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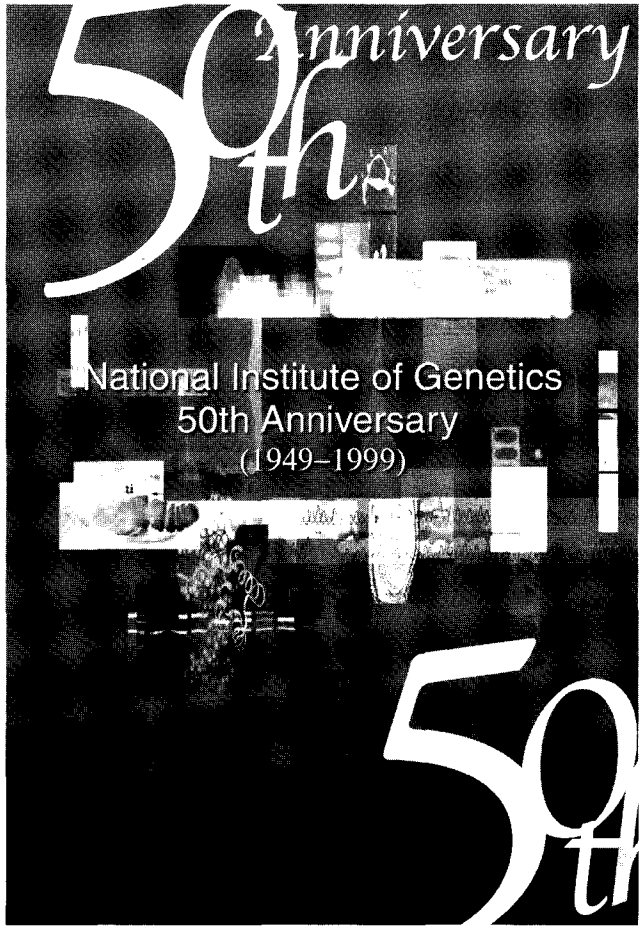

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GENERAL STATEMENT

We had two special events this year. National Institute of Genetics (NIG) was established 50 years ago, and we cerebrated the semi-centennial on June 1st by inviting many distinguished guests and our old friends. To commemorate the anniversary, we started a "Virtual Museum of Genetics" by expanding our home page. We would like to make it as a useful and enjoyable site for citizens and students. At present, it is still in its infancy, but please visit it and give us your feedbacks.

We also had a special day on May 31st, when Emperor and Empress visited us and enjoyed watching our scientific activities. Emperor himself is a biologist and asked many questions. We were very happy to see their relaxed atmosphere.

Major contributions of NIG in the 50-year research in genetics, in particular, plant genetics, population genetics and molecular and developmental genetics, have made it one of the most important centers with worldwide recognition. In 1984, NIG was reorganized into an Inter-university Research Institute to promote collaborative activities. Together with other inter-university research institutes, we founded the Graduate University for Advanced Studies in 1988. We are serving as Department of Genetics of the Graduate School of Life Science. This year, we have 33 graduate students and 10 special research students from other universities, including those from abroad. Nine students obtained Ph.D. this year. In addition, we have the Center Of Excellence (COE) program with which 5 foreign and 7 Japanese postdoctoral fellows conducted research.

We have been carrying out several research-related services. The DNA Data Bank of Japan (DDBJ) is one of the three central data banks in the world that gather, annotate, store and distribute DNA sequence information. It receives data input not only from Japanese institutions but also from institutes in other Asian countries. Recent development of new technology using various

model organisms has enhanced the importance of genetics as the bases of many branches of biological studies. We have Genetic Strains Research Center and Genetic Resource Information Center that are designed to organize and support the use of genetic strains and resources. Our institute also stores and distributes various organisms with genetically characterized traits. Among them, services with Escherichia coli, mice and Drosophila are particularly significant. These activities will continue to develop in the coming years. Our institute is uniquely suited for pursuing cooperative works with scientists of various disciplines by sharing the genetic resources.

Yoshiki Hotta, Director-General

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RESEARCH ACTIVITIES IN 1999

A. DEPARTMENT OF MOLECULAR GENETICS

A-a. Division of Molecular Genetics

(1) Mapping of Protein-protein Contact Network Involving the β and β' Subunits of *Escherichia coli* RNA Polymerase

Tasuku NOMURA, Akira KATAYAMA, Nobuyuki FUJITA and Akira ISHIHAMA

The RNA polymerase holoenzyme of *Escherichia coli* is composed of the core enzyme with the subunit composition of $\alpha_2\beta\beta'$ and one of seven different species of the σ subunit. The core enzyme carries the catalytic activity of RNA polymerization, but the σ subunit is required for the promoter recognition and transcription initiation from the promoter. The core enzyme is assembled sequentially both *in vitro* and *in vivo* under the order: $2\alpha \rightarrow \alpha_2 \rightarrow \alpha_2\beta \rightarrow \alpha_2\beta\beta'$ (premature core) → E (active core). Previously we performed detailed mapping of the subunit-subunit contact sites on the α subunit. Along this line, we carried out the mapping of subunit-subunit contact sites on two large subunits, β and β' subunits. For functional mapping the β subunit (1342 residues), we employed two approaches: (i) comparative analysis of the proteolytic cleavage pattern of the unassembled free β subunit and the intermediate subassembly $\alpha_2\beta$ complex; and (ii) analysis of the complex formation between various β fragments and His_6-tagged α subunit or between various His_6-tagged β fragments and the intact α subunit. The results indicated that the primary and tight contact site on the β subunit with the α subunit is located in the central portion of β polypeptide but in addition, the C-terminal proximal region is needed as the secondary and probably regulatory site for either efficient binding of the α subunit or stabilization of the α-β contact. All the β fragment-α binary complexes isolated were able to bind β' subunit

leading to form pseudo-core complexes, suggesting that the β' subunit-contact site(s) on the β subunit is located near the α-contact sites. For detailed mapping of the subunit-subunit contact sites on the β'subunit (1407 residues) with the σ^{70} subunit and the $\alpha_2\beta$ complex, we employed two approaches: (i) identification of β'tryptic fragments capable of forming complexes with preformed $\alpha_2\beta$ complex or purified σ^{70} subunit; and (ii) systemtaic analysis of complex formation of various β' fragments, with or without His_6-tag, with the $\alpha_2\beta$ complex and the σ^{70} subunit. The results indicated that: two regions, one central region and the other C-terminal proximal region are involved in binding the $\alpha_2\beta$ complex; and one N-terminal proximal region plays a major role in binding the σ^{70} subunit. However, both $\alpha_2\beta$-binding sites have weak activity of the σ^{70} subunit; likewise, the σ^{70} subunit-contact surface has weak binding activity of the $\alpha_2\beta$ complex. The sites involved in the catalytic function of RNA polymerization are all located within two spacer regions sandwiched between these three subunit-subunit contact surfaces.

As an extension of this line of studies, we searched for class-III and -IV transcription factors which interact with the β and/or β'subunits, respectively. For this purpose, we employed an affinity isolation of candidate proteins from whole cell extracts of *E. coli* expressiong a low level of β- or β'-subunts fused with glutathione S-transferase (GST). After N-terminal amino acid microsequencing, we identified 7 proteins as the candidates for class-III (β-contact) and 9 proteins as the candidates for class-IV (β'-contact) factors. The 110 kDa RapA/HepA protein was included in both group of proteins. For details see Refs. 13, 14, 15, 17 and 23.

(2) Structural Organization of the α Subunit of *Escherichia coli* RNA Polymerase

Nobuyuki FUJITA, Shizuko ENDO and Akira ISHIHAMA

The α subunit of *Escherichia coli* RNA polymerase is comprised of two independently folded domains. The N-terminal domain (NTD; residues 8 to 235) is required and sufficient for the assembly of core enzyme with the composition of $\alpha_2\beta\beta'$, while the C-terminal domain (CTD; residues 249 to 329) plays

key roles in transcription activation by interacting with either a large group of transcription factors (class I factors) such as cAMP receptor protein (CRP) and OmpR, or the upstream promoter element (UP element) which is present in a certain class of strong promoters including ribosomal RNA promoters. The intervening sequence connecting these two domains is easily accessible to proteases and has high motional flexibility as revealed by the measurement of NMR relaxation and the comparison of NMR signals between αCTD and intact α subunit. The flexible nature of interdomain linker is also suggested from functional studies. The αCTD is able to interact with CRP bound at widely different positions along the DNA while the RNA polymerase is bound at a fixed position of the promoter. Different surfaces of αCTD can be utilized for the interaction with upstream DNA in different initiation complexes. Three-dimensional structures of αCTD and αNTD of *E. coli* RNA polymerase have been determined by NMR spectroscopy and X-ray crystallography, respectively. The αCTD has a compact globular structure consisting of four helices, two helical turns and a long C-terminal loop with defined structure. The dimer of αNTD has an elongated flat structure. The two αCTDs are supposed to extrude out from the core part of RNA polymerase, probably toward the upstream direction, through the long flexible linkers. Such a molecular architecture would allow αCTDs to behave as independent motional units and interact with a variety of DNA and protein signals while the main part of RNA polymerase stays at the promoter site. Despite the possible importance of the interdomain linker in transcription activation, none of the reported structures contain the linker region.

To elucidate structural requirements, if any, for the interdomain linker, we constructed a set of mutant *E. coli* RNA polymerases, each consisting of mutant α subunit with altered interdomain linker. Deletion of 3 amino acids from the linker exhibited 50% inhibition of CRP-dependent *lac* P1 transcription. Deletion of 6 amino acids completely knocked out the activity. Insertion of 3 amino acids did not affect the activity, whereas 40-60% inhibition was observed after insertion of 1, 2, or 4 amino acids. Substitution of 10 consecutive glycine residues resulted in nearly 90% reduction of the CRP-dependent activity, whereas 50% activity was retained after substitution of 10 proline residues or a sequence expected to form a strong α-helix. Similar

results were obtained with UP element-dependent *rrnB*P1 transcription. These results altogether suggest that: (i) sufficient length of the interdomain linker is required for transcription activation mediated by α C-terminal domain; (ii) the linker directs positioning of the C-terminal domain to a preferred orientation for the interaction with CRP and UP element; (iii) in addition to the overall flexibility, the amino acid sequence context of the linker is important for full function of the C-terminal domain, probably through the formation of loose helix-like structure. See Refs. 6 and 14.

(3) Functional Organization of the α Subunit of *Escherichia coli* RNA Polymerase

Olga N. OZOLINE[1], Nobuyuki FUJITA and Akira ISHIHAMA ([1]on leave of absence from Institute of Cell Physics, RAS, Puschino)

In transcription initiation by the *E. coli* RNA polymerase, exchangeable σ subunits are responsible for recognition of promoter DNA while the C-terminal domain (CTD) of α subunit makes additional contacts with regulatory signals. For instance, the upstream sequence of *rrnB*P1 promoter, generally designated as UP element, directly contacts the αCTD and supports enhanced transcription of rRNA. The αCTD is also responsible for interaction with a set of transcription factors, designated as class-I (or α-contact) factors, regulating transcription efficiency. The most studied factor is cAMP receptor protein (CRP), which regulates transcription of more than 80 genes. Molecular mechanism of transcription regulation by CRP depends on the position of its binding site on the promoter sequence. Promoters which have the CRP-binding site centered between -60 and -100 usually require αCTD for activation.

To get insight into the detailed mechanism of transcription activation by αCTD, we tried to monitor conformational changes within the αCTD upon interactions with DNA UP element or transcription factor CRP. For this purpose, a fluorescent dye, fluorescein mercuric acetate (FMMA), was conjugated at various positions of the αCTD using a newly constructed collection of single Cys-mutant α subunit, and spectral changes were measured after interaction with DNA UP elements or CRP. When FMMA was conjugated at Cys located

in the α-subunit helix-I and the loop between helix-III and -IV, the spectral changes typical for DNA interaction were observed for the RNA polymerase-promoter binary complex with UP element-dependent *rrnB*P1 and the ternary complex with CRP-dependent *uxuAB* promoter in the presence of cAMP-CRP. In addition, we found conformational changes of αCTD upon formation of binary complex with the *uxuAB* (in the absence of CRP) and factor-independent T7D promoters. In these cases, the spectral changes suggested that the C-terminal proximal helix-IV of αCTD approaches the negatively charged phosphate moiety of DNA. This prediction was supported after analysis of contact-dependent DNA cleavage by the chemical nuclease, Fe-(*p*-bromoacetamidobenzyl)-EDTA (Fe-BABE), conjugated to Cys-309 located on the helix-IV. We propose that the helix-IV of αCTD is involved in direct interaction with some promoters. For details see Refs. 13, 14, 24 and 25.

(4) Competition among Seven *E. coli* σ Subunits: Intracellular Concentrations and Binding Affinity to the Core Enzyme

Hiroto MAEDA[1], Miki JISHAGE, Tasuku NOMURA, Akira KATAYAMA, Nobuyuki FUJITA and Akira ISHIHAMA ([1]on leave of absence from Kagoshima Univ., Facul. Fisheries)

The RNA polymerase core enzyme with the subunit composition $\alpha_2\beta\beta'$ has the ability to transcribe the genetic information on DNA into RNA, but for initiation of transcription at specific promoter sites on DNA, an additional component, σ subunit, is required. Seven different molecular species of the σ subunit have been identified in *E. coli*, and replacement of the σ subunit on the RNA polymerase is an efficient way for switching the transcription pattern. In *Escherichia coli*, seven different species of the σ subunit, σ^{70}, σ^{N} (also called σ^{54}), σ^{S} (σ^{38}), σ^{H} (σ^{32}), σ^{F} (σ^{28}), σ^{E} (σ^{24}) and σ^{FecI}, are known to exist, each directing transcription of a specific set of genes. Most of the growth-related and house-keeping genes expressed at the exponential phase of cell growth are transcribed by the holoenzyme containing σ^{70} (the *rpoD* gene product), while the holoenzyme $E\sigma^{S}$ is essential for transcription of some stationary-phase specific genes. The stress response genes are transcribed by RNA

polymerase holoenzymes containing the alternative minor σ subunits. We have purified all seven species of the *E. coli* σ subunit and analyzed their recognition specificity of various *E. coli* promoters. Using specific antibodies raised against the purified σ proteins, we also measured the intracellular concentrations of all seven σ subunits for both exponential and stationary phase cultures of *E. coli* W3110(A). The order of intracellular level was: $\sigma^{70} > \sigma^{F} > \sigma^{N} > \sigma^{S}$, σ^{H}, σ^{E}, σ^{FecI} in exponential phase; and $\sigma^{70} > \sigma^{S} > \sigma^{F} > \sigma^{N} > \sigma^{H}$, σ^{E}, σ^{FecI} in stationary phase.

The global pattern of gene transcription is believed to be determined through competition between available σ subunits, and if this is the case, the replacement of core enzyme-associated σ subunit from one species to another should be the major determinant in the switching of global transcription pattern. To test the σ competition model in the global regulation of transcription among about 4000 genes on the *E. coli* genome, all seven σ subunits have been purified, and compared for the binding affiniy to the same core RNA polymerase (E). In the presence of a fixed amount of σ^{70} (the principal σ for growth-related genes), the level of Eσ^{70} holoenzyme formation increased linearly with the increase in core enzyme level, giving the apparent K_d for the core enzyme of 0.26 nM. Mixed reconstitution experiments in the presence of a fixed amount of core enzyme and increasing amounts of an equimolar mixture of all seven σ subunits indicated that σ^{70} is the strongest in the core enzyme binding, followed by σ^{N}, σ^{F}, σ^{E}/ σ^{FecI}, σ^{H}, and σ^{S} in the decreasing order. This order of core-binding affinity was confirmed by measuring the replacement of one core-associated σ by another σ subunit. Taken together with the intracellular σ levels, we tried to estimate the number of each holoenzyme form in growing *E. coli* cells. Based on the estimation, we propose that, for effective replacement of the core-bound σ^{70} by other minor σ for stress-response gene transcription, it appears that specific intracellular conditions or additional factors are required. See Refs. 13, 14, 15, 16 and 19.

(5) Control of the Activities of σ Subunits by Anti-σ Factors

Miki JISHAGE, Dipak DASGUPTA[1] and Akira ISHIHAMA ([1]on leave of absence from Saha Institute of Nuclear Physics, Calcutta)

The intracellular concentrations of the seven σ subunits change depending on cell growth conditions. In addition to the level control, the activity of σ subunit is controlled. For instance, the subunit σ^{28} (σ^{F}) involved in transcription of the genes for the flagella formation becomes inactive when it forms a binary complex with the *flgM* gene product. Likewise the activity of σ^{E}, a member of the ECF (extracytoplasmic function) family σ subunits, is regulated by the *rseA* (regulator of sigma E or anti-σ^{E} factor) gene product which is associated with the inner membrane and inhibits the activity of σ^{E} by directly interacting with σ^{E}.

Recently we discovered a novel *E. coli* protein, referred to Rsd (regulator of sigma D), which forms a complex with σ^{70} and prevents its function. Purified Rsd protein formed complexes *in vitro* with σ^{70} but not with other σ subunits, and inhibited σ^{70}-dependent transcription *in vitro* to various extents depending on the promoters used. Since Rsd is induced in the stationary phase of cell growth, unused excess σ^{70} subunit, without being involved in transcription cycle, should be trapped by Rsd. Thus, the possibility has arisen that Rsd is an anti-σ factor for the major σ^{70} subunit for its storage in stationary phase. In order to clarify the *in vivo* function of Rsd, we analyzed the influence of both depletion and over-production of Rsd on σ^{70}- and σ^{S}-dependent transcription *in vivo*. On the basis of the results herewith described we propose that Rsd is a regulator that facilitates the switching of σ subunit on RNA polymerase from σ^{70} to σ^{S} during the transition from exponential growth to stationary phase. To understand the *in vivo* role of Rsd level in *E. coli*, we analyzed the transcription organization of the *rsd* gene. Primer extension analysis indicated the presence of two promoters, σ^{S}-dependent P1 and σ^{70}-dependent P2 with the gearbox sequence. The expression of *lacZ* reporter gene fused to either σ^{70}- or σ^{S}-dependent promoter was analyzed in the absence of Rsd or presence of over-expressed Rsd. In the *rsd* null mutant, the σ^{70}- and σ^{S}-dependent gene expression is increased or decreased, respectively. On the other

hand, the σ^{70}- and σ^{S}-dependent transcription was reduced or enhanced, respectively, after over-expression of Rsd. The repression of the σ^{S}-dependent transcription in the *rsd* mutant is overcome by increased production of the σ^{S} subunit. These observations altogether support the prediction that Rsd is involved in replacement of the RNA polymerase σ subunit from σ^{70} to σ^{S} during the transition from exponential growth to stationary phase.

An an extension of this line of studies, we have searched for anti-sigma factors candicates for other minor σ subunts. Using the affinity purification method, we identified, besides the core enzyme subunits, extra components associated with the overexpressed tagged σ subunits. Functional characterization of these σ-associated proteins is in progress. See Refs. 12, 13, 15 and 16.

(6) Contact Site Mapping of σ Subunits and Transcription Factors on the RNA Polymerase Core Enzyme Subunits Using FeBABE

Siva Ramesh WIGNESHWERARAJ[1], Frederic COLLAND[2], Nobuyuki FUJITA, Claude F. MEARES[3] and Akira ISHIHAMA ([1]on leave of absence from Imperial College of Science, Technology and Medicine, London; [2]on leave of absence from Pasteur Institut, Paris; and [3]University of California, Davis)

Previously we developed, in collaboration with Claude F. Meares and colleagues (Univ. California, Davis), a new method of the mapping of protein-nucleid acid and protein-protein contact sites using protein-conjugated (*p*-bromoacetamideenzyl)EDTA-Fe (FeBABE). This useful tool has been successfully used for mapping of the contact sites of RNA polymerase σ and α subunits on promoters and the mapping of α and σ^{70} subunits on the core enzyme. This year we employed this technique for mapping of the contact sites of σ^{S} and σ^{N} subunits on the core enzyme subunits. For this purpose, we constructed sets of single Cys mutant σ^{S} and σ^{N} subunits at various regions along these σ subunits. After conjugation of FeBABE at each Cys residue, holoenzymes were reconstituted by mixing with the core enzyme, and the cleavage sites on the core enzyme subunits were determined upon addition of H_2O_2 and ascorbate. The protein cutting by σ^{S} and σ^{N}tethered FeBABE was observed

in two regions of the β subunit, one including regions C and D, and the other including the region G and the dispensable region 2 (DR2), and one region of the β' subunit, including regions C and D. The cleavage patterns are similar to that observed with σ^{70}-tethered FeBABE, but there were minor but significant differences in the cleavage sites among the three σ subunits.

As an initial attempt for systematic mapping of the contact sites of various transcription factors on the RNA polymerase, we developed a modified method of FeBABE conjugation to protein Lys residues by using 2-iminothiolane (2-IT) as a linker. Using the modified method, we succeded to locate the contact sites on the core enzyme subnits of two elongation factors, NusA and GreA, and an RNA polymerase chaperone ω. For details see Refs. 2, 3, 5, 14, 33 and 37.

(7) Growth Phase-dependent Variation in the Protein Composition of *Escherichia coli* Nucleoid

Ali Azam TALUKDER, Akira IWATA[1], Susumu UEDA[1] and AKIRA ISHIHAMA
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The genome DNA of *Escherichia coli* forms nucleoprotein complexes, often called nucleoid, together with about 10 major DNA-binding proteins, among which Fis, H-NS, HU and IHF have been believed to be the major molecular species. Several lines of evidence indicate that the intracellular levels of the nucleoid proteins and their localization along the genome DNA influence not only the conformation of nucleoid but also the functions of DNA such as replication, recombination, repair and transcription. In certain cases, the regulatory roles of nucleoid proteins in DNA functions are attributed to not only modulation of the genome conformation as a whole but also more direct effect on the local conformation of specific DNA regions or even direct interaction with protein components. Using a quantitative immunoblot method, we perfomed for the first time a systematic determination of the intracellular concentrations for twelve species of the nucleoid protein in *E. coli* W3110, including CbpA (Curved DNA-Binding Protein A), CbpB (Curved DNA-Binding Protein B; or Rob, Right Origin Binding protein), DnaA (DNA-binding protein A), Dps (DNA-binding Protein from Starved cells), Fis (Factor for Inver-

sion Stimulation), Hfq (Host Factor for phage Q_β), H-NS (Histone-like Nucleoid Structuring protein), HU (Heat-Unstable nucleoid protein), IciA (Inhibitor of Chromosome Initiation A), IHF (Integration Host Factor), Lrp (Leucine-Responsive regulatory Protein) and StpA (Suppressor of *td*⁻ Phenotype A). The level is maximum at the growing phase for nine proteins, CbpB (Rob), DnaA, Fis, Hfq, H-NS, HU, IciA, Lrp and StpA, which may play regulatory roles in DNA replication and/or transcription of the growth-related genes. The order of accumulation level, calculated as monomers, in growing *E. coli* is: Fis>Hfq>HU>StpA>H-NS>IHF*>CbpB(Rob)>Dps*>Lrp>DnaA>IciA>CbpA* (stars represent the stationary-phase proteins).

The order of abundance in the early stationary-phase is: Dps*>IHF*>HU>Hfq>H-NS>StpA>CbpB(Rob)>DnaA>Lrp>IciA>CbpA>Fis, while that in the late stationary phase is: Dps*>IHF*>Hfq>HU>CbpA*>StpA>H-NS>CbpB(Rob)>DnaA>Lrp>IciA>Fis. Thus, the major protein components of nucleoid change from Fis and HU in the growing phase to Dps in the stationary phase. The curved DNA-binding protein, CbpA, appears only in the late stationary phase. These changes in the composition of nucleoid-associated proteins in the stationary phase are accompanied by compaction of the genome DNA and silencing of the DNA functions. The intracellular localization of these DNA-binding proteins is being analyzed using indirect immunofluorescence staining. For details see Refs. 12, 13, 30, 31 and 32.

