Goethe University, Germany
Nov. 11	Edward H. Egleman, University of Minnesota, U.S.A.
Dec. 2–4	Kiyoshi Mizuuchi, NIDDK, National Institutes of Health, U.S.A.
Dec. 5	Pavel Pevzner, University of Southern California, U.S.A.
Dec. 9	James A. Weston, University of Oregon, U.S.A.
Dec. 20	Noboru Sueoka, University of Colorado at Boulder, U.S.A.
Dec. 25	Marcy Uenoyama, Duke University, U.S.A.

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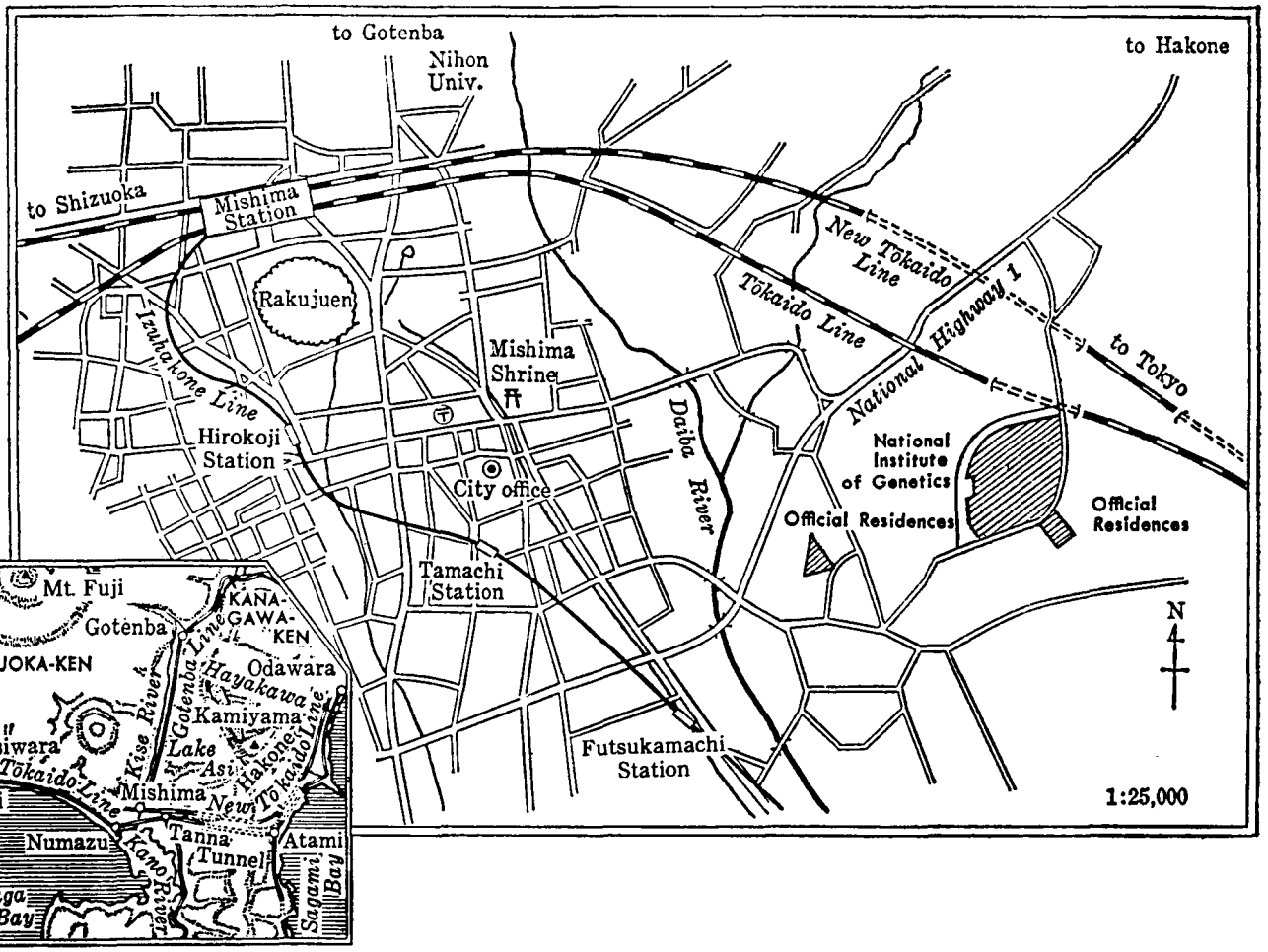
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