(8) Functional Organization of Two Large Subunits of the Fission Yeast *Schizosaccharomyces pombe* RNA Polymerase II

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The RNA polymerase II (Pol II) of the fission yeast *Schizosaccharomyces pombe* is a multifunctional and multisubunit enzyme consisting of 12 putative subunits. In contrast to prokaryotic RNA polymerases, little is known about the molecular architectures of eukaryotic counterparts except that: (i) the two largest subunits, homologous to bacterial β and β' subunits, are

involved in the binding of DNA template, the polymerization of RNA chains, and the association of nascent RNA chains; and (ii) the subunits 3 and 11, homologues of bacterial α subunit, play roles in the assembly of Pol II. Knowledge of the structure and function of the individual subunits is essential for understanding of the molecular mechanisms of transcription and regulation of the protein-coding genes in eukaryotes. As an attempt toward this ultimate goal, we tried to locate the active center for RNA polymerization using an affinity labeling with photo-reactive nucleotide analogues.

The catalytically competent transcription complex of Pol II was affinity-labeled with photo-reactive nucleotide analogues incorporated at 3' termini of nascent RNA chains. To locate the catalytic site for RNA polymerization, the labeled subunits were separated by SDS-PAGE and subjected to partial proteolysis. After microsequencing of proteolytic fragments, a complex multidomain organization was indicated for both of the two large subunits, Rpb1 and Rpb2, with the most available sites of proteolysis in junctions between the conserved sequences among RNA polymerase from both prokaryotes and eukaryotes. The cross-linking studies indicate that: (i) the 3' termini of growing RNA chains are most extensively cross-linked to the second largest subunit Rpb2 between amino acids 825 and 994; and in addition, (ii) the regions 298-535 of Rpb2 and 614-917 of Rpb1 are cross-linked to less extents, suggesting that these regions are situated in the vicinity of the catalytic site. All these regions include the conserved sequences of RNA polymerases, and the catalytic site of Rpb2 belongs to an NH_2-terminal part of its conserved sequence H. For details see Ref. 37.

(9) Twelve Subunits of the Fission Yeast RNA Polymerase II: Intracellular Contents, Transcription Organization and mRNA Levels

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The RNA polymerase II (Pol II) core enzyme of the fission yeast *Schizosaccharomyces pombe* is composed of twelve polypeptides, Rpb1 to

Rpb12, corresponding to RPB1 to RPB12 of the budding yeast *Saccharomyces cerevisiae*. The subunits, Rpb1, Rpb2, Rpb3 and Rpb11, have the sequence similarity with the subunits, β', β, α and α, respectively, of the prokaryotic RNA polymerase core enzyme, and are considered to be the essential for the Pol II assembly and the catalytic function of RNA polymerization. The pathway of subunit assembly also seems to be similar, at least in part, to that of *Escherichia coli* RNA polymerase. The assembly core with the structure Rpb2-Rpb3-Rpb11 corresponds to the $\alpha_2\beta$ complex of *E. coli*. In addition, a larger complex consisting of 7 subunits, Rpb1, Rpb2, Rpb3, Rpb5, Rpb7, Rpb8 and Rpb11 is formed *in vivo* supposedly as an assembly intermediate of the *S. pombe* Pol II. Among the twelve Rpb subunits, five, Rpb5, Rpb6, Rpb8, Rpb10 and Rpb12, are assembled in Pol I (for rRNA synthesis) and Pol III (for tRNA synthesis) as well as Pol II, and thus the processes of rRNA, mRNA and tRNA synthesis should be tightly interrelated through sharing the common subunits. At present, however, little is known on the regulation of synthesis and assembly of the RNA polymerases in eukaryotes.

As an initial attempt to get insight into the regulation of the synthesis and assembly of the Pol II, we have performed a systematic measurement of the intracellular levels of all twelve subunits of the Pol II in *S. pombe* using quantitative immunoblotting. The levels of three subunits, Rpb1, Rpb2 and Rpb3, were found to be low, compared with those of small-sized subunits, which range 2- to 15-fold higher than the levels of the three core subunits. Glycerol gradient centrifugation of *S. pombe* extracts indicated the presence of considerable pools of free unassembled forms (and subassemblies) for these small-sized Rpb subunits. Based on the intracellular concentration of the least abundant Rpb3 subunit, the total number of Pol II in a growing *S. pombe* cell was estimated to be about 10,000 molecules. The intracellular distribution of some Pol II subunits was also analyzed by microscopic observation of the green fluorescent protein (GFP)-fused Rpb proteins.

We also analyzed the transcription organization of the genes for all twelve subunits of the *S. pombe* Pol II using the oligo capping method. Transcription of one group of genes including *rpb3, rpb4, rpb5, rpb6, rpb7* and *rpb10* is initiated mainly at a single site, while that of the other group of genes for *rpb1, rpb2, rpb8, rpb9, rpb11* and *rpb12* is initiated at multiple sites. The promot-

ers of the first group genes contain the TATA box sequence between -26 to -62, while the second group genes carry TATA-less promoters. Several common sequence segments, tentatively designated "Rpb motifs", were identified in the promoter regions of the *rpb* genes. As an extension, we determined the mRNA levels of all twelve *rpb* genes using competitive PCR method. The results indicate that the mRNA levels do not necessarily correspond to those of protein products. mRNAs for Rpb1, Rpb3, Rpb7 and Rpb9 were among the group of low abundance, while the levels of Rpb6, Rpb7 and Rpb10 mRNAs were about 5-fold and that of Rpb2 mRNA was about 40-fold higher than the Rpb3 mRNA level. After comparison between the protein and mRNA levels, we predict that the efficiency of translation is maximal for mRNA species with 5' untranslated region of about 100 nucleotides in length, but becomes low for mRNAs with shorter or longer untranslated regions. The translation efficiency is low for the *rpb1, rpb2, rpb3* and *rpb11* genes, encoding the homologues of subunits β', β, α and α, respectively, of prokaryotic RNA polymerase core enzyme, supposedly because the transcription initiation sites of these genes are all located close to the translation initiation codons. See Refs. 18, 27, 28 and 37.

(10) Involvement of Multiple Subunit-Subunit Contacts in the Assembly of RNA Polymerase II

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RNA polymerase II (Pol II) purified from the fission yeast *Schizosaccharomyces pombe* consists of twelve species of subunits designated as Rpb1 to Rpb12. Among them the largest and the second largest subunits, Rpb1 and Rpb2, have sequence similarities with the β' and the β subunits of prokaryotic RNA polymerase, respectively. The Rpb3 and the Rpb11 show limited similarities with the N-terminal domain of α subunit. The four subunits, Rpb1, Rpb2, Rpb3 and Rpb11, are therefore, supposed to form an enzyme core which corresponds to the core enzyme, $\alpha_2\beta\beta'$, of the prokaryotic RNA polymerase. In the presence of 6 M urea, the purified *S. pombe* Pol II dissociates forming a Rpb2-Rpb3-Rpb11 complex, which is equivalent with the assembly intermediate, $\alpha_2\beta$, of

prokaryotic RNA polymerases.

Function and structure of other eukaryotic Pol II subunits have been studied mainly using the enzyme from *Saccharomyces cerevisiae*. Except for the fragmentary knowledge of the functions of each subunit, the structure-function organization within Pol II complex still remains unclear. To elucidate the functional organization, we have analyzed the subunit-subunit interactions within the *S. pombe* Pol II by chemical crosslinking and Far-western blotting. We also mapped the Rpb5-contact site on the Rpb1 and the Rpb3-contact site on the Rpb2 using yeast two hybrid system. The subunit-subunit interactions were limited in a sense that they only showed interactions between two subunits, except in the case with Rpb5, which stimulates the Rpb3-Rpb11 heterodimer formation. Here we present the formation of multi-protein complexes of Pol II subunits in insect cells expressing the recombinant subunit proteins. We expressed the eleven subunits, except Rpb4, simultaneously in cultured insect cells with baculovirus expression vectors. For the isolation of subunit complexes formed in the virus-infected cells, a glutathione S-transferase sequence was fused to the *rpb3* cDNA to produce GST-Rpb3 fusion protein and a decahistidine-tag sequence was inserted into the *rpb1* cDNA to produce Rpb1H protein. After successive affinity chromatography on glutathione and Ni^{2+} columns, complexes consisting of the seven subunits, Rpb1H, Rpb2, GST-Rpb3, Rpb5, Rpb7, Rpb8 and Rpb11, were identified. Omission of the GST-Rpb3 expression resulted in reduced assembly of the Rpb11 into the complex. Direct interaction between the Rpb3 and other six subunits was detected by pairwise coexpression experiments. Coexpression of various combinations of a few subunits revealed that Rpb11 enhances Rpb3-Rpb8 interaction and consequently Rpb8 enhances Rpb1-Rpb3 interaction to some extent. We propose a mechanism, in which the assembly of RNA polymerase II is stabilized through multiple subunit-subunit contacts. See Refs. 18 and 27.

(11) Mutant Studies of the *Schizosaccharomyces pombe* RNA Polymerase II: Rpb3 and Rpb6 Mutants

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The RNA polymerase II (Pol II) of the fission yeast *Schizosaccharomyces pombe* is composed of twelve subunits and plays a key role in transcription of protein-coding genes. Two large subunits, Rpb1 and Rpb2, have notable sequence homology with the β' and β subunit, respectively, of the prokaryotic RNA polymerases. By catalytic crosslinking, we found that the catalytic site for RNA polymerization is formed by these two subunits. On the other hand, two subunits, Rpb3 and Rpb11, have sequence homology with the amino (N)-terminal domain of prokaryotic RNA polymerase α subunit (αNTD). As expected from the sequence similarity, we found that both Rpb3 and Rpb11 subunits are included in the core assemblies of Pol II.

In order to get insight into *in vivo* roles of individual subunits and furthermore to identify accessory factors which interact each of the twelve subunits, we carried out systematic studies of the *S. pombe* Pol II mutants with mutations in one of the twelve subunits. Up to now, we have isolated temperature-sensitive (Ts) or cold-sensitive (Cs) *S. pombe* mutants with mutations in the *rpb2, rpb3, rpb5, rpb6, rpb7* and *rpb11* genes. This year systematic studies have been carried out for the *rpb3* and *rpb6* mutants. All the *rpb3* mutants showed various degrees of the assembly defect of Pol II. For functional characterization of the *rpb3* mutants, we established an improved system of GAL4-VP16 activator-dependent *in vitro* transcription by *S. pombe* cell extracts. Cell extracts from the *S. pombe* mutants carrying mutations in the eukaryote-specific conserved regions B and C of Rpb3 showed lower activities of the activated transcription compared with other mutants carrying mutations in the regions A and D, implying that the regions B and C are involved in transcription factor-dependent transcription. Since the cell extracts from all the *S. pombe* carrying *rpb3* region A or D mutations showed more reduced thermostability than others, the regiond A and D may play more essential roles in the assembly.

This year we also performed genetic analyses of the Rpb6 subunit of *S. pombe* Pol II. Rpb6 is one of the five common subunits shared among three forms of eukaryotic RNA polymerase. Deletion and truncation analyses of the *rpb6* gene in the *S. pombe* indicated that Rpb6 consisting of 142 amino acid residues is an essential protein for cell viability, and the essential region is located in the C-terminal proximal half between residues 61-139. After ran-

dom mutagenesis, a total of 14 temperature-sensitive (Ts) mutants were isolated, each carrying a single (or double in three cases and triple in one case) mutation. Four mutants each carrying a single mutation in the essential region were sensitive to 6-azauracil (6AU) that inhibits transcription elongation by depleting the intracellular pool of GTP and UTP. Both 6AU sensitivity and Ts phenotypes of these *rpb6* mutants were suppressed by over-expression of TFIIS, a transcription elongation factor. In agreement with the genetic studies, the mutant RNA polymerases containing the mutant Rpb6 subunits showed reduced affinity for TFIIS, as measured by a pull-down assay of TFIIS-Pol II complexes using a fusion form of TFIIS with glutathione S-transferase (GST). Moreover the direct interaction between TFIIS and Pol II was competed by the addition of Rpb6. Taken together we propose that Rpb6 plays a role in the assembly between the Pol II and the transcription elongation factor TFIIS. For details see Refs. 10, 22, 27 and 28.

(12) Two WD Repeat-Containing TAFs in Fission Yeast that Suppress Cell Cycle Arrest in Mitosis

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The general transcription factor TFIID plays a critical role in transcription initiation of protein-coding genes by RNA polymerase II (Pol II). TFIID is a multiprotein complex comprising the TATA-binding protein (TBP) and TBP-associated factors (TAFs), both of which have been well conserved from yeast to humans. TBP specifically recognizes TATA elements, while certain TAFs directly interact with initiator or downstream promoter elements. In addition to a role in core promoter recognition, TAFs have been proposed to function as targets of activators. Subsets of TAFs have also been found in histone acetylase complexes distinct from TFIID.

Ubiquitin-dependent proteolysis has been shown to play a key role in progression through the cell cycle. A ubiquitin-protein ligase complex known as the anaphase-promoting complex or cyclosome (APC/C) promotes the metaphase-to-anaphase transition and the exit from mitosis by mediating

ubiquitination of anaphase inhibitors and mitotic cyclins, leading to their destruction by the 26S proteasome. In the fission yeast *Schizosaccharomyces pombe*, the ubiquitin-conjugating enzyme UbcP4 seems to be involved in APC/C-mediated proteolysis. We have isolated two related TAF genes, *taf72*$^+$ and *taf73*$^+$, from *S. pombe* as multicopy suppressors of a temperature-sensitive mutation in the ubiquitin-conjugating enzyme gene *ubcP4*$^+$. The *ubcP4*^{ts} mutation causes cell cycle arrest in mitosis, probably due to defects in ubiquitination mediated by the APC/C. TAF72 and TAF73 proteins have homology to WD repeat-containing TAFs such as hTAF100, dTAF80/85, and yTAF90. We found that both TAF72 and TAF73 are associated with TBP and other TAFs. We also show that overexpression of TAF72 or TAF73 suppresses cell cycle arrest in mitosis caused by a mutation in the APC/C subunit gene *cut9*$^+$. These results suggest that TAF72 and TAF73 may regulate the expression of genes involved in ubiquitin-dependent proteolysis during mitosis. Our study thus provides genetic evidence for a possible role of WD repeat-containing TAFs in the expression of genes involved in progression through the M phase of the cell cycle.

(13) Identification of the RNA Cap-Binding Site on Influenza Virus RNA Polymerase

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The genome of influenza virus is composed of eight RNA segments of negative polarity, which are transcribed after infection into positive-strand viral mRNAs for viral protein synthesis. The RNA polymerase responsible for the primary transcription is associated, in virions, with each RNA segment at the promoter formed by terminal sequences conserved among eight RNA segments. Transcription is initiated by using 11-13 nucleotide-long capped RNAs which are generated after cleavage of host cell mRNA by the RNA polymerase-associated endonuclease. The RNA polymerase is involved in not only transcription but also replication of the genome RNA. Replication is, however, initiated *de novo* without using primers. Previously we demonstrated that the

RNA polymerase is composed of one molecule each of three viral proteins, PB1, PB2 and PA. All three P proteins are required for the complete cycle of transcription and replication. Since the catalytic site of RNA polymerization is located in the PB1 subunit and since both the PB2 and PA subunits bind to PB1 in the P complex formation, the PB1 is the core subunit in the influenza virus RNA polymerase and plays key roles in both transcription and replication. Genetic and biochemical studies indicated that PB2 is involved in recognition and cleavage of capped RNA for generation of transcription primers.

This year we tried to identify the cap 1-binding site(s) on the PB2 protein. For this purpose, ribonucleoprotein (RNP) cores were purified from virus particles and subjected to UV-crosslinking to RNA with ^{32}P only at the 5'-terminal cap-1 structure. The PB2 protein cross-linked with the radioactive cap structure was isolated by SDS-PAGE, mixed with unlabeled PB2, and digested with V8 in SDS-polyacrylamide gels. Amino acid sequencing of the proteolytic fragments indicated that two regions of PB2 are involved in cap 1 binding, one (N-site) at the N-terminal proximal region between residues 200 and 300, and the other (C-site) at the C-terminal proximal region between residues 520 and 580, suggesting the presence of two capped RNA-binding sites on the PB2 protein. To confirm the results, we expressed two kinds of PB2 fragments, each carrying either the N-site or C-site, in *Escherichia coli*, and tested the capped RNA-binding activity for the PB2 fragments. The results support the conclusion that two capped RNA-binding sites exist on the PB2 protein. See Ref. 8.

(14) Functional Modulation of Influenza Virus RNA Polymerase by Viral RNA: Differential Roles of vRNA and cRNA

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The genome of influenza virus is composed of eight negative-strand viral RNA (vRNA) segments which are transcribed, in virus-infected cell nuclei, into mRNA by virion-associated RNA-dependent RNA polymerase. The influenza virus RNA polymerase is involved in both transcription (vRNA-directed mRNA

synthesis) and replication (vRNA-directed synthesis of complementary RNA (cRNA) and cRNA-directed vRNA synthesis). In transcription, the RNA polymerase catalyzes not only RNA synthesis but also the cleavage of capped host RNA to generate capped RNA primers and polyadenylation at the 3'-termini of mRNA. The RNA polymerase also carries an apparent proof-reading activity for nascent RNA chains.

The RNA polymerase is composed of one molecule each of PB1, PB2 and PA. PB1 plays a central role in RNA polymerase assembly by providing the contact surfaces for both PB2 and PA. In the virus particles, this RNA polymerase is bound to a double-stranded region of viral RNA (vRNA) formed by base-pairing between its 5'- and 3'-termini. Since the RNA polymerase is tightly associated with vRNA, no purification method yielding large amounts of functional RNA polymerase has been established. For detailed analysis of the structure-function relationship of each P protein, large amounts of functional RNA polymerase are required in a template-free form. This year we succeded to purify the RNA polymerase from insect cells, in which three P proteins were simultaneously expressed using recombinant baculoviruses. For purification purpose of the 3P complex, the PA protein was expressed as a fusion with His_6 tag added at its N-terminus. The affinity-purified 3P complex showed the activities of capped RNA binding, capped RNA cleavage, v- and c-sense model RNA binding, model vRNA-directed and ApG- or mRNA-primed RNA synthesis, and polyadenylation of newly synthesized RNA. We conclude that a functional form of influenza virus RNA polymerase with the catalytic specificity of transcriptase is formed in baculovirus-infected insect cells. Moreover, capped RNA cleavage was observed in the presence of vRNA but not of cRNA, indicating that vRNA functions as a regulatory factor for the specificity control of viral RNA polymerase as well as a template for transcription.

(15) Expression of Functional Influenza Viral RNA Polymerase in the Methylotrophic Yeast *Pichia pastoris*

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Influenza virus RNA polymerase with the subunit composition PB1-PB2-PA is a multi-functional enzyme with the activities of both synthesis and cleavage of RNA, and is involved in both transcription and replication of the viral genome. In order to meet the demand for a large amount of the functional influenza virus RNA polymerase in template-free form, an alternative approach has been employed, where all three P proteins were expressed in the same cells, transiently after infection of recombinant baculoviruses (see above). In order to improve the expression levels, we employed the methylotrophic yeast *Pichia pastoris* system as well as the recombinant baculovirus system. Here we describe the first successful expression of negative-strand viral RNA polymerase in yeast. The *P. pastoris* yeast is able to utilize methanol as its sole carbon source and has been developed as a host for the expression of heterologous proteins. The major advantages of this expression system include: (i) a strong, tightly-regulated alcohol oxidase promoter, 5'AOX1, is available; (ii) large scale protein production can be achieved in a large volume fermentor culture; (iii) a secretary pathway allows the product secreted into the medium, separating the foreign protein from most of the host proteins; (iv) the expression system can be easily set up; and (v) the cost is as equally low as the *Escherichia coli* expression system. The results indicate that: (i) an expression system of reasonable amounts of three P proteins in *P. pastoris* was established, after searching for the optimum induction times and the optimum concentration of methanol to give the maximum level of induction; (ii) by adding a histidine-tag to the PB2 protein, the 3P protein complex was isolated from the cell lysates after Ni^{2+}-nitrilotriacetic acid (NTA) agarose affinity chromatography; (iii) by quantitative Western blotting, all three P proteins in the isolated 3P complex were detected in stoichiometric molar ratios; (iv) the catalytic activity of RNA synthesis was detected for the 3P complex only when model vRNA or cRNA templates with the conserved terminal sequences were added; and (v) both synthetic dinucleotides and globin mRNA served as primers, but the 3P protein complex was virtually inactive in the absence of primers. The template recognition specificity was also examined using various kinds of model vRNA or cRNA with or without the terminal conserved sequences. Taken all the results together we conclude that the functional influenza virus RNA polymerase can be produced in the methylotrophic

yeast *P. pastoris*, in amounts sufficient for detailed functional analysis and structural studies. See Ref. 9.

(16) Search for Host Factors Interacting with Influenza Virus RNA Polymerase

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Influenza virus RNA polymerase consisting of three viral P proteins (PB1, PB2 and PA) carries two functions, one for transcription of vRNAs to produce vival mRNAs and the other for replicatin of vRNAs to produce progeny vRNAs via cRNA templates. Transcription of the vRNAs by the viral RNA polymerase is initiated by using host cell capped RNAs as primers. Analysis of the 5'-terminal structure of virus-associated vRNAs indicated that RNA synthesis for replication is initiated *de novo* without using primers. Both purified and reconstituted RNA polymerases require primers for function, while the RNA polymerase in either virus-infected cell extracts or lysates of cells expressing three viral P proteins can catalyze RNA synthesis in the absence of primers. We then proposed that an as yet unidentified host factor(s) is involved in the functional conversion of the RNA polymerase from transcriptase to replicase.

Attempts have been made to identify host proteins, which interact with each of the P proteins, using yeast two hybrid screening system. Several positive clones have been isolated for each P protein, and complete cDNAs for some of these putative PB1-, PB2- and PA-interacting host factors have been isolated. After overexpression in *E. coli*, the purified proteins were used to raise antibodies in rabbits, and the anti-host factors were used for detection of protein complexes in both uninfected and virus-infected cell extracts. The overexpressed and purified host proteins were also tested for the activity of binding to the respective P proteins. Effects of these putative host factors on the functions of influenza virus RNA polymerase are being examined.

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A-b. Division of Mutagenesis

(1) Ubiquitin-conjugating enzymes in fission yeast

JOON-HYUM Park, Hiroaki SEINO and Fumiaki YAMAO

Selective protein degradation in eukaryotic cells is mainly carried out by the ubiquitin system. The many short-lived proteins are subjected to be substrates of ubiquitination. Ultimately causing the destruction of various regulatory proteins, the ubiquitin system plays important roles in many cellular functions, including cell-cycle control, signal transduction, transcriptional regulation, the nuclear transport process, receptor control by endocytosis and so on. The diversity and specificity of ubiquitination are based on the molecular heterogeneity of ubiquitin-conjugating enzymes (Ubc or E2) and ubiquitin-ligases (E3). To elicidate the functional spectrum of Ubc's, we surveyed Ubc's in fission yeast. In our previous works, four Ubc's (UbcP1-P4) were identified through screening by their enzymatic activities. Through homology search in the fission yeast genome data base, other eight Ubc's (UbcP5-P12) have been found. The functional analyses of Ubc's are undergoing through disruption of their genes, showing that some genes are found essential for the cell growth while its ortholog in the budding yeast are not (i.e., UBC11/ubcP4, UBC4/ubcP1). Phenotypic differences between the fission yeast Ubc's and their structural homologues in the budding yeast have been found in many cases. Thus, the whole spectrum of functional diversity in Ubc does not seem to be identical between the two yeast lines, indicating the importance to investigating functions of ubiquitin-conjugating enzymes in fission yeast.

(2) Destruction of S-phase cyclin, Cig2, in fission yeast

Hiroaki SEINO and Fumiaki YAMAO

Ubiquitin-conjugating enzyme UbcP4 in fission yeast, working together with anaphase promoting complex (APC/C), is essential for mitotic progression. They mediate ubiquitin-pathway forwarding Cut2 for destruction to initiate

anaphase in mitosis as well as Cdc13, a mitotic cyclin, to exit from mitosis. Another cyclin, Cig2, functions during S-phase in fission yeast, and is destroyed before mitosis. Since genetic interactions among *cig2, ubcP4* and *cut9* mutant genes were observed, we suggested that Cig2 protein was regulated via proteolysis involving UbcP4 and APC/C. The cyclin boxes of Cig2 and Cdc13 were found interchangeable. The phase-dependent destruction of Cig2 and Cdc13, therefore, was evidently determined by their structures of N-terminus outside the cyclin box. Cig2 has a degradation signal (named destruction box) in the N-terminal region. As expected, Cig2 degradation depended on the destruction box. Since the destruction boxes of Cig2 and Cdc13 are very similar, we are now identifying the determinant for their destruction timing in or near the destruction box. These results indicated that ubiquitin pathway involving UbcP4/APC was active in both G2 phase and anaphase, and that a mechanism for the phase- and substrate-specific regulation of this pathway might exist.

(3) Ubiquitin pathways functioning in mitotic regulatory mechanism

Hiroaki SEINO and Fumiaki YAMAO

Ubc specific for mitotic cyclin ubiquitination has been identified in fission yeast (UbcP4), clam (Ubc-C), Xenopus (UBC-x) and human (UbcH10), which function together with APC/C. During biochemical analysis of M phase cyclin ubiquitination using frog or clam egg extract, an additional ubiquitin conjugating enzyme, a Ubc4 homologue, was found to be capable of ubiquitinating M phase cyclin under the function of APC/C. The functional differences, however, between the two Ubc's have not yet been clarified. We found both of their ortholog in fission yeast, ubcP4 and ubcP1 (ubc4), were essential for cell viability. UbcP1- or UbcP4-depleted cells exhibited M phase defects, accumulating mitotic cyclin in the cells. These results suggest, in accordance with the above observations in egg extract, that the two ubiquitin conjugating enzymes have different and essential functions for ubiquitinating mitotic cyclin. Characterizations of the roles of these ubiquitin pathways in the mitotic regulation are undergoing.

(4) Characterization of SCF^{Grr1} that ubiquitinates G1 cyclins in *Saccharomyces cerevisiae*

Tsutomu KISHI and Fumiaki YAMAO

SCF complexes, composed of Skp1, Cdc53 and one of the F-box proteins, have been implicated in the Cdc34-dependent ubiquitination in Saccharomyces cerevisiae. We have found that Grr1 required for degradation of G1 cyclins, Cln1 and Cln2, as well as for regulation of glucose repression, is an F-box protein and interacts with Skp1 through the F-box motif. Furthermore, we have found that Grr1 also interacts in vitro with phosphorylated Cln1 and Cln2. From these data, we have proposed that Grr1 is required for degradation of Cln2 through linking phosphorylated Cln2 to Skp1 in SCF^{Grr1} complex. To isolate additional genes that are required for the degradation of G1 cyclin Cln2, we isolated mutants that stabilize Cln2. We tentatively named these mutants as dog (Degradation of G1 cyclin) mutants. Characterization of these mutants, and cloning of the genes are underway.

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A-c. Division of Nucleic Acid Chemistry

(1) Proteomic analysis of *Escherichia coli* proteins in the stationary phase by the RFHR 2-D PAGE

Akira WADA (Osaka Medical college, Dept. Physics)

The RFHR (radical-free and highly reducing) 2-D PAGE which we have developed separates basic proteins in addition to neutral and acidic proteins with high resolution. The more commonly used O'Farrell's method can not resolve highly basic proteins, therefore our method is more suitable for comprehensive proteomic analysis.

The total proteins of *E. coli* cultured in the medium E including 2% polypeptone for 7 ± 1 days were separated on the 2-D gels of the RFHR 2-D PAGE. As a result, 6 log-phase-specific proteins and 59 stationary-phase-specific proteins were detected. Among the stationary-phase-specific proteins, 23 proteins were found during the entire stationary phase, while the other 36 proteins were detected only during limited short periods of the stationary phase, respectively. These 36 proteins were classified to 10 groups according to their detected periods. This shows that the *E. coli* cells are surviving for the entire stationary phase by continually changing their protein composition.

Among these stationary-phase-specific proteins, 31 proteins were identified by amino acid sequencing. The protein function distributions are as follows: carbohydrate metabolism 1, amino acid metabolism 1, nitrate metabolism 1, protein folding 1, DNA binding 4, membrane transport 5, translation 4 and hypothetical ORF 14.

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B. DEPARTMENT OF CELL GENETICS

B-a. Division of Cytogenetics

(1) Roles of Mre11 of *Saccharomyces cerevisiae* in Meiotic Recombination and Mitotic Repair Reactions

Daisuke TATSUDA, Hiroyuki OSHIUMI, Tsutomu OHTA, Jun-ichi TOMIZAWA and Tomoko OGAWA

The genetic integrity of vegetative cells is maintained by various DNA repair systems. Among the damage, double strand breaks (DSBs) that were induced by treatment with ionizing radiation or radiomimetic chemicals, such as methyl-methane sulfonate (MMS), are repaired by the homologous recombination or by the non-homologous end-joining. On the other hand, genomic diversity is provided by meiotic recombinatiion. Specific DSBs introduced in the meiotic process are repaired by homologous recombination.

We studied the involvement of the *MRE11* gene of *S. cerevisiae* in homologous recombination [1]. The gene was first identified by isolation of a mutant that is defective in meiotic recombination and repair of DSBs induced by MMS [2]. Mre11 is involved in two temporally coupled processes in the early phase of meiotic recombination, formation of DSBs and their processing. For the DSB formation, Mre11 acts by forming the pre-DSB complex with at least eight proteins including Rad50, Xrs2, Spo11 and Mei4. For processing of the DSB ends, the Mre11 forms the post-DSB complex in which Mre11 holds Rad50 and Xrs2 tightly as a binding core. Such a tight binding among these proteins was not present in the pre-DSB complex. The C-terminal region of the Mre11 binds to four meiotic proteins at least. One of the proteins, Mei4, was identified (Ohta, T. unpublished results). The N-terminal region that contains a phosphodiesterase motif specifies ssDNA endonuclease, 3' to 5' ssDNA exonuclease and 3' to 5' dsDNA exonuclease activities that are collectively required for processing of the DSBs.

Mre11 has two sites to bind to DNA. The central site (Site-A) is required for

the DSB processing, whereas the C-terminal site (Site-B) is involved in the DSB formation in meiosis. Mre11 also binds to recombination hotspots on the chromosomal DNA (Ohta, T. unpublished results). The site-B is not necessary for repair of the DNA damage caused by MMS. Mre11 has two regions to bind to Rad50, one at the N-terminal side and the other at the central region of the protein. Because Rad50 has been shown to bind DNA, Mre11 could bind DNA through Rad50. The presence of two binding sites, each for DNA and for Rad50, permits the protein to bind to DNA differently for formation of DSBs and their processing in meiotic recombination and in repair reactions.

To elucidate further the mechanisms of joining of DSB ends, we studied joining of blunt ended DNA, using the substrate that was made by cutting pUC18 plasmid once with SmaI enzyme. Mre11 protein catalized formation of linear oligomers, mainly dimmers, but not monomeric circular molecules. The results suggested that the formation of oligomers requires homologous sequences at the ends of dsDNA. The reaction depends on Mn^{++}, but not on Mg^{++} and ATP, and is enhanced by Rad50 protein. The joining is unstable against the heating and the alkaline treatment, indicating binding by hydrogen bonds. These results show that this end-joining activity depends on the unwinding of the ends of DNA and on annealing with the counter molecules. The reaction is independent of the nuclease activities, because the mutant protein that is defective in the nuclease activities can join the ends.

We then examined the unwinding activity of Mre11, using various substrates that were made by annealing of M13 single-stranded circular or linear viral DNA with various nucleotide sequences of oligonucleotides in order to create ends with different structures. Mre11 released the oligonucleotide from the substrates that has blunt ends with 3' overhung and also that have no overhanging at the annealed oligonucleotides. On the other hand, both overhanging ends at the annealed oligonucleotides was not unwound. These results suggested that, by Mre11, the substrates that have blunt ends were unwound. The substrate with 5'overhung released the fragments, but those with 3' overhung were not, suggesting that 5' overhung enhances the release of the fragment from the blunt ends, but 5' overhanging end could not be unwound. Thus, the unwinding activity is dependent on the structures of the ends of the substrates. The activity was dependent on Manganese, but not

ATP.

Mre11 also has an activity to anneal heat denatured linear plasmid DNA. Therefore, homologous blunt ends by Mre11 must be carried out through unwinding of the ends followed by annealing of complementary ssDNA.

The ends of linear DNA were joined covalently by T4 DNA ligase. We then examined whether the Mre11 enhances the joining. We used the condition where a formation of oligomers and circular molecules from blunt ended linear dsDNA was almost undetactable due to the very small concentration of the ligase. However, when the Mre11 was also present, substantial amount of linear oligomers were formed. For the reaction, homology of the ends of linear dsDNA molecules was not required. The Rad50 enhanced the reaction. The joint formed from DNA fragmetns that had 3' or 5' overhanging ends was cleaved by the restriction enzymes which were used for the preparation of the fragments, indicating that the joining is accurate. On the other hand, the joint formed by blunt ended fragments by the wild-type Mre11 and Mre11-6 was inaccurate. Because Mre11-6 that has no nuclease activity joins the ends inaccurately, the inaccuracy by wild-type may results from processing ends and also unwinding the ends.

These results showed that Mre11 participates in two types of end joining reactions, homology-dependent end-joining and the enhancement of covalent end-joining of non-homologous ends by DNA ligase. Mre11 joins homologous blunt ends through unwinding and annealing activities. It also facilitates covalent joining of non-homologous ends by bringing the ends of two molecules close and/or holding them together. Rad50 participates in the bindings of Mre11 to DNA in this reaction. Because Mre11 uses different DNA binding sites for different Mre11 reactions, the difference in the mode of binding of Mre11 to DNA is critical for different roles of the protein in different reactions. Thus, Mre11 plays a key role in selecting repair process that is suitable to repair each particular structure at the DSB site.

(2) Roles of Rad52, Rad55 and Rad57 Proteins in Rad51-mediated Homologous Recombination in *Saccharomyces cerevisiae*

Koichi WATANABE, Shimako MATSUDA, Tsutomu OHTA and Tomoko OGAWA

RecA protein in *Escherichia coli* has been known to play a pivotal role in searching for homologous DNA sequences, their pairing and exchange of DNA strands. The RecA forms a typical right-handed helical filament in the presence of ATP, in which the DNA strand is extended 1.5-1.6 times compared with B-form DNA that is called a nucleoprotein filament or a pre-synaptic filament. The structure is essential for recombination and repair of damaged DNA. We then searched for mutants that show similar phenotypes as *E. coli recA* mutant among *rad* mutants of *S. cerevisiae*, which are defective in the repair of DNA damage. The *RAD51* and *RAD52* genes were found. Mutants of either gene are defective in mitotic and meiotic recombination and repair of DNA damage induced by MMS. The amino acid sequence of Rad51 protein has a weak similarity to the RecA protein [3] and found that Rad51 forms nucleoprotein filament [4]. On the other hand, Rad52 and RecA have no sequence homology [5]. Another *recA* homologue, *DMC1* of *S. cerevisiae* was also identified [6]. Both *RAD51* and *DMC1* genes are required for the meiotic recombination. Using the homologous sequences of *E. coli* RecA and *S. cerevisiae* Rad51, *RAD51* homologues from higher eukaryotes were isolated [7], suggesting that the *recA* like genes are involved in homologous recombination in all living cells. Further characterization of *RAD51* homologues showed that several eukaryotic gene products, Rad52, Rad54, Rad55, Rad57, Dmc1 and RPA, which is functional homolog of SSB of *E. coli*, act for formation of the Rad51 filament [8, 9].

To understand the roles of various proteins that are involved in Rad51-mediated strand exchange reaction, we analyzed the role of Rad52 for the Rad51-mediated strand exchange reaction. We found that the Rad52 eliminates RPA from ssDNA [10] and thus facilitating formation of Rad51 filaments in both the strand-exchange and ATP hydrolysis reactions. We then analyzed the biochemical properties of a protein from *rad52* mutant cells, Rad52-1, the mutant strain shows severe defects in both mitotic repair reaction and homologous recombination. The wild-type of Rad52 protein binds to

both single and double stranded DNA, while the Rad52-1 protein has weaker binding ability. Nevertheless, Rad52-1 can anneal complementary single-stranded DNA similar to that of the wild-type. Although Rad52-1 can bind to both Rad51 and RPA, Rad52-1 could not help the Rad51-mediated strand exchange reaction in the presence of RPA. These results indicate that the binding of Rad52 to ssDNA is more important than the binding of the protein to RPA or Rad51 for the enhancement of formation of Rad51 filament.

Rad55 and Rad57 proteins form a complex and bind to ssDNA. A mutation in the putative ATP binding consensus amino acid sequence of *RAD55* gene inactivated the function for repair of DNA damage induced by MMS and for meiotic recombination. The mutant protein prevents the complex formation with Rad57, whereas the DNA binding activity was not affected, showing that the complex formation of Rad55 with Rad57 is important for these reactions. The analysis of the *rad55* mutant in meiosis demonstrated that DSBs were introduced at the same timing as the wild-type diploid, but that commitment to recombination and completion of the formation of synaptonemal complexes delayed in the mutant. These results suggest that Rad55-57 complex acts in formation of the Rad51 filaments on exposed ssDNA tail at DSB ends and that the pairing with homologous DNA molecules may stimulate formation of synaptonemal complexes by stabilizing axial association between synaptic chromosomes.

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Oral Presentations

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2. OGAWA, T., TATSUDA, D., OSHIUMI, H., KAWANE, K., OGAWA, H. and OHTA, T.: Homology-dependent and -independent end-joining reactions by Mre11. FASEB Summer Conference Snowmass, USA August.
3. OHTA, T., TATSUDA, D., OSHIUMI, H., TANAKA, S.and OGAWA,T.: Roles of Mre11 protein in yeast recombination and repair. The 16th Radiation Biology Center International Symposium Bioregulation of Radiation Response: Evolutional Dynamism of Damage Tolerance Kyoto December.
4. OHTA, T., OSHIUMI, H. and OGAWA, T.: Roles of Mre11 protein in yeast recombination and repair. FASEB Summer Conference Snowmass USA August.
5. OSHIUMI, H., OGAWA, H. and OGAWA, T.: Functional domains of Mre11. FASEB Summer Conference Snowmass USA August.
6. OHTA, T., TANAKA, S., and OGAWA, T.: Roles of *MRE11* protein in yeast recombination and repair. 3R Symposium Miki November.
7. TANAKA, S. and OGAWA, T.: Functional analysis of *MRE11* on the telomere maintenance. 3R Symposium Miki November.
8. WATANABE, K., MATSUDA, S. and OGAWA, T.: Biochemical characterization of Rad55-57 complex. 3R Symposium Miki November.
9. TATSUDA, D., KAWANE, K., OSHIUMI, H., MATSUDA, S., OHTA, T., OGAWA, H. and OGAWA,

T.: Homology-dependent and independent end-joining reaction by *MRE11*. 3R Symposium Miki November.

10. OSHIUMI, H., TSUBOUCHI, H., OGAWA, H. and OGAWA, T.: Functional domains of Mre11. 3R Symposium Miki November.
11. ALEXEEV, A. and OGAWA, T.: Role of the CBF1 protein in *RAD51* transcriptional regulation in response to the DNA damage. 3R Symposium Miki November.

B-b. Division of Microbial Genetics

(1) Dpb11 controls the association between DNA polymerases α and ε and the autonomously replicating sequence region of budding yeast

Hiroshi MASUMOTO and Hiroyuki ARAKI

Chromosomal DNA replication in eukaryotic cells initiates from multiple origins which fire sequentially throughout the S phase; some fires early and others late. The pre-replicative complex (pre-RC) starts to assemble at origins from late M phase and DNA polymerases are recruited onto origins to initiate DNA synthesis during the S phase. The Dpb11 protein which genetically interacts with essential DNA polymerase ε (Pol ε), is required for DNA replication and the S-phase checkpoint. The physical interaction between Pol ε and Dpb11 had not been detected. Using a cross-linker, we detected a complex of Pol ε and Dpb11 (Dpb11-Pol ε complex). During the S phase of the cell cycle, Dpb11 associated preferentially with DNA fragments containing autonomously replicating sequences (ARSs), at the same time as Pol ε associated with these fragments. Association of Dpb11 and Pol ε with these fragments was mutually dependent, suggesting that the Dpb11-Pol ε complex associates with the ARS. Moreover, Dpb11 was required for the association of the Pol α-primase with the fragments. Thus, it seems likely that association of the Dpb11-Pol ε complex with the ARS fragments is required for the association of the Pol α-primase complex. Hydroxyurea inhibits late-origin firing in *S. cerevisiae*, and the checkpoint genes, *RAD53* and *MEC1*, are involved in this inhibition. In the presence of hydroxyurea at temperatures permissive for cell growth, Pol ε in *dpb11-1* cells associated with early- and late-origin fragments. In wild-type

cells, however, it only associated with early-origin fragments. This indicates that Dpb11 may also be involved in the regulation of late-origin firing. Overall, these results suggest that Dpb11 controls the association between DNA polymerase α and ε, and the ARS (Masumoto et al., 2000).

(2) Functional characterization of Sld3 and Sld4 that genetically interact with Dpb11

Yoichiro KAMIMURA and Hiroyuki ARAKI

To identify factors interacting with Dpb11, we have isolated 10 *sld* (synthetic lethality with *dpb11-1*) mutations which fall into 6 complementaion groups (*sld1~6*). So far, we have cloned *SLD1~6* and found that *SLD1* is identical to *DPB3* encoding the third largest subunit of Pol ε, *SLD4* is identical to *CDC45* required for the initiation of chromosomal DNA replication and *SLD6* is identical to *RAD53* required for cell cycle checkpoints. The *SLD2, -3* and *-5* genes were novel genes essential for the cell growth. We have shown that Sld2 forms complex with Dpb11 and this complex is involved in the early step of DNA replication.

To know the function of Sld3, we have isolated a thermosensitive *sld3-5* mutation. *Sld3-5* cells were defective in DNA replication at the restrictive temperature. We further analyzed the DNA replication defect of *sld3-5* cells by 2-D agarose gel electrophoresis. In *sld3-5* cells, the bubble arc in ARS region that is a signal indicative of the initiation of DNA synthesis, reduced quickly after temperature shift-up, suggesting that Sld3 is required for the early step of DNA replication. When *sld3-5* cells were arrested at permissive temperature in S phase by hydroxyurea and were released at the restrictive temperature, cells did not complete DNA replication, suggesting that Sld3 also functions in the elongation step of DNA replication. Thermosensitive growth of *sld3-5* mutant was suppressed by high copy *CDC45* / *SLD4*. Moreover, 2-hybrid analysis showed an interaction between Sld3 and Cdc45. These results suggest that Sld3 and Cdc45 form a complex.

We analyzed association of Sld3 and Cdc45 with ARS using chromatin immunoprecipitation assay. Both Sld3 and Cdc45 associated with early-firing

ARSs from G1 while their association with late-firing ARSs occurred in the S phase. Their association with ARSs depended on Mcm5, a component of the pre-RC (see section (1)) and was mutually dependent, consistent with complex formation of Sld3 and Cdc45. When the ARS is activated, it has been thought that double-stranded DNA in the ARS is unwound to single-stranded DNA that binds to single-stranded DNA binding protein, RPA. We, thus, examined RPA association with ARSs in *sld3-5* cells at the restrictive temperature and found that RPA does not bind ARSs. Therefore, Sld3 is required for RPA association and probably functions before unwinding of ARS region.

(3) Function of the Sld5/Psf1 complex which interacts with DNA polymerase ε and Dpb11 in *Saccharomyces cerevisiae*

Yuko TAKAYAMA, Yoichiro KAMIMURA and Hiroyuki ARAKI

The *SLD5* gene encodes an essential 34 kDa protein. To elucidate the function of Sld5, we isolated the *PSF1* (Partner of *SLD* Five) gene as a multicopy suppressor of the *sld5-12* mutation. *PSF1* encodes a 24 kDa protein essential for cell growth. In two-hybrid analysis and co-immunoprecipitation experiment, Sld5 interacts with Psf1 in yeast cells. The Sld5-Psf1 complex was detected throughout the cell cycle. We also isolated a thermosensitive *psf1-1* mutation by the plasmid shuffling method. *Psf1-1* mutant cells arrested with a dumbbell shape at the restrictive temperature as did *sld5-12* cells. We did not detect the Sld5/Psf1 complex in *psf1-1* cells. By two hybrid assay, Psf1-1 was defective in an interaction with Dpb11. These results suggest that the Sld5/Psf1 complex, together with Dpb11, is required for the early step of DNA replication.

(4) Novel mutations of *DPB11*

Sachiko MURAMATSU and Hiroyuki ARAKI

Dpb11 has four BRCT (*BR*CA1 *C*-terminus) repeats that occur in several proteins involved in cell cycle checkpoints, and has been suggested to be a domain for

interaction between proteins. Since *dpb11-1* is a nonsense mutation that occurs at C-terminus of the fourth BRCT repeats, the function of each BRCT repeats is obscure. We, therefore, isolated novel *DPB11* mutations to know the function of each BRCT repeats. A mutagenized *DPB11* gene library was created by random PCR mutagenesis and was screened for Ts (thermosensitive), HU (hydroxyurea)-sensitive or MMS (methyl methanesulfonate)-sensitive alleles by using a plasmid shuffling strategy. So far, we have isolated eight Ts (*dpb11-3* ~ *-9*, *-11*), two MMS-sensitive (*dpb11-12*, *-13*) and one HU, MMS-sensitive (*dpb11-14*) alleles.

We first examined the Dpb11 protein level by tagging Dpb11 and western blotting. The Dpb11 protein levels were reduced to 10 ~ 30 % of the wild type level in the Ts mutants except for two which have mutations in first BRCT repeats (*dpb11-3*, *-4*). Since most of Ts mutants grew normally at permissive temperature, it was suggested that small amount of Dpb11 is sufficient for the cell growth.

The novel Ts mutants lost viability quickly after temperature shift-up when population of cells having large bud increased during S-phase as *dpb11-1* cells do. Multicopy *SLDs* and *PSF1* that were isolated as factors interacting with Dpb11, were introduced to each *dpb11* mutants. Multicopy *SLD2*, whose products form a complex with Dpb11, strongly suppressed the growth defects of the Ts mutations occurring in C-terminal half, but not of the Ts mutations in N-terminal half. This result suggests that Sld2 interacts with C-terminal half of Dpb11, and further suggests that all the novel mutations do not have the defect in the same process although phenotypes are similar.

All the Ts mutants (*dpb11-3*~ *-9*, *-11*) were slightly sensitive to HU and MMS, while six Ts mutants (*dpb11-3* ~ *-6*, *-9*, *-11*) were sensitive to UV and two mutants (*dpb11-7*, *-8*) were not. *Dpb11-12* and *-13* mutants showed semi-dominant phenotype in sensitivity to MMS, but not to HU and UV. *Dpb11-14* mutant was slightly sensitive to HU and MMS but not to UV.

To know whether *dpb11* mutants are defective in the checkpoint, we examined the Rad53 modification in response to damage. It was reported that Rad53 modification in response to MMS treatment was reduced in *dpb11-1* cells at semi-permissive temperature. The Rad53 modification after MMS treatment, however, was not reduced in *dpb11-1* cells at semi-permissive tem-

perature by our hands. Surprisingly Rad53 was modified in *dpb11-1* more than WT in response to low level MMS at permissive temperature. This result suggests that Dpb11 is not major determinant of Rad53 modification in the DNA damage checkpoint pathway, and Dpb11 may be involved in repair of damaged DNA.

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B-c. Division of Cytoplasmic Genetics

(1) Studies on behavioral disorders of knockout mice

Hiroaki NIKI (Lab. for Neurobiology of Emotion, Brain Science Institute, RIKEN)

The research is aimed at elucidating the behavioral abnormalities of Fyn-deficient mice and their pharmacological and physiological correlates. These mice were more fearful in a variety of fear tests (light aversion test, novelty preference test, elevated plus maze test, and passive avoidance test). Decreased offensive aggression and increased defensive aggression were noted in these mice.

We also found that these mice were more susceptible to seizures induced by intense sound and convulsive drugs. Related to these abnormalities of emotional behavior and seizure susceptibility Fyn-deficient mice were found to have a lower density of central benzodiazepine receptor binding sites in brain tissues. We have recently found that these mice were hypersensitive to the hypnotic effect of ethanol along with the loss of acute tolerance to ethanol

inhibition of NMDA receptor mediated EPSPs in the hippocampus slice experiment. In wild-type mice administration of ethanol enhanced tyrosine phosphorylation of the NMDA receptor in the hippocampus, but such a change was not noted in Fyn-deficient mice. In 1999 we found a novel target of ethanol in Xenopus oocyte protein expression system: ethanol activates G-protein activated inwardly rectifying potassium channel (GIRK channel). In addition, physiological and behavioral analyses of weaver mutant mice having mutant GIRK channel in the brain revealed that the activation of GIRK channel by ethanol is important for ethanol-induced analgesia.

Publication

1. KOBAYASHI, T., IKEDA, K., KOJIMA, H., NIKI, H., YANO, R., YOSHIOKA, T. and KUMANISHI, T.: Ethanol opens G-protein-activated inwardly rectifying K^+ channels. Nature Neurosci. **2**:1091-1097, 1999.

C. DEPARTMENT OF DEVELOPMENTAL GENETICS

C-a. Division of Developmental Genetics

(1) Regulation of neural induction by negative regulators of the ras/MAPK signaling pathway

Masaki IWANAMI, Masataka OKABE, Yasushi HIROMI

Induction is a fundamental strategy in cell fate decisions during development, and is often used to generate cellular diversity. During neural development, however, neurons or their precursors often induce their neighbors to take a fate similar to their own. This phenomenon, called "homeogenetic induction", occurs in at least two processes in Drosophila; photoreceptor neuron development in the adult retina, and chordotonal organ formation in the embryonic peripheral nervous system. In both cases, the inducing factor is an EGF-like molecule Spitz, which acts though EGF-receptor and ras/MAPK signaling pathway to phosphorylate ETS transcription factor PointedP2. To prevent unlimited spread of the induction process, induction must be negatively regulated. We showed that in both eye and chordotonal organs, three negative regulatory system operate simultaneously to inhibit induction. Two of them produces either cell autonomous or non-autonomous factors that inhibit ras/MAPK signaling, in response to the inducing signal. While Argos had been known to act non-autonomously to antagonize EGF-receptor function, we showed that Sprouty acts autonomously to inhibit Spitz-mediated induction. Furthermore, we found a third mechanism; signal-dependent inhibition of inducing ability. PointedP2 activated by the inducing signal inhibits the ability to induce, thereby ensuring that cells, once induced, does not act as a secondary source of induction. These negative regulatory systems are all necessary, and none of the three is sufficient.Production of constant outcome by induction must therefore involve interaction of these negative regulatory systems. In fact, *argos* and *sprouty* exhibit genetic interactions. We are cur-

rently analyzing mechanisms of interaction between three negative regulatory systems. For details, see ref. 1.

(2) Positional information required for sensory organ identity in the peripheral nervous system

Masataka OKABE and Yasushi HIROMI

The Drosophila larval peripheral nervous system consists of many types of sensory organs, such as external sensory (Es) organ (mechanosensory organ) and chordotonal (Ch) organ (stretch receptor). We are investigating how each organ forms in a specific segment, at specific positions within each segment. Formation of sensory organs require activities of organ-specific proneural genes that encode bHLH proteins; Es organ depends on proneural genes in the *achaete-scute complex* (*AS-C*), while Ch organ require *atonal*. A down stream target of *AS-C* is *cut*, which encodes a homeobox transcription factor that acts as a genetic switch between Es and Ch organs. Although the prevailing view is that organ-specific bHLH transcriptional factors determine the distinction between Es and Ch organs, we found that even in the absence of organ-specific bHLH proteins, correct organ formation and Es-specific *cut* expression can be achieved by an organ-nonspecific bHLH protein Asense. This indicates that there must be positional information that activates *cut* specifically in the Es organ precursors, independently from organ specific bHLH proteins.

With the aim of identifying positional information that regulates *cut* expression, we began analysis of *cis*-elements of the *cut* gene, responsible for its expression in Es organ precursors. We have identified a minimal enhancer of 200bp that directs Es organ specific expression. In near future, we hope to identify factors that act through this element and regulate *cut* expression in Es organ precursors.

(3) Spatio-temporal constraints of organogenesis in Drosophila imaginal discs

Nao NIWA, Masataka OKABE and Yasushi HIROMI

The Drosophila *eyeless* (*ey*) gene has been identified as a master control gene for the compound eye development. Ey is required for eye formation and is expressed in the entire eye imaginal disc. However, not all of *ey*-expressing cells form the compound eye. For example, peripheral region of the eye disc form the head cuticle surrounding compound eye. Furthermore, while *ey* is ubiquitously expressed throughout the eye disc, retinal differentiation starts only from the posterior edge of the disc. Therefore, in addition to *ey* expression, there must be other conditions required for the initiation of eye formation.

We are investigating the spatio-temporal constraints on eye formation using the ectopic eye formed by the *ey* gene expression as an experimental paradigm. We found that the ectopic eye formation was most frequent when *ey* was expressed at about 80 hours after egg laying (AEL), and the positions of ectopic eyes in all imaginal discs were restricted to several domains near the anterior-posterior compartment borders where *decapentaplegic* (*dpp*) or *wingless* (*wg*) gene is expressed. To test whether *dpp* and *wg* have a regulatory role in the responsiveness to *ey*, *ey* was expressed under the conditions where Dpp or WG signaling is activated. Coexpression of *ey* and a constitutive active form of a Dpp receptor could induce ectopic eyes where Dpp is normally absent. On the other hand, coexpression of *wg* with *ey* blocked ectopic eye formation everywhere in the imaginal discs. These results suggest that the Dpp signal is required for the ectopic eye formation, and that Wg expression negatively regulates eye formation. In addition, we found that the period of competence to *ey* was precisely the stage when endogenous retinal development starts at the posterior edge of the eye imaginal disc. At this time, *dpp* is expressed at the posterior edge of the eye disc and is required for the initiation of retinal differentiation. These results indicate that spatial and temporal conditions for ectopic eye formation by *ey* are quite similar to those for the endogenous eye formation.

(4) Mechanism of action of a nuclear receptor Seven-up

Motomi MATSUNO, Hiroyuki KOSE[1], Yasushi HIROMI ([1]University of Tokushima, School of Medicine)

Nuclear receptor Seven-up (SVP) is expressed in a subset of photoreceptor neurons and functions as a genetic switch between photoreceptor subtypes. The ligand binding domain (LBD) of SVP is extremely well conserved during evolution, suggesting that it functions by interacting with other molecules that are also conserved through evolution. Using chimeric molecules of the GAL4 DNA binding domain and the SVP LBD, we showed that SVP LBD has transcriptional repression activity. To identify molecules that participate in SVP-mediated gene regulation, we searched for proteins that interact with SVP LBD using the yeast two-hybrid screen . We identified two proteins that could act in transcriptional regulation. One is a protein that contain the SAM domain similar to the one found in a Polycomb group protein Polyhomeotic. Another is a p52 subunit of basal transcription factor TFIIH. An intriguing possibility is that SVP exerts its repression function by interacting with the p52 subunit of TFIIH. We have identified four strains that harbor nonsense mutation or deletion in the p52 ORF. Currently, we are studying the developmental role of p52 and the genetic/molecular interactions between p52 and SVP.

(5) Hydra Peptide Project: Epitheliopeptides which are involved in Hydra morphogenesis

Toshio TAKAHASHI, Naoe HARAFUJI, Seungshic YUM, Masayuki HATTA, Hiroshi SHIMIZU, Osamu KOIZUMI[1], Yoshitaka KOBAYAKAWA[2] and Toshitaka FUJISAWA ([1]Fukuoka Women's University, [2]Faculty of Science, Kyushu University)

In our ongoing "Hydra Peptide Project" in which peptide signaling molecules are systematically purified and identified, we found several peptides which are produced and/or localized in epithelial cells. They were referred to as epitheliopeptides. Among them, two (Hym-323 and Hym-346) are involved in foot formation, one (Hym-301) in tentacle/head formation and another one

(Hym-330) in budding. These peptides have entirely different amino acid sequences and are encoded in independent cDNAs. It has been shown that morphogenesis in hydra is primarily controlled by epithelial cells. This is particularly obvious by the fact that epithelial hydra which is composed of only epithelial cells still maintains its morphology and polarity, buds and regenerates normally. Thus, important morphogenetic molecules are expected to be epithelial cell origin. We have shown that both Hym-323 and Hym-346 altered the positional values along the body column when applied exogenously and enhanced foot regeneration. It is one of the important features attributed to morphogens. Whether Hym-301 and Hym-330 affect positional values remains to be seen. In the Project, we expect to find more peptides which are involved in morphogenesis and hope to find true morphogens.

(6) Expression of nanos related genes in Hydra

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Hydra contains undifferentiated multipotent stem cells which give rise to three somatic cell types as well as germ cells. In order to analyze germ cell differentiation, the molecular markers specific to germline cells are essential. For this reason, we cloned two nanos (nos) related genes, Cnnos1 and Cnnos2 from Hydra magnipapillata. Using whole mount in situ hybridization, expression of Cnnos1 and Cnnos2 was examined. Cnnos1 was weakly expressed in multipotent stem cells and strongly expressed in germline cells but not in somatic cells. Cnnos2 appears to be also expressed in germline cells. However, it was expressed more conspicuously in the endoderm of the hypostome and suggested to be involved in head morphogenesis. In addition to structural conservation in the zinc finger domain of nos related genes, functional conservation of Cnnos1 was also shown by that a Cnnos1 transgene can rescue, although partially, nosRC phenotype that is defective in the activity of germline stem cell maintenace in Drosophila. (Sano and Kobayashi, personal comm.) Thus, the function of nos related genes in germline appears to be well

conserved from primitive to highly evolved metazoans. Cnnos genes are the first molecular genes specific to germline cells in Hydra.

(7) Feeding and wounding responses of hydra: nervous system of tentacles show unidirectional conduction of stimulus

Hiroshi SHIMIZU

Diffuse nervous system is primarily found in freshwater hydra as nerve net which is dispersedly distributed throughout the animal. It has been assumed that external stimulus is conducted in all directions through the nerve net, which is in contrast with central nervous system where the stimulus is conducted unidirectionally into or out of brain. I examined feeding and wounding responses of hydra and obtained evidence that hydra tentacle nervous system responds in a polarized manner to feeding and wounding stimulus. Tissue located proximal to the site of stimulus contracted enormously whereas not at all or very little in the distal part of it. Further analyses using animals which is devoid of nerve cells showed that this polarized response does not occur in the tissue where there are no nerve cells thus suggesting that nervous system is responsible for the response. This is so far the first demonstration that polarized nervous system as the most primitive form of central nervous system was first established in hydra, the most primitive organism having nervous system.

(8) Evolution of reef-building corals

Masayuki HATTA

Crossing experiments were carried out systematically using mass-spawning corals of the genus Acropora in Okinawa, Japan. Interspecific fertilization was observed in specific combinations of species, even between species that have very different morphologies. Molecular phylogenetic analysis of the minicollagen gene, rDNA and histone gene clusters suggested the species were divided to three related groups. Within the groups, the genetic relationships

of the species are intermixed and hybridization was observed. These charcteristics are explained by interspecific gene introgression via the hybrids produced by mass spawning, and are consistent with the concept of a species complex under the processes of the fusion of multiple species.

Surveyrance of alternative reproductive behaviors and genetic relationships suggested sympatric speciation by shifting spawning timing from mass-spawning as an alternative mechanism of speciation in sympatric Acropora corals (Hatta et al. 1999).

Phylogeny of the coral family Acroporidae based on mitochondrial genes suggested that divergence of extant species in each genus is recent events after extinction during palaeoclimatic changes. Mass-spawning appeared to be the ancestral reproductive behavior (Fukami et al. in press).

(9) Differentiation mechanisms of neurons and glia

Takeshi SASAMURA[1], Yoshihiko UMESONO[2], Kazunaga TAKIZAWA[1, 2] and Yoshiki HOTTA[2, 3] ([1]Graduate School of Science, University of Tokyo, [2]CREST, JST, [3]National Insitute of Genetics)

The transcriptional regulation of the gcm gene was examined. In the peripheral nervous system (PNS), dorsal bipolar dendritic (dbd) lineage is the most simple lineage, in which the precursor once divides asymmetrically to give rise to a neuron and a glia. gcm is transiently expressed in the glial precursor and it presence is required for induction of the glial fate, whereas its absence is required for the neuronal fate in the dbd lineage. Notch signaling pathway is sufficient for the induction of gcm in this process. Loss of Notch function results in the loss of glial differentiation as a consequence of no gcm expression. Conversely, gain of Notch function leads to the glial transformation of the neuron through the ectopic gcm expression. Notch signaling becomes activated only in the glial lineage and its neuronal inactivation requires Numb. Thus, we propose a simple mechanism that asymmetric activation of Notch directs asymmetric expression of gcm to establish a binary decision of the neuron/glia fate in the dbd lineage.

Glial differentiation mechanisms were analyzed by investigating the func-

tions of genes expressed in glial cell lineage. Longitudinal glia (LG) are generated from a single precursor cell called longitudinal glioblast (LGB). LGB generates 6 to 8 LGs, which migrate medially eventually to ensheath longitudinal tract. We showed that gcm genetically upregulates some glial specific makers. Investigation of glial morphology in these mutants showed that glial differentiation proceeds in some different pathways such as migration, process formation, induction of expression of neuronal antigen. Next we studied the relationship between glial differentiation and axon pathfinding and find that migration of glial cells are abnormal in the mutants that have defects in pioneer pathfinding.

To identify genes that regulate neural cell differentiation, we screened about 3,000 tau-lacZ enhancer trap lines. One line, Z477, whose embryos show CNS axon defect, was selected for further analysis, and named as solo (snapped outer longitudinals). We cloned solo and showed that it encodes a transcription factor that has BTB/POZ domain and zinc finger motifs. The solo gene was found to be most related to Drosophila tramtrack gene. The solo mRNA was detected in all neurons, but not glial cells by in situ hybridization to embryos. We found that solo mutant embryos have defects in the formation of one of the CNS axon fascicles and dendrites of the DBD neuron in the PNS. These data suggest that solo is required for neural differentiation, especially for axon guidance.

(10) Axon guidance mechanisms

Kazunaga TAKIZAWA[1,2], Masaki HIRAMOTO[1,2] and Yoshiki HOTTA[2,3] ([1]Graduate School of Science, University of Tokyo, [2]CREST, JST, [3]National Insitute of Genetics)

We investigated functions of glial cells in axonal pathfindings by examining axonal behavior in the gcm mutants of Drosophila that lack glial cells in the nervous system. In this study, we showed that the initial extension of pioneer neurons and target recognition of motoneurons are normal in the absence of glial cells. We also demonstrated that formation of axon bundles and pathfindings are disrupted at later stages in the absence of glial cells and

abnormal migration of glial cells leads to misrouting of axons, indicating that glial cells are required for correct pathfinding at later stages. From these observations, we propose that formation of the nervous system proceeds in multisteps with respect to glial cell functions.

Netrin is a secreted protein that has properties of a chemotropic axon guidance molecule. In Drosophia, Frazzled is a member of the DCC class of Netrin receptors, and its phenotype in the embryonic CNS is consistent with the idea that it mediates Netrin signals. Here we demonstrate a novel form of Netrin-dependent axon patterning by Frazzled: Frazzled controls the spatial distribution of Netrin, 'presenting' this ligand molecule for recognition by other receptors. Moreover, Frazzled itself is actively relocalized within axons by means of its cytoplasmic domain, thereby precisely rearranging Netrin into a spatial pattern that is completely different from the pattern of Netrin source. Finally, we show that Frazzled-dependence of one axon guidance decision, the longitudinal turning of the pioneer neuron dMP2, can be accounted for solely on the basis of this ability of Frazzled to redistribute Netrin and not by Frazzled signaling. We propose a novel patterning mechanism in which controlled distribution of a receptor creates positional information for other receptors by rearranging secreted ligand molecules.

(11) Function of the gcm-related transcriptional regulators

Toshihiko HOSOYA[1,2], Yoshiki HOTTA[1,3] ([1]National Insitute of Genetics, [2]PRESTO, JST, [3]CREST, JST)

The gcm protein is a member of a novel protein family, the gcm-family. Members of this family share the gcm-motif, a novel protein motif that is of about 150 amino acids in length. It has been shown that the gcm-motif has sequence-specific DNA-binding activity and that the *gcm* protein has transcriptional regulation activity. The gcm-binding DNA sequences are found in tandem in the upstream of a glia-specific protein whose expression is dependent on *gcm*. Thus it is strongly suggested that the gcm protein is a novel transcriptional regulator and that the gcm-family is a novel family of transcriptional regulators.

Drosophila has at least another gcm family gene, *gcm2*, which is expressed

in a subset of glial cells and early hemocytes. *gcm* is also expressed in hemocytes and required for their differentiation. Two deficiency strains that lack both *gcm* and *gcm2* have smaller number of hemocytes than wild-type. These hemocytes show defects in taking up corpses and in movement. Preliminary experiments indicated that either misexpression of *gcm* or *gcm2* can rescue these phenotypes. Thus it was suggested that *gcm* and *gcm2* have overlapping function in the hemocyte differentiation. Two gcm family genes have been isolated from Mouse, which show tissue specific expression. β -gal knock-in chimera mice have been generated.

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C-b. Division of Gene Expression

(1) Functional dissection of DNA supercoiling factor : EF-hand domains and C-terminal HDEF motif are essential for its activity

Masatomo KOBAYASHI and Susumu HIROSE

DNA supercoiling factor (SCF) was first identified in the silkworm as a protein capable of generating negative supercoils into a relaxed DNA in conjunction with eukaryotic topoisomerase II. *Drosophila melanogaster* SCF localizes to puffs on polytene chromosomes, implicating its role in gene expression. The factor is a Ca^{2+}-binding protein with four EF-hand domains and possesses a tetrapeptide sequence HDEF at its C-terminus.

To clarify the roles of the domains of SCF, we carried out a functional dissection of the factor. A glutamic acid to glutamine substitution at the end of the loop in EF-hand domain II or III reduced both the Ca^{2+}-binding and supercoiling activities; simultaneous substitutions at both sites abolished these activities. During native polyacrylamide gel electrophoresis, SCF migrated more rapidly in the presence of Ca^{2+} than in the presence of Mg^{2+} or EGTA. SCF binds directly to topoisomerase II. Deletion of the C-terminal HDEF sequence destroyed the binding and supercoiling activity.

From these results, we conclude that two regions of SCF play critical roles in the supercoiling activity. The C-terminal HDEF is essential for the factor binding to topoisomerase II. The EF-hand domains II and III are functional for the Ca^{2+} binding that induces a mobility change in the factor upon gel electrophoresis. For details, see Ref. 1.

(2) The Role of Human MBF1 as a Transcriptional Coactivator

Yasuaki KABE[1], Masahide GOTO[1], Daisuke SHIMA[1], Takeshi IMAI[1], Tadashi

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Multiprotein bridging factor 1 (MBF1) is a coactivator which mediates transcriptional activation by interconnecting the general transcription factor TATA element-binding protein and gene-specific activators such as the *Drosophila* nuclear receptor FTZ-F1 or the yeast basic leucine zipper protein GCN4. The human homolog of MBF1 (hMBF1) has been identified but its function, especially in transcription, remains unclear. Here we report the cDNA cloning and functional analysis of hMBF1. Two isoforms, which we term hMBF1 α and hMBF1 β, have been identified. hMBF1 α mRNA was detected in a number of tissues, whereas hMBF1 β exhibited tissue-specific expression. Both isoforms bound to TBP and Ad4BP/SF-1, a mammalian counterpart of FTZ-F1, and mediated Ad4BP/SF-1-dependent transcriptional activation. While hMBF1 was detected in the cytoplasm by immunostaining, coexpression of the nuclear protein Ad4BP/SF-1 with hMBF1 induced accumulation of hMBF1 in the nucleus, suggesting that hMBF1 is localized in the nucleus through its binding to Ad4BP/SF-1. hMBF1 also bound to ATF1, a member of the basic leucine zipper protein family, and mediated its activity as a transcriptional activator. These data establish that the coactivator MBF1 is functionally conserved in eukaryotes. For details, see Ref. 2.

(3) Identification of the core domain and the secondary structure of the transcriptional coactivator MBF1

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Multiprotein bridging factor 1 (MBF1) is a transcriptional coactivator necessary for transcriptional activation caused by DNA binding activators, such as FTZ-F1 and GCN4. MBF1 bridges the DNA-binding regions of these activators and the TATA-box binding protein (TBP), suggesting that MBF1 functions by recruiting TBP to promoters where the activators are bound. In addition, MBF1 stimulates DNA binding activities of the activators to their recognition sites. To date, little is known about structures of coactivators that bind to TBP.

The two-dimensional (2D) ^{1}H-^{15}N correlation spectrum of ^{15}N labeled MBF1 indicated that MBF1 consists of both flexible and well structured parts. Limited digestion of MBF1 by α-chymotrypsin yielded a $\sim$ 9 kDa fragment. N-terminal sequence analysis and NMR measurements revealed that this fragment originates from the C-terminal 80 residues of MBF1 and form a well structured C-terminal domain of MBF1, $MBF1_{CTD}$. As previous deletion analyses have shown that $MBF1_{CTD}$ is capable of binding to TBP, it is suggested that $MBF1_{CTD}$ is the TBP binding domain of MBF1. Sequential assignments have been obtained by means of three-dimensional (3D) and four dimensional (4D) heteronuclear correlation spectroscopies, and then the secondary structure of $MBF1_{CTD}$ was determined. As a result, $MBF1_{CTD}$ was shown to contain four amphipathic helices and a conserved C-terminal region. Asp106 which is assumed to be responsible for the binding to TBP is located at the hydrophilic side of the third helix.

From these results we conclude that MBF1 consists of two structurally different domains. A N-terminal region is indispensable for the binding to activators, and does not form a well defined structure. In contrast, the C-terminal 80 residues, which is capable of binding to TBP by itself, form a well-structured domain, $MBF1_{CTD}$. $MBF1_{CTD}$ is made up of four amphipathic helices and a conserved C-terminal tail. A putative TBP binding residue is located on the hydrophilic surface of the third helix. For details, see Ref. 3.

(4) Upstream element of the sea urchin arylsulfatase gene serves as an insulator

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Insulator DNAs functionally isolate neighboring genes by blocking interactions between distal cis-regulatory elements and promoters. Here we report that a DNA fragment located in the upstream region of sea urchin, *H. pulcherrimus*, arylsulfatase (HpArs) gene blocks the interaction of the *Ars* enhancer when positioned between the enhancer and the target promoter, in an orientation dependent manner. The *Ars* insulator works only 3' to 5' direction and has no significant stimulatory or inhibitory effects on its own promoter. In transgenic *Drosophila*, the *Ars* insulator blocks the interaction between *even-skipped* stripe enhancer and its target promoter. The insulation mechanism operates also unidirectionally in *Drosophila*. We also show that the efficiency of transformation of HeLa cells is enhanced when the integrated gene is flanked by the *Ars* insulator, suggesting the sea urchin insulator overcomes the position-dependent transgene expression in mammalian cells. These results demonstrate that the mechanism of action of the insulator has been conserved throughout evolution. For details, see Ref. 4.

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C-c. Division of Early Embryogenesis

(1) Mesoderm induction and its dorsoventral specification in zebrafish

Toshiro MIZUNO[1], Etsuro YAMAHA[2] and Hiroyuki TAKEDA ([1]School of Science, Kagoshima University, [2]Faculty of Fisheries, Hokkaido University)

In the previous study, we have demonstrated that zebrafish yolk cell is responsible for induction and dorsoventral patterning of the mesoderm (Mizuno et al., Nature383: 131, 1996). In order to examine the presence and localization of cytoplasmic determinants for axis formation in zebrafish, we have developed a method with which we removed vegetal yolk hemisphere of early fertilized eggs (vegetal-removed embryos). When the vegetal half of the yolk mass was removed at 1-cell stage, the embryos showed severe dorsal deficiencies and bilaterally symmetrical morphology in later development. Furthermore, vegetal-removed embryos examined at early gastrula stage (50%-epiboly) did not express a dorsal marker, *goosecoid* (*gsc*), while the expression of a ventral marker, *eve1*, was expanded dorsally. These results suggest that the vegetal yolk cell mass contains the cytoplasmic determinants responsible for dorsal specification (dorsal determinants) of the zebrafish embryos. Furthermore, we found that the frequency of abnormalities decreased as the age at which the vegetal yolk hemisphere was removed increased: The embryos operated before 1-cell stage showed the highest frequency of abnormalities while most of the embryos operated after 8-cell stage developed normally. This result suggests a translocation of the dorsal determinants from the vegetal pole toward the

animal pole during early cleavage stages in zebrafish.

To determine which tissue, the yolk cell or blastomere, requires the dorsal determinants for development of dorsal fate, recombination experiments between normal and vegetal-removed embryos were carried out. Normal yolk cell, when transplanted in the animal-pole region of intact host embryo, induced ectopic expression of *gsc* in the host blastoderms. By contrast, the yolk cell obtained from vegetal-removed embryo lost the ability to induce *gsc* in normal blastomeres. Furthermore, the blastomeres of vegetal-removed embryo expressed *gsc* when combined with normal yolk cell. These results indicate that the dorsal inducing ability of the yolk cell is highly dependent on the dorsal determinants and that the blastomeres without receiving these determinants are competent to express the dorsal specific gene in response to the signals from the yolk cell.

(2) Cell-cell interaction in zebrafish floor-plate development

Minori SHINYA and Hiroyuki TAKEDA

The floor plate is located at the ventral midline of the neural tube in vertebrates. The floor-plate development is severely impaired in zebrafish *one-eyed pinhead* (*oep*) mutants. *oep* encodes a membrane-bound protein with a EGF motif and functions autonomously in floor-plate precursors. To understand the cell behavior and cell-cell interaction during floor plate development, we examined the distribution and gene expression of wild-type and *oep* mutant cells in genetic mosaics. When mutant shield cells were transplanted into a wild-type host, an ectopic neural tube with a floor plate was induced. However, the floor plate of the secondary axis was consistently devoid of mutant cells while its notochord was composed entirely of mutant cells. This indicates that *oep* shield cells adopt only a notochord fate in a wild-type environment. In reciprocal transplants (wild -> *oep*), however, grafted shield cells frequently contributed to part of the floor-plate region of the secondary neural tube and expressed floor-plate markers. Careful examination of serial sections revealed that a mutant neural cell, when located next to the wild-type cells at the ventral midline, inhibited floor-plate differentiation of the adja-

cent wild-type cells. This inhibition was effective over an area only one or two cells wide along the anteroposterior axis. Since the cells located at the ventral midline of the *oep* neural tube are thought to possess a neural character, similar to those located on either side of the floor plate in a wild-type embryo, this inhibition may play an important role during normal development in restricting the floor-plate region into the ventral-most midline by antagonizing homeogenetic signals from the floor-plate cells.

(3) Roles of *mesp* gene family in zebrafish segmentation

Atsushi SAWADA, Andreas FRITZ[1], Yun-Jin JIANG[2], Yumiko SAGA[3] and Hiroyuki TAKEDA ([1]Department of Biology, Emory University, [2]Vertebrate Development Laboratory, Imperial Cancer Research Fund, [3]Cellular and Molecular Toxicology, National Institute of Health Sciences)

Segmentation of a vertebrate embryo begins with the subdivision of the paraxial mesoderm into somites through a not-well-understood process. Recent studies provided evidence that the Notch-Delta and the FGFR (fibroblast growth factor receptor) signalling pathways are required for segmentation. In addition, the Mesp family of bHLH transcription factors have been implicated in establishing a segmental prepattern in the presomitic mesoderm (PSM). In this study, we have characterized zebrafish *mesp-a* and *mesp-b* genes that are closely related to *Mesp* family genes in other vertebrates. During gastrulation, only *mesp-a* is expressed in the paraxial mesoderm at the blastoderm margin. During the segmentation period, both genes are segmentally expressed with one to three stripes in the anterior parts of somite primordia. In *fused somites* (*fss*) embryos, in which all early somite boundary formation is blocked, initial *mesp-a* expression at the gastrula stage remains intact, but the expression of *mesp-a* and *mesp-b* is not detected during the segmentation period. This suggests that these genes are downstream targets of *fss* at the segmentation stage.

Ectopic expression of Mesp-b in embryos causes a loss of the posterior identity within the somite primordium, leading to a segmentation defect. These injected embryos show a reduction in expression of the posterior genes, *myoD*

and *notch5*, with uniform expression in the anterior genes, *FGFR1*, *papc* and *notch6*. These observations suggest that zebrafish *mesp* genes are involved in anteroposterior specification within the presumptive somites, by regulating the essential signalling pathways mediated by Notch-Delta and FGFR.

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C-d. Division of Physiological Genetics

(1) Codon usage diversity of bacterial genes: An application of self-organization mapping for investigating origin of horizontal transfer genes

Shigehiko KANAYA and Toshimichi IKEMURA[1] ([1]Division of Evolutionary Genetics)

Multivariate analysis such as factor corresponding analysis and principal component analysis (PCA) have been used to study heterogeneous codon usage systematically in various species. To characterize the species-specific heterogeneity of genes in codon usage, we developed previously a measure denoted by Z1 based on the widest range of axis obtained by PCA of codon frequency patterns of genes analyzing a wide range of bacterial species. Extent of codon bias for each gene was found to be associated with the level of protein production in the respective bacterial species.

In the present study, we attempted to comprehend species-specific codon usage of genes in intra- and inter-species by self-organizing mapping. This neural networks have been proposed to visualize high-dimensional systems. Using the modern multivariate analysis, we analyzed codon usage heterogeneities for intra- and inter-bacterial species with special emphasis on distinct codon usage of horizontal transfer genes involving bacteriophages, restriction enzymes, and transposons, and searched for the original species from which the transferred genes were derived.

Publications

1. KANAYA S., FUKAGAWA T., ANDO A., INOKO H., KUDO Y. and IKEMURA T.: Distribution of polypurine/polypyrimidine tract sequences in the human MHC region and their possible functions. In: Major Histocompatibility Complex, Springer-Verlag Berlin / Heidelberg / New York / Tokyo, 131-145, 2000.
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D. DEPARTMENT OF POPULATION GENETICS

D-a. Division of Population Genetics

(1) Causes of the irregular synonymous substitution rates in Drosophila lineages

Toshiyuki TAKANO-SHIMIZU

Significant locus-lineage interaction exists in the synonymous substitution rates among Drosophila lineages (Takano 1998; Zeng et al. 1998). I have studied its causes by using four species of the *D. melanogaster* species subgroup. Changes in effective population sizes have generally been considered a genome-wide effect, but this is not always the case. Faster replacement- and synonymous-substitution rates in *D. melanogaster* as compared with *D. simulans* were observed in many genes; no difference in synonymous substitution rates between the two species was observed in the four *achaete-scute* complex (*AS-C*) genes and *ci* gene, however. The *AS-C* and *ci* genes are located in regions of severely reduced recombination rates: *AS-C* on the tip of the X and *ci* on the fourth chromosome. A lack of between-species difference in substitution rates at these loci is most likely due to the low efficacy of natural selection, regardless of their effective population sizes. Thus, coupled with weak selection against synonymous changes, fluctuation in effective population sizes can contribute to the heterogeneous synonymous substitution rates among genes to some degree. This study also provides evidence of remarkable local changes in substitution patterns and in recombination rates on Drosophila chromosomes. It is posited that these between-region variability and between-lineage fluctuations of mutation pressures and recombination rates affected evolutionary patterns of genes in the regions involved. In sum, the present results suggest that changes in mutation, recombination, and effective population size all contribute to the significant locus-lineage interaction in the synonymous substitution rates among the Drosophila lineages, but changes in

mutation pressures presumably have the greatest effects. I should add that Ms Yuriko Ishii has contributed significantly to this work. For details, see publications 1.

(2) Study of species difference as observed as interspecific hybrid anomaly in Drosophila: genetic screens for genes involved in the bristle loss of hybrids

Toshiyuki TAKANO-SHIMIZU

Artificial selection for quantitative characters changes the phenotype far beyond the range of variation in the original base population in most cases. Environmental stresses (phenocopy) and mutant backgrounds also uncover a surprising amount of hidden variation. Cryptic variation probably exists because of stabilizing selection and genetic buffering ability, and cryptic variation and selection may provide an scenario for discontinuous evolutionary changes in morphological characters. The clarification of the genetic basis underlying the cryptic variation can shed a light on long-standing question of how morphology evolves while maintaining its homeostasis.

Interspecific-hybrid analysis is another powerful means to uncover hidden within- and between-species variation in populations. One example is a bristle loss phenotype of hybrids between *Drosophila melanogaster* and *D. simulans*, though both the pure species have exactly the same pattern of bristle formation on the notum. There exists a large amount of genetic variability in the *simulans* populations with respect to the number of missing bristles in hybrids, and the variation is largely attributable to *simulans* X chromosomes. I have screened the *simulans* X chromosome for genetic factors affecting the bristle phenotype in *melanogaster-simulans* hybrids. Hybrid bristle-loss anomaly is potentially caused by several distinct classes of genetic factors: (1) factors responsible for the within-*simulans* variation in the number of missing bristles, (2) fixed *melanogaster-simulans* differences involved in the hybrid bristle loss, and (3) factors that show dose-sensitive effects, especially in a hybrid background, without a between-species functional difference. Because the pattern of notum bristles is fixed within each species and identical be-

tween species, these factors do not cause the anomaly in the original pure species, but only the combination of the factors in hybrids does. Thus, we have to make interspecific hybrids to screen for genetic factors responsible for the bristle loss phenotype. On the other hand, both male and female hybrids between the two species are completely sterile [but see Davis et al. (1996) for an exception], allowing only F1 hybrid screening. I used two screening methods: (i) quantitative trait loci (QTL) mapping of X-chromosome factors affecting the hybrid-bristle number by using a pair of *simulans* lines with high (H) and low (L) missing bristle numbers and (ii) deficiency screening. The former is one method for identifying class (1) factors; the second method is based on phenotypic effects in *melanogaster*-deficiency-carrying hybrids. It has the potential to identify factors of the three classes.

The QTL mapping, together with duplication-rescue experiments, recovered a single major X-chromosome QTL (or block of QTL). Importantly, this region accounted for most of the differences between H and L lines in three other independent pairs. This suggests segregation of H and L alleles (but not necessarily the same alleles) at the same locus in different populations. On the other hand, a deficiency screening uncovered several regions with factors that potentially cause the hybrid bristle loss due to epistatic interactions with the other factors [class (2) or (3) factors].

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D-b. Division of Evolutionary Genetics

(1) Fine replication-timing map of the entire human chromosome 11q at a genome sequence level: genome-wide approach to put landmarks with biological significance along the human genome sequence by defining transition zones for the replication-timing during S phase and GC% level

Yoshihisa WATANABE, Asao FUJIYAMA[1,2], Masahira HATTORI[2], Yoshiyuki SAKAKI[2] and Toshimichi IKEMURA ([1]Division of Human Genetics, [2]Human Genome Research Group, RIKEN Genomic Sciences Center)

The human genome is composed of large-scale compartmentalized structures including replication-timing zones and long-range G+C% (GC%) mosaic domains, that are thought to be related to chromosome bands. Using PCR-based quantification of newly-replicated DNA from cell-cycle fractionated human THP-1 cells, replication timing during S phase for the entire human chromosome 11q (ca. 80 Mb) was determined at a genome sequence level, analyzing 212 sequence-tagged sites (STSs). This fine mapping could put approximately twenty five transition zones of replication timing from the early to late S period, mainly in the regions where ambiguous or inconsistent FISH mapping data have been reported. Global correlation of the replication timing with GC% level was also found, disclosing several characteristic exceptions. We proposed that genome-wide replication-timing map at a sequence level could provide fundamental knowledge of the human genome sequence inevitable for comprehensively understanding a wide range of genome functions and evolutionary processes to have built up the present-day genome organization and therefore that the map construction is an indispensably important genome study that has now become possible.

(2) Triplex-forming sticky DNAs in human interphase nuclei; with special emphasis on trinucleotide repeat (GAA/TTC)n

Toshimichi IKEMURA, Mizuki OHNO[1], Tatsuo FUKAGAWA ([1]Inst. Genetic Information, Kyushu Univ.)

The (GAA/TTC)n repeat with the length equivalent to the expanded trinucleotide repeat in Friedreich's ataxia (FRDA) patients was reported to form a novel DNA structure (designated sticky DNA) through triplex formation. An excellent correlation between the repeat length found in patients (n > 59) and that required for formation of the novel DNA structure indicated that the structure may be formed in living human nuclei and cause odd biological activity resulting in FRDA. Many copies of polypurine/polypyrimidine (PuPy) tracts such as (GAA/TTC)n are known to be present in the human genome and to scatter among and within chromosomes. In PuPy-tract sequences, triplex formation occurs readily under physiological conditions, leaving single-stranded DNAs available for hybridization with other unpaired DNAs and RNAs. Triplex-forming DNAs in the human interphase nucleus were analyzed by combining immunodetection by antitriplex antibodies and fluorescence *in situ* "nondenaturing" hybridization employing PuPy-tract probes. The "nondenaturing" hybridization technique, which is used to detect RNA, can detect single-stranded DNAs in nondenatured nuclei. The probes such as (GAA/TTC)n gave sequence-specific signals that overlapped with or were closely associated with triplexes immunolocalized by known antitriplex antibodies. Pretreatment of nuclei with antitriplex antibodies blocked the probe-signal formation. The signal formation was resistant to pretreatment of nuclei with RNases but sensitive to singe-strand-specific nucleases. These findings showed triplex formation to occur in human interphase nuclei. For details, see Ref. 7.

(3) Distribution of polypurine/polypyrimidine tract sequences in the human MHC region and their possible functions

Shigehiko KANAYA[1,2], Tatsuo FUKAGAWA, Asako ANDO[3], Hidetoshi INOKO[3], Yoshihiro KUDO[2], Toshimichi IKEMURA
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To characterize distribution of polypurine/polypyrimidine (pur/pyr) tract sequences in the human MHC region, we searched 100-nt sequences with the A+G% (AG%) higher than 85% for either DNA strands. In all human genome

sequences registered in DDBJ (ca. 32 Mb in total), we obtained 6247 A+G tracts. There exists one tract per 51 kb and therefore roughly one tract per one replicon size. 117 tracts (one tract per 33 kb) was found in the MHC region, that shows the level is significantly higher than levels of usual human genome portions. One of the A+G tracts is found in DNA-replication switch region at the junction of MHC classes II and III. Other tracts were discussed in connection with polymorphism levels of several MHC genes. We also investigated evidently long A+G tracts in the human genome. The longest A+G tract found so far was the 2798-nt tract found in the 3' downstream of rDNA. The biological significance of long A+G tracts was discussed in connection with triplex formation, pausing of DNA replication and, enhancement of recombination. For details, see Ref. 1.

(4) Functional analyses of centromere proteins of higher vertebrate cells

Tatsuo FUKAGAWA, Atsushi OKAMURA, Toshimichi IKEMURA

The centromere is essential for the accurate segregation of eucaryotic chromosomes at cell division. CENP-C is an evolutionarily conserved centromeric protein and has been predicted to play an important role in kinetochore assembly and function. We have used the chicken DT40 cell line to test the idea that CENP-C is sufficient as well as necessary for the formation of a functional centromere. Firstly, we have used gene targeting in the DT40 cell line to create a cell line which expresses a fusion between CENP-C and a mouse steroid receptor and which behaves as a conditional loss of function mutant of CENP-C. We also have engineered the DT40 cell line that over-expressed CENP-C using the tetracycline-repressible promoter system. This cell line over-expressed CENP-C in the absence of tetracycline, and expression was extinguished by the addition of tetracycline.

We have compared the effects of disrupting the localization of CENP-C with those of inducibly over-expressing the protein. Removing CENP-C from the centromere cause disassembly of the centromere protein complex and blocks cells at the metaphase-anaphase junction. Over-expressed CENP-C is associ-

ated with an increase in errors of chromosome segregation and inhibits the completion of mitosis. However the excess CENP-C does not detectably disrupt the native centromeres and does not associate with another conserved centromere protein, ZW-10. The distribution of the excess CENP-C changes during the cell cycle. In metaphase the excess CENP-C coats the chromosome arms. At the metaphase anaphase transition the excess CENP-C clusters and during interphase it is present in large bodies that form around pre-existing centromeres which are also clustered. These results indicate that CENP-C is necessary but not sufficient for the formation of a functional centromere and suggest that the structure of CENP-C may be regulated during the cell cycle. For detail, Fukagawa et al., EMBO J., 18, 4196-4209 (1999).

Recently, we attempt to make temperature-sensitive mutants for CENP-C in DT40. When we put several point mutations into CENP-C, we can isolate some temperature-sensitive mutants of CENP-C. These cells grow well at 34℃, but die at 43℃ with chromosome missegragation. We also tried to isolate a suppressor for these mutations using a retrovirus system. We are now characterizing suppressor genes.

In addition, we are trying to create knockout cell lines of several centromere proteins, including ZW10, CENP-H, Meteor and Mis6. From phenotype analyses of these cell lines, we can understand how centromere works.

(5) Development of mammalian artificial chromosomes

Tatsuo FUKAGAWA and Toshimichi IKEMURA

There are two alternative approaches to the construction of mammalian artificial chromosomes (MACs). In the first, "bottom-up", approach, homologous recombination in the yeast is used to assemble MACs from candidate sequences that have been cloned in YACs. In the second, "top-down", approach, assembly of a chromosome from cloned constituents is avoided and instead, a natural mammalian chromosome is whittled down to a mini-chromosome by telomere-directed chromosome breakage that is developed by Dr William Brown in Oxford. Fukagawa has been developing a minichromosome vector in Brown Lab. In this year, we created two counter selectable hprt-/- DT40 derived cell

lines. One of these cell lines contains a stably integrated gene encoding a conditionally active cre recombinase and thus allows efficient manipulation of targeted loci by site-specific recombination. These cell lines will cell lines will enhance the utility of the hyper-recombinogenic DT40 cell line as a tool for mammalian chromosome engineering. For detail, Fukagawa et al., Nucl. Acids Res., 27, 1966-1969 (1999).

We are now modifying mini-chromosomes by double strand breaks induced by the presence of a target site for a rare-cutting endonuclease, such as I-Sce-I.

(6) Molecular genetic analysis of remains of a 2,000-year-old human population in China-and its relevance for the origin of the modern Japanese population

OOTA Hiroki[1], SAITOU Naruya, UEDA Shintaroh[1] ([1]Department of Biological Sciences, Graduate School of Science, University of Tokyo)

We extracted DNA from the human remains excavated from the Yixi site (approximately 2,000 years before the present) in the Shandong peninsula of China and, through PCR amplification, determined nucleotide sequences of their mitochondrial D-loop regions. Nucleotide diversity of the ancient Yixi people was similar to those of modern populations. Modern humans in Asia and the circum-Pacific region are divided into six radiation groups, on the basis of the phylogenetic network constructed by means of 414 mtDNA types from 1,298 individuals. We compared the ancient Yixi people with the modern Asian and the circum-Pacific populations, using two indices: frequency distribution of the radiation groups and genetic distances among populations. Both revealed that the closest genetic relatedness is between the ancient Yixi people and the modern Taiwan Han Chinese. The Yixi people show closer genetic affinity with Mongolians, mainland Japanese, and Koreans than with Ainu and Ryukyu Japanese and less genetic resemblance with Jomon people and Yayoi people, their predecessors and contemporaries, respectively, in ancient Japan. For details, see ref. 12.

(7) Phylogenetic relationship of muscle tissues deduced from superimposition of gene trees

OOTA Satoshi and SAITOU Naruya

Muscle tissues can be divided into six classes; smooth, fast skeletal, slow skeletal and cardiac muscle tissues for vertebrates, and striated and smooth muscle tissues for invertebrates. We reconstructed phylogenetic trees of six protein genes that are expressed in muscle tissues and, using a newly developed program, inferred the phylogeny of muscle tissues by superimposition of five of those gene trees. The proteins used are troponin C, myosin essential light chain, myosin regulatory light chain, myosin heavy chain, actin, and muscle regulatory factor (MRF) families. Our results suggest that the emergence of skeletal-cardiac muscle type tissues preceded the vertebrate/arthropod divergence (ca. 700 MYA), while vertebrate smooth muscle seemed to evolve independent of other muscles. In addition, skeletal muscle is not monophyletic, but cardiac and slow skeletal muscles make a cluster. Furthermore, arthropod striated muscle, urochordate smooth muscle, and vertebrate muscles except for smooth muscle share a common ancestor. On the other hand, arthropod nonmuscle and vertebrate smooth muscle and nonmuscle share a common ancestor. For details, see ref. 14.

(8) Alpha1,3-fucosyltransferase IX (Fuc-TIX) is very highly conserved between human and mouse; molecular cloning, characterization and tissue distribution of human Fuc-TIX

KANEKO Mika, NISHIHARA Shoko[1], INOKO Hidetoshi[2], SAITOU Naruya, NARIMATSU Hisashi[1]([1]Division of Cell Biology, Institute of Life Science, Soka University, [2]Department of Molecular Biology, Tokai University Medical School)

The amino acid sequence of Fuc-TIX is very highly conserved between mouse and human. The number of non-synonymous nucleotide substitutions of the Fuc-TIX gene between human and mouse was strikingly low, and almost equivalent to that of the alpha-actin gene. This indicates that Fuc-TIX is under a

strong selective pressure of preservation during evolution. The human Fuc-TIX (hFuc-TIX) showed a unique characteristics, i.e. hFuc-TIX was not activated by Mn2+ and Co2+, whereas hFuc-TIV and hFuc-TVI were activated by the cations. The hFuc-TIX transcripts were abundantly expressed in brain and stomach, and interestingly were detected in spleen and peripheral blood leukocytes. For details, see ref. 15.

(9) Population Genetic Studies on Nine Aboriginal Ethnic Groups of Taiwan. I. Red Cell Enzyme Systems

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Population genetic study of nine aboriginal ethnic groups of Taiwan (Ami, Atayal, Bunun, Paiwan, Puyuma, Rukai, Saisiat, Tsou, and Yami) was carried out. Twelve red cell enzymes (AcP, AK, CA1, CA2, EsD, GLO, GPT, GOT, LDH, MDH, PGD and PGM1) were analyzed by isoelectric focusing method and starch gel electrophoretic method. Six loci (AcP, EsD, GLO, GPT, PGD, and PGM1) were polymorphic. Three alleles (PGM1*6, GPT*6, and EsD*7) were in relatively higher allele frequencies in Taiwan aboriginal populations, and we found homozygotes for those alleles. Phylogenetic relationship based on genetic distances among those ethnic groups more or less fit to their geographical distribution, but not to linguistic classification. For details, see ref. 17.

(10) Evolution of Rh Blood Group Genes Have Experienced Gene Conversions and Positive Selection

KITANO Takashi and SAITOU Naruya

There are two tightly linked loci (D and CE) for the human Rh blood group. Their gene products are membrane proteins having 12 transmembrane domains and form a complex with Rh50 glycoprotein on erythrocytes. We constructed phylogenetic networks of human and nonhuman primate Rh genes, and the network patterns suggested the occurrences of gene conversions. We therefore used a modified site-by-site reconstruction method by using two assumed gene trees and detected 9 or 11 converted regions. After eliminating the effect of gene conversions, we estimated numbers of nonsynonymous and synonymous substitutions for each branch of both trees. Whichever gene tree we selected the branch connecting hominoids and Old World monkeys showed significantly higher nonsynonymous than synonymous substitutions, an indication of positive selection. Many other branches also showed higher nonsynonymous than synonymous substitutions; this suggests that the Rh genes have experienced some kind of positive selection. For details, see ref. 19.

(11) Phylogenetic Analysis of Pseudomonas syringae Pathovars Suggests the Horizontal Gene Transfer of argK and the Evolutionary Stability of hrp Gene Cluster

SAWADA Hiroyuki[1] and SAITOU Naruya ([1]National Institute of Agro-Environmental Sciences)

Pseudomonas syringae are differentiated into approximately 50 pathovars with different plant pathogenicities and host specificities. To understand its pathogenicity differentiation and the evolutionary mechanisms of pathogenicity-related genes, phylogenetic analyses were conducted using 56 strains belonging to 19 pathovars. gyrB and rpoD were adopted as the index genes to determine the course of bacterial genome evolution, and hrpL and hrpS were selected as the representatives of the pathogenicity-related genes located on the genome (chromosome). Based on these data, NJ, MP, and ML phylogenetic trees were constructed and thus 3 trees for each gene and 12 gene trees in total were obtained, all of which showed three distinct monophyletic groups: Groups 1, 2 and 3. The observation that the same set of OTUs constitute each

group in all four genes suggests that these genes had not experienced any intergroup horizontal gene transfer within P. syringae but have been stable on and evolved along with the P. syringae genome. These four index genes were then compared with another pathogenicity-related gene, argK (the phaseolotoxin-resistant ornithine carbamoyltransferase gene, which exists within the argK-tox gene cluster). All 13 strains of pv. phaseolicola and pv. actinidiae used had been confirmed to produce phaseolotoxin and to have argK, whose sequences were completely identical, without a single synonymous substitution among the strains used. On the other hand, argK were not present on the genomes of the other 43 strains used other than pv. actinidiae and pv. phaseolicola. Thus, the productivity of phaseolotoxin and the possession of the argK gene were shown at only two points on the phylogenetic tree: Group 1 (pv. actinidiae) and Group 3 (pv. phaseolicola). At test between these two pathovars for the synonymous distances of argK and the tandemly combined sequence of the four index genes showed a high significance, suggesting that the argK gene (or argK-tox gene cluster) experienced horizontal gene transfer and expanded its distribution over two pathovars after the pathovars had separated, thus showing a base substitution pattern extremely different from that of the noncluster region of the genome. For details, see ref. 20.

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D-c. Division of Theoretical Genetics

(1) Effect of gene conversion on polymorphic patterns at major histocompatibility complex loci

Tomoko OHTA

The pattern of polymorphisms at MHC loci was studies by computer simulations and by DNA sequence analyses, with special reference to synonymous

and non-synonymous divergence among alleles. A model of overdominance plus short-term selection was simulated for a gene family with allelic and non-allelic gene conversion. It was found that the ratio of non-synonymous-to-synonymous divergence among alleles decreases as the non-allelic conversion becomes more frequent. On the other hand, the ratio of synonymous divergence at the antigen recognition site to that at non-coding region increases by non-allelic conversion. By comparing such a pattern with the observed values of mammalian genes, it is suggested that non-allelic conversion is less frequent in human genes than in genes of other mammals. A quantitative analysis on the patchwork pattern was attempted by using identity excess among nucleotide sites of a gene. Comparison of simulation results with DNA data analysis shows that the rate of allelic conversion is high. For details, see Immunological Reviews 167, 319-325.

Publication

1. Effect of gene conversion on polymorphic patterns at major histocompatibility complex loci. Immunological Reviews **167**, 319-325, 1999.

Oral presentations

1. Near neutrality and the molecular clock. Symposium, "Molecular Clock," at the American Society for the Study of Evolution meeting, June, Madison, US.
2. Near neutrality in the interactive systems. Symposium, "Neutralism and Selectionism: the end of a debate?" at the European Society of Evolutionary Biology meeting, August, Barcelona, Spain, and at the seminar of the Uppsala University, September, Uppsala, Sweden.
3. Usefulness of identity coefficient for analysing gene diversity. At the Malecot Symposium, August, Paris, France, and at the seminar of the Lund University, September, Lund, Sweden.

E. DEPARTMENT OF INTEGRATED GENETICS

E-a. Division of Human Genetics

(1) Cloning and structural analysis of the mouse 7F4/F5 imprinted domain

Hiroyuki SASAKI, Reiko KATO[1], Chikako SUDA, Hisao SHIROHZU, Wahyu PURBOWASITO[2], Takaaki YOKOMINE[2], Shin-ichi MIZUNO[2], Tsunehiro MUKAI[3], Masahira HATTORI[1] and Yoshiyuki SAKAKI[1] ([1]Genome Science Center Riken, [2]Inst. of Genetic Information, Kyushu Univ., [3]Dept. of Biochemistry, Saga Medical College)

Genomic imprinting, an epigenetic gene marking phenomenon, causes parental-origin-specific monoallelic expression of a subset of mammalian genes. Imprinted genes tend to form clusters in specific regions of the genome, which may be related to the mechanism of imprinting or the reason for the evolution of imprinting. As a step to understand the structural and functional characteristics of the imprinted genome domains, we are studying an imprinted domain in mouse chromosome band 7F4/F5. This region is syntenic to human 11p15.5, which contains genetic loci responsible for Beckwith-Wiedemann syndrome and childhood and adult tumors. To date, at least eight imprinted genes have been mapped to this mouse region. Therefore, YAC, BAC, and cosmid contigs covering the 1-Mb region were constructed and a physical map of the region was produced (Ref. 1). Large-scale sequencing of selected BAC clones is now ongoing. The obtained sequence information will be used to identify new imprinted genes and their regulatory elements.

(2) Regulation of imprinting of the mouse *Igf2/H19* sub-domain

Hiroyuki SASAKI, Ko ISHIHARA, Reiko KATO[1], Hiroyasu FURUUMI, Mizuki OHNO[2], Naoya HATANO[2] and Tohru IWAKI[3] ([1]Genome Science Center Riken, [2]Inst. of

Genetic Information, Kyushu Univ., [3]Dept. of Neuropathology, Neurological Inst., Faculty of Medicine, Kyushu Univ.)

At the centromeric end of the above imprinted domain, there are two reciprocally imprinted genes, *Igf2* and *H19*: *Igf2* is paternally expressed and *H19* maternally expressed. It was previously shown that the allele-specific expression of these genes occurs through a competition for a set of endoderm-specific enhancers located downstream of *H19*. To look for enhancers responsible for the imprinted expression in other tissues, we sequenced both the mouse and human *H19* regions over 40 kb. A comparison of the sequences from the two species revealed a total of ten evolutionarily conserved non-coding segments, two of which precisely coincided with the known endoderm enhancers. We then tested the function of the remaining eight segments in transgenic mice and found that at least five of them possessed tissue-specific enhancer activity. We also identified a conserved 39-bp element that appeared repeatedly within the upstream differentially methylated region (DMR) and formed complexes with specific nuclear factors. Binding of one of the factors was inhibited when the target sequence contained methylated CpGs. These complexes may contribute to the presumed boundary function of the unmethylated DMR, which is proposed to insulate maternal *Igf2* from the enhancers.

(3) Cloning of a family of novel mammalian methyltransferases

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In the imprinting process, DNA methylation works as an important marking mechanism to distinguish the parental alleles of imprinted genes. We performed a TBLASTN search of the dbEST database using the catalytic domain of the known mammalian DNA(cytosine-5) methyltransferase *Dnmt1*, and identified cDNAs that potentially codes for novel types of methyltransferase. We cloned full-length cDNAs for the mouse proteins and confirmed their methyltransferase activity using an in vitro assay. It will be

interesting to investigate the roles of these novel methyltransferases in genomic imprinting.

(4) Is the chicken genome imprinted?

Hiroyuki SASAKI, Takaaki YOKOMINE[1], Yoichi MATSUDA[2] and Masaoki TSUDUKI[3] ([1]Inst. of Genetic Information, Kyushu Univ., [2]Chromosome Research Unit, Hokkaido Univ., [3]Faculty of Animal Production, Hiroshima Univ.)

Although it is generally thought that genomic imprinting has evolved only in mammals, this has not been tested in other vertebrate species. We have asked whether the homologs of the mammalian imprinted genes are imprinted in chicken. Using DNA plymorphisms identified between various breeds and strains, it was shown that both *IGF2* and *MPR* are expressed biallelically in chicken embryos. We are currently looking at other genes.

(5) Are the polycomb group genes involved in genomic imprinting?

Hiroyuki SASAKI, Naomi TSUJIMOTO and Haruhiko KOSEKI[1] ([1]Chiba Univ. Graduate School)

Polycomb group proteins (PcGs) are the components of chromatin and involved in gene silencing and insulator functions in *Drosophila*. Previous experiments using transgenic flies suggested that mammalian PcGs may be involved in the allele-specific repression of imprinted genes. We have examined the allele-specific expression of *Igf2* and *H19* in mice disrupted with two PcG genes *Mel-18* and *Bmi-1*. In both single homozygotes ([*Mel-18*$^{-/-}$] and [*Bmi-1*$^{-/-}$]) and double homozygotes ([*Mel-18*$^{-/-}$, *Bmi-1*$^{-/-}$]), the proper imprinted expression patterns of both *Igf2* and *H19* were maintained, suggesting that imprinting was not influenced by these PcG genes.

(6) X-inactivation in the mouse embryo deficient for DNA methyltransferase 1 (Dnmt1)

Takashi SADO, Martin H. FENNER[1], Seon-Seng TAN[2], Patric P. L. TAM[3], Toshi SHIODA[1], En LI[4] ([1]Cancer Center., MGH, USA, [2]Melbourne Univ., Aus., [3]CMRI, Aus., [4]CVRC, MGH, USA)

It has been suggested that DNA methylation plays a crucial role in genomic imprinting and X-inactivation. Using DNA methyltransferase 1 (Dnmt1) deficient mouse embryos carrying X-linked *lacZ* transgenes, we studied effects of genomic demethylation on X-inactivation. Based on the expression pattern of *lacZ*, the imprinted X-inactivation in the visceral endoderm, a derivative of the extraembryonic lineage, was found unaffected in *Dnmt1* mutant embryos at the time other imprinted genes showed aberrant expression. Random X-inactivation in the embryonic lineage of *Dnmt1* mutant embryos, however, was unstable as a result of hypomethylation, causing reactivation of, at least, one *lacZ* transgene that had initially been repressed. Our results suggest that maintenance of imprinted X-inactivation in the extraembryonic lineages can tolerate extensive demethylation while normal levels of methylation are required for stable maintenance of X-inactivation in the embryonic lineage.

(7) Analysis of X-inactivation center (Xic) in mouse

Takashi SADO, Asuka MIURA, Hiroyuki SASAKI, En LI[1] ([1]CVRC, MGH, USA)

It has been shown that the region called X-inactivation center (Xic) on an X chromosome is essential for X-inactivation to occur in cis. Available evidence suggests that the *Xist* gene, which is mapped to Xic and exclusively expressed from the inactive X chromosome, is one of components of Xic. In our attempt to isolate a gene(s) involved in X-inactivation other than *Xist* in Xic region, we found antisense transcripts to *Xist*, which cover whole the transcription unit of Xist and apparently do not encode for a protein. To elucidate the function of this novel RNA in X-inactivation, we disrupted the antisense transcripts in ES cells by targeted mutagenesis. Production of the knockout mouse is currently in progress.

(8) Human Genome Resources and Their Application to the Completion of Human Chromosome 21 Sequencing

Asao FUJIYAMA, Akiko ENDO and Ayuko MOTOYAMA[1] ([1]Genome Science Center Riken)

The goal of human genome analysis is not only sequencing entire genome nor cataloging protein coding regions, but to understand functions retained in the human genome and chromosomes. Since most of human chromosomes can be purified by means of dual-laser cell sorting system, such isolated chromosomes are good resources for the studies to understand biological functions retained in individual chromosome. Using purified chromosomes, we have constructed human mono-chromosomal cosmid libraries (except for CM#9 - 12) and #21 fosmid library, and BAC library. Unlike other libraries, our cosmid and fosmid libraries employed random fragmentation/cloning protocol that enabled us to efficiently construct highly randomized libraries. Using these resources, sequencing of human chromosome 21 long arm has been accomplished. A total of 33,546,361 bp of DNA, distributed over four contigs, were sequenced with very high accuracy. The largest of these contigs is 28,515,322 bp long. Only three small clone gaps remain, which together comprise approximately 100 kb. Thus, we achieved a coverage of 99.7% of 21q. In addition, 281,116 bp from the short arm were also sequenced. The chromosome 21-specific libraries were particularly useful for mapping the centromeric and telomeric regions which contain repeats, as well as sequences showing homology with other human chromosomes.

(9) Whole Genome Analysis of Signal-Transduction Pathways in Fission Yeast

Yong-Sik BONG, Inaho DANJO[1], Masahiro INOUYE and Asao FUJIYAMA ([1]Radiation Effect Research Foundation, Hiroshima, Japan)

In fission yeast, *Schizosaccharomyces pombe*, deficiency of *ras1* gene causes abnormal cell shape and abolishes mating ability. However, the signaling pathway in the cell and its target genes are largely unknown because of the lack of appropriate analysis system. To overcome this problem, we categorized genes based on their expression levels in the presence or absence of the *ras1* gene product under different growth conditions. We utilized arrays of clones covering entire genome of the fission yeast. Here, we demonstrate the detection of low molecular weight heat shock protein gene, *hsp16*, and show that it is regulated by a *ras*-mediated signaling pathway, not by the heat shock response in fission yeast.

Publications

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E-b. Division of Brain Function

(1) Mechanisms of Collateral Branching and Target Invasion of Mitral Cell Axons during Development

Tatsumi HIRATA

During development, mitral cell axons, the major efferents of the olfactory bulb, exhibit a protracted "waiting period" in the lateral olfactory tract (LOT) before giving off collateral branches and innervating the target olfactory cortex. To investigate the target invasion mechanism, a series of heterochronic and heterotopic co-cultures of olfactory bulbs with various olfactory cortical strips were conducted. These experiments indicated that development of collateral branches is triggered by environmental cues but not by intrinsic mechanisms in mitral cells. The collateral-inducing cues are apparently different

from the cues directing outgrowth of primary mitral cell axons. Co-culture experiments also indicated that the target olfactory cortex undergoes a developmental change to become accessible to mitral cell fibers. Primary mitral cell axons, however, still preferred the LOT position over such accessible piriform cortex when encountered both the locations. These results suggest that mitral cell projection comprises multiple steps which are controlled by various environmental cues.

Publication

1. HIRATA, T. and FUJISAWA, H.: Environmental control of collateral branching and target invasion of mitral cell axons during development. J. Neurobiol. **38**, 93-104, 1999.

E-c. Division of Applied Genetics

(1) Characterization of β-amylase and its deficiency in various rice cultivars

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β-amylase deficiency in various cultivars of rice was examined. Although β-amylase is a starch-hydrolyzing enzyme, the β-amylase gene is expressed differently from α-amylase gene, i.e. (1) β-amylase is synthesized only in aleurone cells, (2) the enzyme production in the embryo-less half-seeds is not under hormonal control. We identified several cultivars that are deficient for β-amylase activity. We present new evidence that the synthesis is blocked at the level of mRNA synthesis. For details, see Ref. 1.

(2) Differential in situ expression of three ABA-responsive genes of rice, Rab16A, REG2 and OSBZ8, during seed development

Kazumaru MIYOSHI[1], Eijirou NAKATA[1], Yasuo NAGATO[1] and Tsukaho HATTORI[2] ([1]Graduate School of Agricultural and Life Sciences, University of Tokyo, [2]Center for Molecular Biology and Genetics Mie University)

The spatial and temporal expression pattern of three rice abscisic acid (ABA)-responsive genes, Rab16A, REG2 and OSBZ8, which are expressed during seed maturation, were studied by in situ hybridization. Tissue-specificity of these three genes in embryo was much different from each other. The onset of Rab16A and REG2 expression was observed after organogenesis had started. By contrast, the expression of OSBZ8 was already observed in the whole embryo at the globular stage when organogenesis had not been initiated. While the expression of OSBZ8 at the earlier stages was uniform throughout the embryo, a clear tissue specificity was established at the later stage. Interestingly, an exactly opposite relationship in the spatial pattern of expression was observed between OSBZ8 and REG2. For details, see Ref. 2.

(3) Initiation of shoot apical meristem in rice: characterization of four SHOOTLESS genes

Namiko SATOH[1], Soon-Kwang HONG[2], Asuka NISHIMURA[2], Makoto MATSUOKA[2], Hidemi KITANO[3] and Yasuo NAGATO[1] ([1]Graduate School of Agricultural and Life Sciences, University of Tokyo, [2]BioScience Center, Nagoya University, [3]Faculty of Agriculture, Nagoya University)

We characterized nine recessive mutations derived from four independent loci (SHL1-SHL4) causing the deletion of the SAM. Concomitant with the loss of SAM, two embryo-specific organs, coleoptile and epiblast, were lost, but the scutellum was formed normally. Therefore, the differentiation of radicle and scutellum is regulated independently of SAM, but that of coleoptile and epiblast may depend on SAM. An in situ hybridization experiment using a rice homeobox gene, OSH1, as a probe revealed that shl1 and shl2 modified the

expression domain of OSH1, but normal expression of OSH1 was observed in shl3 and shl4 embryos. Accordingly, SHL1 and SHL2 function in the upstream of OSH1, and SHL3 and SHL4 in the downstream or independently of OSH1. These shl mutants are useful for elucidating the genetic program driving SAM initiation. For details, see Ref. 3.

(4) Population Genetic Studies on Nine Aboriginal Ethnic Groups of Taiwan. I. Red Cell Enzyme Systems

Feng JIN[1], Naruya SAITOU[2], Takafumi ISHIDA[3], Cheih-Shan SUN[4], I-Hung PAN[5], Keiichi OMOTO[6] and Satoshi Horai[7] ([1]Chinese Academy of Sciences, Beijing, China, [2]National Institute of Genetics, Mishima, [3]University of Tokyo, Tokyo, [4]Taidong Hospital,Taiwan, [5]National Taiwan University, Taiwan, [6]International Research Center for Japanese Studies, Kyoto, [7]The Graduate University for Advanced Studies, Hayama)

Population genetic study of nine aboriginal ethnic groups of Taiwan (Ami, Atayal, Bunun, Paiwan, Puyuma, Rukai, Saisiat, Tsou, and Yami) was carried out. Twelve red cell enzymes (AcP, AK, CA1, CA2, EsD, GLO, GPT, GOT, LDH, MDH, PGD, and PGM1) were analyzed by isoelectric focusing method and starch gel electrophoretic method. Six loci (AcP, EsD, GLO, GPT, PGD, and PGM1) were polymorphic. Three alleles (*PGM1*6, GPT*6*, and *EsD*7*) were in relatively higher allele frequencies in Taiwan aboriginal populations, and we found homozygotes for those alleles. Phylogenetic relationship based on genetic distances among those ethnic groups more or less fit to their geographical distribution, but not to linguistic classification. For the details, see Ref. 7.

(5) The presence of ancient human T-cell lymphotropic virus type I provirus DNA in an Andean mummy

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The worldwide geographic and ethnic clustering of patients with diseases related to human T-cell lymphotropic virus type I (HTLV-I) may be explained by the natural history of HTLV-I infection. The genetic characteristics of indigenous people in the Andes are similar to those of the Japanese, and HTLV-I is generally detected in both group. To clarify the common origin of HTLV-I in Asia and the Andes, we analyzed HTLV-I provirus DNA from Andean mummies about 1,500 years old. Two of 104 mummy bone marrow specimens yielded a band of human β-globin gene DNA 110 base pairs in length, and one of these two produced bands of HTLV-I-*PX* (open reading frame encoding $p40^x$, $p27^x$) and HTLV-I-LTR (long terminal repeat) gene DNA 159 base pairs and 157 base pairs in length, respectively. The nucleotide sequences of ancient HTLV-I-*PX* and HTLV-I-LTR clones isolated from mummy bone marrow were similar to those in contemporary Andeans and Japanese, although there was micro-heterogeneity in the sequences of some mummy DNA clones. This result provides evidence that HTLV-I was carried with ancient Mongoloids to the Andes before the Colonial era. Analysis of ancient HTLV-I sequences could be a useful tool for studying the history of human retroviral infection as well as human prehistoric migration. For the details, see Ref. 8.

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F. GENETIC STRAINS RESEARCH CENTER

F-a. Mammalian Genetics Laboratory

(1) Phenotype characterization of male sterility of a consomic strain B6.MSM-ChrX and fine mapping of the causative gene(s) in the X chromosome

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Hybrid sterility is the mechanism that safeguards the integrity of species by preventing a free flow of genetic information between related species. The infertility of inter-specific hybrids usually affects the heterogametic sex, which in the case of mammals is the male. In the mouse, male infertility has been found in hybrids between *Mus spretus* and laboratory mouse strains (*Mus musculus*). Understanding hybrid sterility might give us an insight into not only how a new species evolves, which is one of the fundamental questions about the evolution, but also how terminal differentiation of germline cells is operated.

Since several years ago, we have set out to construct a series of consomic strains that substitute each chromosomes of Japanese wild mouse-derived MSM strain (*Mus musculus molossinus)* for the chromosomes of the standard laboratory strain C57BL/6J (*Mus musculus domesticus*). The consomic strains would be of great use to study all kind of multigenic traits. During the process of production of X-chromosome consomic strain, in which the X chromosome of C57BL/6J (B6) was replaced by the MSM-derived one, we noticed that males of the strain have lowered fertility. It seemed attributable to the disturbance of coordination between the X chromosome of MSM (X-MSM) and the autosome background gene(s) from B6 strain, which has separately evolved over a long period, approximately one million years.

Previously the severe reproductive disadvantage and morphological anomaly in spermatozoa of B6 strain that carries the X chromosome of MSM strain has been reported (Takagi et al., 1994). The decline in the fertility was emphasized as backcross generations proceeded. Unlike $X^{-}_{MSM}Y$males, $X^{-}_{B6}Y$ males in the same backcross generation were fully fertile. Thus, the X chromosome from MSM is responsible for the reduced fertility of the consomic males. We inferred that certain autosomal or Y chromosomal gene(s) of B6 could not coordinate with a gene(s) on X chromosome of MSM strain.

In this study, we first examined whether the X-chromosome consomic strain shows male sterility that was previously reported. We observed that most epididymal spermatozoa from B6 and $X^{-}_{B6}Y$ males had a well-balanced, hook-like head. The distal part of the hook tended to be slightly short and less curved in $X^{-}_{MSM}Y$ males of N2 (BC1) generation. Malformation of the distal part of the sperm head was accelerated in $X^{-}_{MSM}Y$ males of N3 (BC2) and N4 (BC3). At the N3 or N4 generation, certain sprmatozoa from $X^{-}_{MSM}Y$ males did not have the hook-like heads, instead almost the entire distal part were lost, and in extreme cases they had small and round heads. In order to evaluate the fertility of the consomic males, in vitro fertilization (IVF) and IVF after partial zona pellucida dissection (PZD) were performed with capacitated sperms of $X^{-}_{MSM}Y$ and $X^{-}_{B6}Y$ males, and the number of progeny was counted in natural mating with B6 females for 4-8 months. Significant low fertility of IVF was observed in $X^{-}_{MSM}Y$ males (5.9%; N=6), though the $X^{-}_{B6}Y$ males showed high frequency (79%; N=6). In the IVF-PZD, fertility of the $X^{-}_{MSM}Y$ males was recovered slightly, but it remained to be still lowered (32.9%; N=4). In addition, significantly small testis were observed in the $X^{-}_{MSM}Y$ males, which were histologically normal (76.4 ± 13.5mg; $p<0.05$). In the natural mating, the number of live birth and offspring of the $X^{-}_{MSM}Y$ males were smaller than those of the $X^{-}_{B6}Y$ males. The defect of the sperms of the consomic males seemed to be much severer in the IVF experiment than that in the natural mating. In the TYH medium for IVF, most of the capacitated spermatozoa of the $X^{-}_{MSM}Y$ males was no longer active after 2 hours, whereas sermatozoa from the $X^{-}_{B6}Y$ males kept on swimming for several hours. An environment of the female reproductive tract is different from that of *in vitro*, which is possibly much milder.

We intended to map gene(s) responsible for this morphological anomalies. QTL(quantitative trait loci) analysis with 120 recombinants on the X Chromosome, whose autosomes were mostly replaced by ones from B6, demonstrated a tight linkage of X chromosomal loci and the anomaly in spermatozoa. The most intense linkage with *DXMit166* and the weaker linkage with an interval between *DXMit97* and *DXMit217* were shown. It is of interest to note that the locus that controls the testis weight was linked with an interval between *DXMit217-DXMit160*, which was possibly separated from the loci that control sperm morphology.

(2) Phenotype characterization and fine mapping of a preaxial polydactylous mouse mutantion, X-linked polydactyly (*Xpl*)

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The anteroposterior axis patterning on vertebral limb morphogenesis is controlled by the formation of the zone of polarizing activity (ZPA), which is located at the posterior margin of limb buds. It is established that the Sonic hedgehog (*Shh*) gene that is expressed at the posterior margin of limb bud and encodes a secreted signaling molecule mediates the ZPA activity. However, it is poorly understood how the polarized expression of *Shh* in developing limb buds is regulated.

X-linked polydactyly (*Xpl*) is a spontaneous mouse mutation that exhibits preaxial polydactyly only on the hindlimb and was mapped to X chromosome. *in situ* hybridization with the *Xpl* embryos revealed ectopic expression of *Shh*, *Fgf4*, *Gre* and *Hoxd11* genes at the anterior margin of the hindlimb bud. Such ectopic ZPA activity may results in the preaxial polydactyly of *Xpl* mutants.

To isolate the *Xpl* gene, we have carried out a linkage analysis using 1,262 backcross progeny generated from cross between B6-*Xpl*/+ and Japanese wild mouse-derived MSM strain. As a result, *Xpl* gene was mapped to a 0.48cM region between microsatellite markers, *DXMgc39* and *DXMit5*. In the human syntenic region, chromosome Xp22, a congenital face and limb deformity, Oral-facial-digi-

tal syndrome type1 (OFD1: OMIM311200), has been mapped. Therefore, *Xpl* is likely to be a mouse model for OFD1. Now we are constracting YAC and BAC contigs covering the causative gene for *Xpl*.

(3) Developmental genetics of a mouse mutant, mesenchymal dysplasia (*mes*)

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mesenchymal dysplasia (*mes*) is a recessive mouse mutant that exhibits abnormality of mesenchyme-derived tissues, including preaxial polydactyly, shortened face, bifurcate sternum and shortened kinky tail. To determine the chromosomal location of *mes*, we carried out a linkage analysis. As a result, *mes* was mapped to a region between *D13Mit318* and *D13Mit187*, where *patched* (*ptc*) has been mapped. To examine a possibility that *ptc* is a candidate for *mes*, we investigated the nucleotide sequence of *ptc* in the *mes* mutation. In this study, we identified a 32 bp-deletion in the C-terminal cytoplasmic domain of *ptc*. Ptc is a transmembrane receptor protein for Shh that is expressed in the organizing centers in developing mouse embryo, such as notochord, floor plate, ZPA of limb buds and lung, and it mediates a key signaling for cell growth and differentiation in developing embryos. Ptc functionally antagonizes the Shh activity. Subsequently, we performed allelism test of *mes*, using a *ptc* knockout mutant (*ptc*$^{-}$) (Goodrich et al., 1996). As a consequence, the compound heterozygotes (*ptc*$^{-/+}$,*mes*$^{-/+}$) exhibited severe polydactyly, though neither *ptc*$^{-/+}$ nor *mes*$^{-/+}$ mice exhibited such visible phenotype. This result showed that *mes* is hypomorphic allele for *ptc*, and that the cytoplasmic domain plays an indispensable role in antagonistic activity of Ptc for Shh signaling.

ptc$^{-/mes}$ mice died soon after birth because of inability of breathing. Histological analysis demonstrated that lung mesenchyme overgrew after about 17.5 dpc. With the observation of increased body weight in *ptc*$^{-/mes}$, the results suggested that Ptc has a function of negative regulation in mesenchymal cell growth.

In addition, whole mount *in situ* hybridization at 11.5 dpc showed that *shh* and *fgf4* were ectopically expressed at the anterior margin of the limb buds. This result suggested that ectopic expression of *shh* was induced by subtle activation of Shh signaling evoked by weakened Ptc activity.

(4) Positional cloning of the mouse skeletal mutation, Tail-short (*Ts*)

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A mouse mutation Tail-short (*Ts*) exhibits shortened kinky tail and numerous skeletal abnormalities including homeotic anteroposterior patterning problem along the axial skeleton. *Ts* gene was mapped to the teromeric region of the chromosome 11. To elucidate the function of the *Ts* gene in mouse embryogenesis, we intended to clone the gene by means of positional cloning. First, we employed a fine mapping of this gene based on a large scale inter-subspecific backcross between the mutant stock TsJ/Le-*Ts*/+ and Japanese wild mouse-derived MSM strains. *Ts* gene was mapped to a 0.16cM region between two microsatellite markers, *D11Mit128* and *D11Mit256*. We screened mouse YAC and BAC libraries with microsatellite markers tightly linked to the *Ts* locus and have obtained YAC and BAC clones. Further chromosome walking with the isolated clones allowed us to construct a complete BAC contig covering the *Ts* causative gene, and the critical region of the *Ts* was narrowed down to an interval between two new STSs, *D11Rin56* and *D11Nig17*. This contig consists of 3 BAC clones, spanning a 250kb DNA fragment.

We have isolated several cDNA clones as *Ts* candidate genes from the critical region by directed cDNA selection using the corresponding BAC and mouse embryonic cDNA library. Among these candidate genes, expression level of *Rpl38* gene, which encodes a subunit of the large component of ribosomal pro-

tein complex, was reduced in *Ts* mutant mouse. Analysis of the genomic structure of *Rpl38* in *Ts* mutant revealed that it has an 18kb long deletion containing *Rpl38*. Other two mutations, Rabo torcido (*Rbt*) and Tail-short shionogi (*Tss*), were reported to have phenotype similar to *Ts* and to be mapped to the same region in the chromosome 11. We found that *Rbt* and *Tss* have a frame shift mutation and an insertional mutation in *Rpl38*, respectively. In order to confirm that *Rpl38* is the causative gene for *Ts*, we tried to rescue the lethality of *Ts* mutant by transgenesis with the wild-type gene of *Rpl38*. Over expression both of the BAC containing *Rpl38* and the *Rpl38* cDNA appeared to restore the viability of the *Ts* mutant in a certain genetic background and completely rescued the morphological phenotype. Thus, it appeared that the lethality and the morphological anomalies of *Ts* mutants, including their homeotic transformation, are caused by the *Rpl38* mutation.

(5) Positional cloning of the preaxial polydactyly mutation, hemimelic extra toes (*Hx*)

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The zone of polarizing activity (ZPA) located at the posterior limb margin controls patterning of the vertebrate limb along the anterior-posterior axis. One of the hedgehog family member, Shh has been shown to be able to mediate the function of the ZPA. Several preaxial polydactyly mutations of mouse, exhibit ectopic expression of *shh* at the anterior limb margin. One of the mutations, hemimelic extra-toes (*Hx*) have been mapped to the region very closely linked to *shh* locus. In the human syntenic region of Chr7q36, a complex poly-syndactyly mutation has been mapped. The data of our physical mapping using YAC and BAC clones localized *Hx* to a telomelic region approximately 1Mb away from the *shh* locus. By genetic mapping based on several intra-subspecies crosses and use of newly isolated STS markers, we were able to narrow down the *Hx* critical region to a single BAC clone. To identify the transcripts in the approximately 140kb of the BAC, we carried out cDNA selection with mouse

embryonic cDNA library. We obtained many cDNA clones. Recently, another group has identified eleven transcripts in the human syntenic region 7q36. From a search of sequence homology, we found the two mouse homologs of the transcripts in compiling several cDNA fragments. Because one homolog was located in the *Hx* critical region, we anticipated that it should be a good candidate of *Hx*. We cloned the full-length cDNA of this transcript, and compared the coding sequence of *Hx* mutant with the wild type sequence. We found no difference in the two sequences. It is likely that there is limb specific isoforms through alternative splicing of the same gene, and there are unknown genes in the *Hx* critical region. Now, we are employing genomic sequencing of the whole BAC clone .

(6) Gasdermin (*Gsdm*) localizing to mouse chromosome 11 is predominantly expressed in upper gastrointestinal tract but significantly suppressed in human gastric cancer cells

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Amplification of proto-oncogenes associated with their over-expression is one of the critical carcinogenic events identified in human cancer cells. In many cases of human gastric cancer, a proto-oncogene *ERBB-2* is co-amplified with *CAB1* genes physically linked to *ERBB-2*, and both genes are over-expressed. The amplified region containing *ERBB-2* and *CAB1* was named 17q12 amplicon from its chromosomal location. The syntenic region corresponding to the 17q12 amplicon is well conserved in mouse. We isolated and characterized a novel mouse gene that locates telomeric to the mouse syntenic region. Northern blot analysis using the mouse cDNA and a cloned partial cDNA of human homologue disclosed unique expression pattern of the genes. They are expressed predominantly in the gastrointestinal (GI) tract

and in the skin at a lower level. Moreover, in the GI tract, the expression is highly restricted to the esophagus and stomach. Thus, we named the mouse gene Gasdermin (*Gsdm*). Interestingly, in spite of its expression in normal stomach, no transcript was detected by Northern blot analysis in human gastric cancer cells. These data suggest that the loss of the expression of the human homologue is required for the carcinogenesis of gastric tissue and that the gene has an activity adverse to malignant transformation of cells. The function of *Gsdm* is unknown. To investigate the function of this gene that posses unique feature, we are preparing a knock-out mouse mutant with disrupted *Gsdm* gene.

(7) Behavioral study of inbred strains established from wild mice. III. Active avoidance test

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Many aspects of mouse behavior have been studied using only a relatively small sample of laboratory strains. An inherent problem in analyzing mouse behavior is that genetic diversity is limited among currently available strains. In this respect, the use of strains that are derived from a variety of wild mice should provide a means to identify novel behavioral phenotypes. We have been investigating several behavioral phenotypes using females of a number of mouse strains, BFM/2, NJL, BLG2, HMI, CAST/Ei, KJR, SWN and MSM, which were derived from wild mice of different subspecies. A fancy mice derived strain JF1 as well as two standard laboratory strains, C57BL/6 and DBA/1, have been also used in our study.

In the previous report, we have shown that the two strains, CAST/Ei and BLG2, are poor learners, while five strains, BFM/2, KJR, SWN, MSM and C57BL/6 are good learners in the passive avoidance test. In order to investigate the ability for learning and memory in different ways, we conducted an active avoidance test. The test (fear-motivated learning test) was performed in a shuttle avoidance apparatus (O'hara & Co. Ltd., Tokyo, Japan) made of polyvinyl chloride (300 x 90 x 150 mm). The conditioned stimulus (CS) consisted of a signal

light (30 V, 0.1 A, 3 W) placed on the lid and sound (600 Hz, 60 dB) for 5 seconds, preceding the unconditioned stimulus (US; continuous electric stimulation, 30V constant; 0.05 mA average, through the grid floor) for 3 seconds. For the US, mild electric stimulation was carefully chosen to minimize the occurrence of freezing. Both CS and US were terminated when the animal ran to the other side of the apparatus. The mice were tested individually. Each animal received 60 trials per day for 4 consecutive days, a total of 240 trials with an inter-trial interval of 22 sec. The JF1 strain was not included in this analysis due to a hearing defect associated with a coat color mutation. Significant differences were noted in the learning curves between the strains. The two poor performers, NJL and MSM strains, exhibited rearing or jumping (R-type response), while the other strains showed an increase in horizontal locomotion (L-type response) following the electric shock in the active avoidance test. Therefore, it is possible that the poor learning ability shown by the NJL and MSM strains in this test would be improved if the test is modified such that the R-type response effectively terminates the CS signal and electric shock. The wild mouse derived BLG2 strain also performed relatively poorly in this test, but did not show an R-type response. Instead, increased L-type activity was observed. The data obtained from two tests for passive and active avoidance behavior suggesting that BLG2 strain is impaired for the ability of learning and memory, contrasting to two other strains, KJR and SWN, which are good performers in both tests.

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F-b. Plant Genetics Laboratory

(1) Analysis of genetic mechanisms controlling embryogenesis and regeneration of rice (*Oryza sativa*)

(1)-a. Regulation of expression of *KN1*-type homeobox genes in rice

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To elucidate genetic programmes that control embryogenesis and regeneration of rice, we conducted cloning, structural and functional analyses of genes which encode transcription factors and protein kinases. We previously identified eight *KN1*-type homeobox genes. Previous studies of our and others' indicated that *KN1*-type homeobox genes are specifically expressed in a shoot meristem and function in formation or maintenance of the shoot meristem. To understand a shoot meristem specific expression of *OSH1*, a *KN1*-type homeobox gene of rice, GUS or GFP reporter genes driven by the *OSH1* promoter were introduced into rice. In the transgenic rice GUS or GFP activities were detected in the leaves in addition to the shoot meristem. Thus, the *OSH1* promoter region alone does not confer the endogeneous expression of *OSH1* and other regions are necessary for the precise expression. To gain insights into the function of *OSH1*, the *OSH1* cDNA driven by the CaMV 35S promoter in the antisense orientation was introduced into rice. Transgenic rice showed an abnormal leaf phenotype similar to those in which *OSH1* was

overexpressed. RT-PCR analysis indicated that the *OSH1* sense mRNA was expressed in such leaves. The expression of *OSH1* in the leaves does not depend on the 35S promoter and deletion of the 5' half of the cDNA lost such effects. These results suggest that exons of *OSH1* contain certain activity on the *OSH1* expression in the leaves. Our current hypothesis is that the *OSH1* promoter region is active both in a shoot meristem and in leaves, and the coding region has a repressor activity in leaves. By these two activities the expression of *OSH1* is restricted in the shoot meristem and the introduction of the extra copy of the coding sequence somehow interfares its repressor activity.

(1)-b. Isolation of a mutant for a *KN1*-like homeobox gene in rice

Yukihiro ITO, Hirohiko HIROCHIKA[1], Nori KURATA ([1]National Institute of Agrobiological Resources, MAFF)

The most effective method to isolate a mutant of a gene with known sequence in rice is to screen insertional lines of *Tos17* retrotransposon by PCR with primers specific for *Tos17* and for the target gene. We identified a line in which *Tos17* was inserted in a 3' untranslated region of *HOS59*, a *KN1*-type class 2 homeobox gene. Characterization of this line will be carried out.

(1)-c. Generation and screenig of retrotransposon insertion lines in rice

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To obtain a large number of mutations affecting the steps of embryogenesis, we also used transposon-induced mutagenesis system of rice using retrotransposon *Tos17*. To isolate valuable genes taking roles for early embryogenesis, we screened for segregation of embryo defect mutation with each 20 M2 seeds of about 2800 *Tos17* lines. We obtained about 100 candidate lines defective in early embryogenesis. Twenty individuals from each 100 embryo defect lines were grown. DNAs of all individuals were extracted and Southern hybridization using *Tos17* probe was conducted. However, no link

between *Tbs17* insertion and embryo defectiveness was seen in about 30lines screened so far. Further investigation for screening insertional mutations has been continuing.

(1)-d. Isolation and structural analysis of *OSLEC1* genes of functional importance in embryogenesis

Kazumaru MIYOSHI, Nori KURATA

LEC1 (LEAFY COTYLEDON1) gene is thought to be a central regulator of seed development in *Arabidopsis thaliana*. *LEC1* encodes HAP3 subunit protein, a component of transcriptional factor, CBF (CCAAT-binding factor), which is ubiquitously present in many eukaryotes. Most striking feature of this gene is that the overexpression of *LEC1* protein causes ectopic embryogenesis on leaves of transgenic plant. This is the first case of ectopic induction of plant embryogenesis by gene manipulation. It must be significant to analyse the signaling pathway before and after the *LEC1* homologous gene expression during embryogenesis of rice. We have isolated several rice cDNA clones showing high similarity to the *LEC1* gene from a 3DAP seed cDNA library. Sequencing analysis revealed that at least three different *LEC1* homologues (*OSLEC1a, OSLEC1b, LEC1*-EST) are expressed during early seed development in rice. The temporal and spatial expression pattern analysed by *in situ* hybridization revealed that *OSLEC1a* transcripts are detectable in developing globular embryo as early as at 3 days after pollination, in which no organ differentiation was observed. *OSLEC1a* expression was also detected in the regenerating calli at 1 day after transplantation, whereas the morhological changes of regenerated cells were observed at 4-5 days after transplantation. The early onset of the gene expression in both zygotic embryogenesis and somatic regeneration process might suggest that *OSLEC1a* plays important role in early rice embryogenesis like as *Arabidopsis LEC1* gene. Overexpression experiment and search for *OSLEC1* knock-out plants would unravel the real function of these genes.

(1)-e. Isolation and structural analysis of genes for receptor-like kinases in rice

Kazuhiko TAKAYA, Yukihiro ITO, Nori KURATA

In plant development, positional information and communication with neighbouring cells are assumed to be important. Receptor-like protein kinases are suitable candidates to transduce such signals and mutations of some of these genes are reported to cause developmental defects. We cloned a cDNA for a receptor-like kinase gene from a 3DAP (3 days after pollination) embryo cDNA library using a carrot *SOMATIC EMBRYOGENESIS RECEPTOR KINASE (SERK)* gene as a probe. Southern blot of rice genomic DNA probed with the cloned cDNA showed several bands and three independent genomic sequences that hybridize with the cDNA were obtained from a rice genomic DNA library. These results suggested that several *SERK*-like genes exsist in a rice genome. We analyse functions of these genes with a reverse genetic technique.

(2) Positional cloning of a heterochronic gene, Pla1, regulating the plastochron and the duration of vegetative phase in rice

Byoung-Ohg AHN, Kazumaru MIYOSHI, Jun-Ichi ITOH[1], Yasuo NAGATO[1] and Nori KURATA([1]Graduate School of Agricultural and Life Science, Univ. Tokyo)

Heterochronic mutations affecting the timing of developmental events may be of major significance in ontogeny and evolution. In plants, several heterochronic mutations that affect step wise development of vegetative tissue and therefore alter shoot architecture have been identified. The recessive mutations at the rice *Plastochron 1 (Pla1)* locus cause the short plastochron and ectopic expression of vegetative programs in the reproductive phase. To understand the molecular aspects of *Pla1* during plant development, we have started to isolate the *Pla1* gene by map-basedcloning.

To generate mapping population, the indica variety Kasalath was crossed by the japonica variety Fukei 71 carrying *pla1-1* mutation. Since the *pla1-1* homozygous plants are sterile, the heterozygous plants were used in this crossing. Among 579 F_2 segregants, *pla1-1* homozygous plants, which have narrower leaves and a shorter plastochron than wild type siblings, were selected.

Preliminary studies for rough mapping using 30 *Pla1* homozygotes and 24 RFLP markers located on all 24 chromosomes arms indicated that the *Pla1* locus was relatively close to both marker R2174 and R1629 on the short arm of chromosome 10. And, By using the 117 pooled sampling method which each leaf of five individuals is one pool to save cost and laborious, it showed that *Pla1* locus is in between the marker C961 and R1738A on chromosome 10.

To construct a high-resolution genetic map of *Pla1* locus, we isolated genomic DNA of 279 plants occurred recombinant on two marker above and linkage analysis was conducted. These results indicated that *Pla1* is closely linked between the marker C961(0.9cM) and R1738A(3.0cM) on centromere region of chromosome 10.

(3) Study for the Chromosome and Nucleus Organization in Rice

(3)-a. Structural analyses for the centromeric region of rice chromosome 5 and construction of rice artificial chromosomes

Ken-Ichi NONOMURA, Nori KURATA

One of profitable features of plant cells for biotechnology is to induce the reproductive adults easier than animal cells. Fusing this feature with molecular biotechnology such as construction of stable plant artificial chromosomes will introduce us to tremendous progression of chromosome technology. In this study, we analyzed the structure of the centromeric region of rice chromosome 5, ultimate goal of the analysis is to construct the cereal artificial chromosome.

We selected Y6514 (220kb), one of rice yeast artificial chromosome (YAC) clones derived from the centromere region of rice chromosome 5 (Saji *et al.*1995). This clone includes two kinds of centromeric repeats RCS2 and RCE1, which was 160bp-short tandem repeats and 1.9-kb dispersed repeats localizing on all centromeres, respectively. The contiguous clones including Y6514 was mapped just in the centromere region by the analysis of secondary trisomics (Harushima *et al.* 1998). Furthermore, the crossing over events within the region was severely reduced. These facts indicate that the contig region would be a functional center for the chromosome 5 centromere.

The contig was composed of at least four YAC clones including Y6514 and occupied about 860kb length in the minimum path. It was also revealed that RCS2 repeats formed two tandem arryed clusters within 100 kb length and at least nine copies of RCE1 were scattered within 600 kb in this path. Many copies similar to long terminal repeat (LTR) of RIRE3, which was Ty3/gypsy type retrotransposon of rice, were found out to be distributed in this contig. Recent research revealed that a part of RCE1 repeat unit was identical with LTR sequence of RIRE4, another retroposon of rice.

Structural and functional importance of the detected centromere repetitive sequences is waiting to be resolved in the analysis of centromere organization through the processes of construction, intruduciton and maintenance of rice artificial chromosomes in rice. Retrofitting the vector sequences of the centromeric YAC clones to those with rice reporter genes and transferring into rice cells are now in progress.

(3)-b. Analysis of chromosome dynamics in the nucleus by using GFP-tags

Nori KURATA

Focusing to the chromosome movement in the nucleus of critical stages of development would show some rationales for controlling nuclear organization. To analyse such chromosome movements, I constracted and introduced a GFP-Lac repressor fusion gene (GFP-LR) into 17 rice plants and a Lac operator repeats (LOR) into another 6 rices. After self-pollination of the transgenic heterozygotes, GFP-LR and LOR homozygous lines were crossed each others to generate GFP-tagged chromosomes. IF this system will work well in the rice cells, we could analyse GFP-tagged chromosome movements under the circumstances of specific differentiation stages and under mutants phenotypes, aneuploids and genomic hybrids conditions. Then we could expect to extract principals necessary for establishing nucleus organization and chromosome dynamics.

(4) Analysis of Genetic Factors causing Segregation Distortion

Yoshiaki HARUSHIMA, Nori KURATA

Genetic mechanisms for isolation of "species" are called as reproductive barriers and these include hybrid incompatibility, hybrid inviability, hybrid sterility, hybrid breakdown, etc. These reproductive barriers cause segregation distortion of linked marker genotypes in the population derived from hybrid of inter- or intra- species cross. In the last year, we developped a new method to conduct a genome-wide survey of reproductive barriers by a multi-response non-liner regression analysis. This method attempts to explain segregation distortions of a linkage map by adopting mathematical models and to estimate the number, position and strength of the reproductive barriers on each chromosome. We succeed to explain the segregation distortions on all chromosomes in F_2 population derived from a cross between a japonica cultivar, Nipponbare, and an indica cultivar, Kasalath by 34 reproductive barriers (in preparation for publication).

In this year, we have been constructing linkage maps using reciprocal backcross populations between Nipponbare and F_1 of Nipponbare and Kasalath to confirm whether the detected each reproductive barrier affects male gametophyte, female gametophyte or zygote. The most pronounced segregation distortion was observed at C582 on chromosome 3 in the F_2 population and we have aimed to isolate the causal reproductive barrier by positional cloning. However, segregation distortion of C582 was not observed either backcross populations. This situation of the C582 segregation in the F_2 and the backcross populations can be explained by the interaction between the male gametophyte reproductive barrier and genotype of mother plant. This explanation was confirmed by the progeny test of the back crossed populations. The C582 segregation in self pollinated progenies from the back crossed plants those were heterozygote at C582 were segregated into not-distorted and distorted depending on the genotype of the interactive mother locus. The interactive locus in mother plant was located on the long arm of chromosome 6.

To isolate the above male gametophyte gene on chromosome 3, we have

selected 61 plants with recombination that occurs heterozygote to homozygote in 1.9 cM regions near the gene, from 473 backcross plants and 1000 F_2 plants and then obtained their self pollinated progenies for detailed mapping the gene.

(5) Generation of enhancer trap lines of rice

Yukihiro ITO, Mitsugu EIGUCHI, Nori KURATA

To isolate valuable mutants defective in various steps of rice development and to clone the corresponding genes, we are generating enhancer trap lines of rice. We employ an enhancer trap system used in *Arabidopsis* with some modifications. This system is based on the *Agrobacterium*-mediated transformation using *Ac/Ds* transposable elements of maize. The enhancer trap construct contains, in the T-DNA region, the *Ds* element that harbours a GUS coding region with a P35S minimal promoter and a hygromycin resistance gene. We also use a P35S-Ac transposes (AcTPase) gene together with a bialaphos resistance gene to supply transposase which is essential and sufficient for transposition of the *Ds*. We generated more than two hundreds transgenic rice with *Ds* (*Ds* line) and six with P35S-Ac (AcTPase line). The four *Ds* lines were clossed with each of the six AcTPase lines and somatic excision of the *Ds* in leaves of the F_1 plants were examined. The excision of the *Ds* was detected in most of the combinations but in one *Ds* line the excision was not detected in any combinations with the AcTPase lines and one AcTPase line could not excise the *Ds* in any combinations with the *Ds* lines. This result indicates that this enhancer trap system is functional in rice. Screening of germinal transposants is now in progress.

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F-c. Microbial Genetics Laboratory

(1) The *kdsA* mutations affect the FtsZ-ring formation in *Escherichia coli* K12

Kana NISHIMORI[1], Hideyuki TAKAGI, Masaaki WACHI[1], Hiroshi FUJISHIMA, Takeshi KAWABATA, Ken NISHIKAWA, Akiko NISHIMURA ([1]Tokyo Institute of Technology)

The outer membrane of Gram negative bacteria, such as *Escherichia coli,* is integral in controlling the interaction of the bacteria with the external environment, serving as a barrier, controlling the absorption of ions and nutrients, and transmitting signals to the inside of cell. Instabilities of the outer membrane adversely affects multiple cellular responses. Proper cell division requires both the construction of division machinery, physically separating internal cellular components, and the division of cell membrane structures. Both processes are closely related; instabilities in membranes may affect both the construction of division machinery and the expression of gene(s) essential for cell division. The mechanism, however, controlling the relationship between cell division and the nature of the cell membrane remains unknown. No one has, as yet, addressed the relationship between the nature of the outer membrane and cell division. We have created seven unique *fts/kdsA* mutants allowing analysis of the discrimination mechanism governing membrane con-

struction and cell division.

The outer membrane of Gram negative bacteria is comprised principally of lipopolysaccharide (LPS) and phospholipids. LPS, a major component of the outer membrane, comprises approximately 30% of the outer membrane by gross weight. LPS, comprised of hydrophorbic Lipid A, the hydrophilic core oligosaccharide chain and 3-deoxy-D-manno-octulosonic acid (KDO), connecting Lipid A to the oligosaccharide chain, is important in determining outer membrane barrier function.

KDO is synthesized by the condensation of D-arabinose-5-phosphate and phosphoenol pyruvate, followed dephosphorylation. The first step of the reaction is catalyzed by KDO 8-phosphate synthetase, encoded by the *kdsA* gene in both *Salmonella typhimurium* and *E. coli*. *E. coli kdsA*, however, was first identified as a gene complementing the *kdsA* mutation of *Salmonella*, although no similar mutants have yet been identified in *E. coli*. Mutations in *kdsA* of *Salmonella typhimurium* causes the accumulation of Lipid A in the periplasm, also resulting in the disappearance of LPS from the outer membrane. LPS is an essential membrane component; only conditional lethal mutants in KDO biosynthesis can be isolated in. The biosynthesis of LPS is growth phase regulated at the transcriptional level in *E. coli*; no evidences, however, has uncovered a relationship between LPS biosynthesis and cell division. In this study, we isolated temperature sensitive mutants of *kdsA*, to determine that membrane instability resulting from the defect in KDO biosynthesis affected the FtsZ-ring formation.

Seven temperature sensitive mutants, *fts/kdsA*, contained mutations mapped at approximately 27 min in the *E. coli* genetic map. These mutants stopped cell division at 41°C, producing filamentous cells lacking FtsZ-rings. The mutations were co-transduced with *trp* into the wild type strain, MG1655, at an efficiency of 13%. All seven mutants contained missence mutations in *kdsA*, to be complemented only by the plasmid-born, wild type *kdsA*. The mutations affected the production of KDO; the defect was reversed by the plasmid-born, wild type *kdsA*. Western blotting analysis, however, demonstrated that the amount of FtsZ protein was not affected by the mutation. Therefore, we concluded that the instability of the outer membrane affects the FtsZ-ring formation. The FtsZ-ring, however, is constructed onto the inner membrane,

maintaining a position at the leading edge of invaginating septum; the protein(s) governing this cell division at the outer membrane have not been found as yet. As E. coli can divide without outer membrane, it is not likely the structure of outer membrane would affect the construction of FtsZ-rings. The mechanism whereby the instability of outer membrane results in the loss of FtsZ-ring formation, therefore, remains to be established.

While the mutants also displayed sensitivity to hydrophobic materials, including Novobiosine, Eosine Y and SDS, at 36°C, Methylene blue, however, restored the colony forming ability of the mutants, indicating that the instability of the outer membrane creates the altered cellular responces to their environment. The expression of a subset of genes may overcome the sensitivity to the hydrophobic materials. The kdsA mutation may also affect the transcription of a cell division gene(s), required for FtsZ-ring formation. Membrane instability is expected to affect the transcription of genes, including those involved in cell division. Because decreases in lipopolysaccharide bare phospholipids to the external environment, and phospholipids are important in the transfer of signals from the extracellular environment to the transcription machinary.

Publication

None

F-d. Invertebrate Genetics Laboratory

(1) Proximal to distal cell communication in the *Drosophila* leg provides a basis for an intercalary mechanism of limb patterning

Satoshi GOTO and Shigeo HAYASHI

Proximodistal patterning in the *Drosophila* leg is elaborated from the circular arrangement of the proximal domain expressing *escargot* and *homothorax*, and the distal domain expressing *Distal-less* that are allocated during embryogenesis. The distal domain differentiates multiply segmented distal appendages by activating additional genes such as *dachshund*. Secreted sig-

naling molecules Wingless and Decapentaplegic, expressed along the anterior-posterior compartment boundary, are required for activation of *Distal-less* and *dachshund* and repression of *homothorax* in the distal domain. However, whether Wingless and Decapentaplegic are sufficient for the circular pattern of gene expression is not known. Here we show that a proximal gene *escargot* and its activator *homothorax* regulate proximodistal patterning in the distal domain. Clones of cells expressing *escargot* or *homothorax* placed in the distal domain induce intercalary expression of *dachshund* in surrounding cells and reorient planar cell polarity of those cells. *Escargot* and *homothorax*-expressing cells also sort out from other in the distal domain. We suggest that inductive cell communication between the proximodistal domains, which is maintained in part by a cell-sorting mechanism, is the cellular basis for an intercalary mechanism of the proximodistal axis patterning of the limb. For detail, see ref 2.

(2) Interplay of Notch and FGF signaling restricts cell fate and MAPK activation in the *Drosophila* trachea

Tomoatsu IKEYA and Shigeo HAYASHI

The patterned branching in the *Drosophila* tracheal system is triggered by the FGF-like ligand Branchless that activates a receptor tyrosine kinase Breathless and the MAP kinase pathway. A single fusion cell at the tip of each fusion branch expresses the zinc-finger gene *escargot*, leads branch migration in a stereotypical pattern and contacts with another fusion cell to mediate fusion of the branches. A high level of MAP kinase activation is also limited to the tip of the branches. Restriction of such cell specialization events to the tip is essential for tracheal tubulogenesis. Here we show that Notch signaling plays crucial roles in the singling out process of the fusion cell. We found that Notch is activated in tracheal cells by Branchless signaling through stimulation of Delta expression at the tip of tracheal branches and that activated Notch represses the fate of the fusion cell. In addition, Notch is required to restrict activation of MAP kinase to the tip of the branches, in part through the negative regulation of Branchless expression. Notch-mediated lateral in-

hibition in sending and receiving cells is thus essential to restrict the inductive influence of Branchless on the tracheal tubulogenesis. For detail, see ref 3.

(3) Repression of the wing vein development in *Drosophila* by the nuclear matrix protein Plexus

Hitoshi MATAKATSU, Ryosuke TADOKORO, Sumiko GAMO[1] and Shigeo HAYASHI ([1]Osaka Prefecture University.)

The wing of *Drosophila* is separated into several sectors by the wing veins. Vein primordia are specified by the positional information provided by *hedgehog* and *decapentaplegic* in the wing imaginal disc and express the key regulatory gene *rhomboid*. One model of this process is that boundaries of gene expression regulated by *hedgehog* or *decapentaplegic* provide reference points where *rhomboid* transcription is activated. We present an analysis of the gene *plexus*, whose loss of function causes an excess vein phenotype. Molecular cloning revealed that *plexus* encodes a novel 1990-amino acid protein with cysteine-rich motifs. Plexus protein was ubiquitously expressed and was tightly associated with the nuclear matrix. In *plexus* mutant wing imaginal discs, an anteroposterior positional coordinate was established normally as revealed by the wild-type pattern of *spalt major* and *knirps* expression. However, the expression of several vein-specific and intervein-specific genes was misregulated, as if they had neglected the positional coordinate. These results suggest that Plexus is an essential component of a global repressor of vein differentiation. Although Plexus protein was expressed in vein primordia of the wing disc, it does not appear to interfere with vein differentiation in the normal position. A genetic epistasis test between *px* and *knirps* suggests that *plexus* acts downstream of *knirps*. We propose that the vein differentiation takes place by inactivation of the *plexus*-mediated repression by prepattern genes such as *knirps*. Plexus may regulate transcription of vein- and intervein-specific genes by tethering transcriptional regulators to specific locations in the nucleus. For detail, see ref 4.

(4) Snail-type zinc finger proteins prevent neurogenesis in *Scutoid* and transgenic animals of *Drosophila*

Naoyuki FUSE, Hitoshi MATAKATSU, Misako TANIGUCHI and Shigeo HAYASHI

Scutoid is a classical dominant gain-of-function mutation of *Drosophila*, causing a loss of bristles and roughening of the compound eye. Previous genetic and molecular analyses have shown that *Scutoid* is associated with a chromosomal transposition resulting in a fusion of *no-oceli* and *snail* genes. How this gene fusion event leads to the defects in neurogenesis was not known until now. Here have found that *snail* is ectopically expressed in the eye-antennal and wing imaginal discs in *Scutoid* larvae, and that this expression is reduced in *Scutoid* revertants. We have also shown that the expressivity of *Scutoid* is enhanced by *zeste* mutations. *snail* and *escargot* encode evolutionarily conserved zinc-finger proteins involved in the development of mesoderm and limbs. Snail and Escargot proteins share a common target DNA sequence with the basic helix-loop-helix (bHLH) type proneural gene products. When expressed in the developing external sense organ precursors of the thorax and the eye, these proteins cause a loss of mechanosensory bristles in the thorax and perturbed the development of the compound eye. Such phenotypes resemble those associated with *Scutoid*. Furthermore, the effect of ectopic Escargot on bristle development is antagonized by coexpression of the bHLH gene *asense*. Thus, our results suggest that the *Scutoid* phenotype is due to an ectopic *snail* expression under the control of *no-oceli* enhancer, antagonizing neurogenesis through its inhibitory interaction with bHLH proteins. For detail, see ref 5.

(5) Enhancer trap screen for genes involved in pattern formation

Satoshi GOTO, Misako TANIGUCHI, Yukiko SADO and Shigeo HAYASHI

To identify novel genes and gene functions in the pattern formation of the imaginal disc and the trachea, we are conducting an enhancer trap screen using the Gal4-UAS system. About 4500 lines were established in collabora-

tion with groups in Japan and were examined for the activity of enhancers flanking the inserts in embryos, larvae and adults. In order to identify genes responsible for those expression, we are mapping insertion sites of each transposons onto the genomic sequence of Drosophila.

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G. CENTER FOR GENETIC RESOURCE INFORMATION

G-a. Genetic Informatics Laboratory

(1) ORYZABASE--INTEGRATED RICE SCIENCE DATABASE--

Takahiro YAMAKAWA, Kazu MITSUI, Nori KURATA, Atsushi YOSHIMURA[1], Yasuo NAGATO[2] and Yukiko YAMAZAKI([1] Kyushu University, [2] University of Tokyo)

The Oryzabase is a comprehensive rice science database established in the collaboration with rice researcher's committee in Japan. The database is originally aimed to gather as much knowledge as possible ranging from classical rice genetics to recent genomics and from fundamental information to hot topics.

The current Oryzabase consists of five parts, which are (1) genetic resource stock information, (2) gene dictionary, (3) chromosome maps, (4) mutant images, and (5) fundamental knowledge of rice science.

The database includes more than 10000 collections of germplasms collected or developed by classical breeding and/or new molecular biological method as a result of long history of rice breeding in Japan. The Oryzabase map represents the integration of seven different maps from classical linkage map to the latest YAC physical map provided by Rice Genome Project. We've completed the internal cross-linking of related information such as common markers on different maps, genes and the respective mutant images, strains and their marker genes, and so on. Using Web-based good interface, the rice gene dictionary has been constantly updated by the specialists coupled with the activity of Rice Gene Cooperative.

Oryzabase uses an object-oriented database management software with Java2D for application.

We are planning to do more extensive cross-referencing of Oryzabase to the major DNA sequence database, literature database and other rice database such as Ricegenes in order to provide the wealth of information to rice re-

searchers.

Oryzabase is available from http://www.shigen.nig.ac.jp/rice/oryzabase/

(2) PEC:Profiling of Escherichia coli Chromosome

Yukiko YAMAZAKI, Toru IKEGAMI[1], Takehiro YAMAKAWA, Kazu MITSUI, Takeshi KAWABATA, Ken NISHIKAWA, Tadahiro MORI[2], Akiko NISHIMURA and Junichi KATO
([1] University of Tokyo, [2] Nara Institute of Science and Technology)

Profiling of Escherichia coli chromosome (PEC) is a database that compiles all necessary information for functional analysis of E. coli genes. The Japanese E. coli genetic resource committee has extensively contributed to the development of the database, which provides a comprehensive interface for experimental researchers. Available from the database are (i) deletion mutant information obtained from experimental research, (ii) annotated gene information including gene classification, (iii) genetic stock information, (iv) similarity search results for each genes/orfs, (v) structural information proposed by bioinformatics researchers and (vi) a tool for graphical display of different gene classes in different colors. PEC is available at http://www.shigen.nig.ac.jp/ecoli/pec/

Since the whole genome sequence of E.coli was published in 1997, enormous efforts have been made to analyze the functions of the genes/orfs. Further innovative research is still needed to complete those tasks. Many different E.coli databases are now available through the Internet, but most databases have been constructed by computer scientists with the contents derived from sequence analysis. Experimental biologists, however, are not always satisfied with such databases. On the other hand, computational scientists face problems on how to annotate their analytical results generated and/or calculated from sequence data.

To resolve the dilemma, we've developed the PEC aiming to provide experimental scientists a chance to meet and communicate with computer scientists and vice versa through the Internet. The database includes original deletion mutant data and genetic stock available through the Internet. Most genes/orfs in the database are classified into three groups, essential, non-essential,

and unknown according to either experimental results or data published in journal articles. The basic information of genes/orfs are acquired from resources available to the public and annotated by researchers before their incorporation into the database. BLAST/PSI-BLAST homology search and motif search using PROSITE and Pfam database have also been performed for all genes/orfs periodically and the results incorporated into PEC. The database also provides analytical data such as nucleotide composition proposed by computer scientists with a comprehensive interface. To create an overview, we are planning to develop off-line tool by which the user can create an original image file by applying his/her own data set.

Publication

None

G-b. Genome Biology Laboratory

(1) Analysis of gene expression patterns in *Caenorhabditis elegans*

Yuji KOHARA, Tadasu SHIN-I, Ikuko SUGIURA, Tokie OBA, Masumi OBARA, Takami SUZUKI, Sayuri KITAYAMA, Tomoko MOTOHASHI, Keiko HIRONO, Tamami NAGAOKA, Masahiro ITO, Yohei MINAKUCHI, Jean and Daniell THIERRY-MIEG[1]([1]CNRS, Montpellier, France)

The nematode *C.elegans* is a good model system to study functional genomics with respect to animal development, nervous system and behavior at the level of single cells. Although C.elegans has the basic structure of animals, it has only about 1,000 somatic cells. This simplicity has led to the description of the entire cell from embryo to adult, which has allowed us to study gene functions in individual cells. The genome consists of six chromosomes whose total size is about 100 Mbp and total number of genes is estimated to be about 19,000. Almost the entiret of the genome had been sequenced by the consortium of the Sanger Centre and Washington University by the end of 1998.

We think the main targets in the post genomics era are as follows:(1) to integrate the information on the expression and the function of all the genes of

the genome in the context of development, (2) to extract the rules that govern the molecular mechanisms of development that are carried out by a finite number of genes, (3) ultimately to reconstruct the developmental process in the computer.

We have identified about 10,000 cDNA species (more than a half of all of the genes) of C.elegans through our EST project. About 4,000 cDNA species mostly from chromosome 3 (autosome) and X (sex chromosome) have been analyzed by use of whole mount in situ hybridization for mRNA distribution throughout the life of the worm. (Generation time is about 3 days). The mRNA patterns were classified into several categories: maternal expression, zygotic expression, expression in a specific cell lineage but at different times, expression in specific cell(s) and so on. Based on the information, we are performing clustering analyses on subsets of the genes to elucidate the network of gene regulation. As a trial, focusing on the early embyrogenesis, we selected a subset of 100 genes on the criteria that the mRNA was expressed maternally and disappeared quickly before gastrulation, which seemed to play important roles in this period. For these genes, we are analyzing (i) the phenotypes caused by the systematic RNAi (dsRNA mediated interference) experiments, (ii) the protein distribution through the systematic raising of antibodies, and (iii) interacting genes by the yeast two hybrid technology. To integrate and analyze the information, first we have established a WWW-based database NEXTDB (http://watson.genes.nig.ac.jp:8080/db/index.html), and recently we have been constructing a computer graphics based 4 dimensional (3D + time course) database that covers the early embryogenesis.

(2) Studies on translational regulation of maternal mRNA

Ken-ichi OGURA, Yuji KOHARA

Accumulation of maternal messenger RNAs in oocytes is widely observed in many species, and the temporal and spatial control of their translation in early embryos is crucial for development. Although extensive studies of various maternal mRNAs, including *glp-1*, have shown that the 3' UTR (untranslated region) is important for the translational control, the molecular

mechanism is largely unknown. Previously we found that a *C.elegans* maternal gene, *pos-1*, which has two CCCH zinc finger motifs, plays an important role in the translational control of the maternal polarity gene *apx-1*. We further characterized the function of the protein POS-1 and of its interacting proteins that we identified by the yeast two hybrid method. Here we report on PIP-1, a novel RNP type RNA binding protein that interacts with POS-1. We show that PIP-1 is a positive regulator and POS-1 is a negative regulator of the translation of the maternal *glp-1* mRNA.

(3) Application of cDNA array of *C.elegans*

Makoto MOCHII[1], Satoru YOSHIDA[1], Kiyokazu MORITA[1], Hiroko UESUGI, Yuji KOHARA, Naoto UENO[1]([1]National Institute of Basic Biology)

Members of the transforming growth factor-b family play critical roles in body patterning, in both vertebrates and invertebrates. One transforming growth factor-β-related gene *dbl-1*, has been shown to regulate body length and male ray patterning in *Caenorhabditis elegans*. We screened arrayed cDNAs to identify down-stream target genes for the DBL-1 signaling by using differential hybridization. C.elegans cDNAs representing 7,584 independent genes were arrayed on a nylon membrane at high density and hybridized with 32P-labeled DNA probes synthesized from the mRNAs of wild-type, *dbl-1*, *sma-2* and *lon-2* worms. Signals for all the spots representing hybridized DNA were quantified and compared among strains. The screening identified 22 and 2 clones, which were positively and negatively regulated, respectively, by the DBL-1 signals. Northern hybridization confirmed the expression profiles of most of the clones, indicating good reliability of the differential hybridizaiton using arayed cDNAs. In situ hybridization analysis revealed the spatial and temporal expression patterns of each clone and showed that at least four genes, including the gene for the type I receptor for DNB-1, *sma-6* were transcriptionally regulated by the DBL-1 signal.

(4) Functional analysis of the *C. elegans* T-box gene *tbx-9*

Yoshiki ANDACHI

Members of the T-box family that share a DNA binding motif play critical roles in developmental phenomenon in metazoans. Some of the members have been shown to encode transcription factors. The complete sequence of the *C. elegans* genome indicates 20 T-box genes in this organism, and nearly half of them have been identified by the *C. elegans* EST project. I have been studying one of the *C. elegans* T-box genes, the cDNA group CELK02736 or *tbx-9*, which is the first T-box gene found by the EST project and seems to be the most highly expressed T-box gene as deduced from the number of isolated cDNA clones corresponding to each gene. I previously showed that *tbx-9* encodes a transcription activator, that *tbx-9* is expressed in a few cells in embryogenesis, and that the *tbx-9* deletion mutant generated by gene disruption shows aberration of morphogenesis predominantly in the posterior body, including abnormality of body-wall muscle.

To further elucidate the expression pattern of *tbx-9*, I performed *in situ* hybridization double-staining analysis using the *pos-1* and *hlh-1* genes as markers. The onset of the *tbx-9* expression is at the 8-cell stage in the E cell, the ancestor cell of gut, and the expression lasts until daughters of the E cell at the 26-cell stage. At this stage, the Ca and Cp cells start to express *tbx-9*, which produce body-wall muscle and hypodermis. On the other hand, at about the 200-cell stage four MS descendant cells express both *tbx-9* and *hlh-1*. *hlh-1* is a homolog of vertebrate *MyoD* and is expressed in the cells whose clonal descendants give rise only to striated muscle cells. This indicates that *tbx-9* is also expressed in precursors of MS-lineage body-wall muscle cells. Among these tissues whose precursor cells express *tbx-9*, body-wall muscle that shows abnormality in the *tbx-9* mutant was examined with respect to formation by immunostaining with an antibody against body-wall muscle myosin. In wildtype embryos, the muscle cells move and form four rows along the length of the animal by the 1.5-fold stage. In *tbx-9* mutant embryos, some of the muscle cells that differ form worm to worm turned out to be positioned apart from the rows at this stage, suggesting that *tbx-9* is involved in the proper arrangement of the body-wall muscle cells.

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H. STRUCTURAL BIOLOGY CENTER

H-a. Biological Macromolecules Laboratory

(1) Single Molecule Imaging of Biological Functions

Makio TOKUNAGA

Single molecule techniques have been recently developed, and importance of visualization, manipulation, as well as force measurement of single biological molecules are being realized. These new techniques were born mainly at *in vitro* researches of motor proteins, and we are trying to spread out this new technique from *in vitro* to *in vivo* and from motor proteins to other biological molecules. Moreover, by using green fluorescent protein (GFP), realtime imaging of biological molecules has been achieved in live cells quite recently. Conjunction of these new techniques, on which much attention is now focused, will provide new powerful and universal tools for researches of molecular mechanisms of biological functions in the post-genome era. With which molecule does a molecule interact? When? Where? And How? In this project, we will answer these questions by directly visualizing not only molecules themselves but also their interactions.

Imaging of single fluorescent molecules has been achieved in a relatively simple manner using objective-type total internal reflection fluorescence microscopy (TIRFM). It is found that single molecule imaging *in vivo* is realized using the objective-type TIRFM. Furthermore, I'm developping new microscopy to achieve followings: 1) Quantitative imaging of molecular numbers or concentrations *in vivo*. The key points are minimized background light, high sensitivity and wide dynamic range. 2) Imaging *in vivo* of interacting molecules and activated molecules.

(2) Single molecule imaging of nuclear transport

Naoko IMAMOTO[1] and Makio TOKUNAGA ([1]Department of Anatomy III, Osaka University Medical School)

Using objective-type total internal reflection fluorescence microscopy (TIRFM), it has been found that singe molecules can be visualized *in vivo*. This method allowed visualization of single molecules up to the depth of about 10 μm from the glass-water interface. We are now trying to visualize molecules involved in nuclear transport of macromolecules. Recent years have seen considerable progress in researches of macromolecule import into the nucleus and export from the nucleus. NLS proteins are transported into the nucleus by importin alpha/beta heterodimer. Importin alpha binds the NLS, while importin beta mediates translocation through the nuclear pore complex. After translocation, RanGTP, whose predicted concentration is high in the nucleus and low in the cytoplasm, binds importin beta and displaces importin alpha. Importin alpha must then be returned to the cytoplasm, leaving the NLS protein behind.

NLS proteins bound to the nuclear pore have been well visualized using our single molecule technique. This method is useful to elucidate molecular mechanisms of nuclear transport.

(3) Single molecule measurement of intermolecular and intramolecular interactions using subpiconewton intermolecular force microscopy

Michio HIROSHIMA and Makio TOKUNAGA

For a detailed understanding of the interactions between biological macromolecules and of intramolecular interactions, it is required to obtain information about the properties of these forces at the molecular level. We have developed intermolecular force microscopy (IMF) by refining atomic force microscopy (AFM) to improve the sensitivity of force detection and control of probe position. Force resolution of subpiconewton has been achieved, which is over 100-fold more sensitive than that of conventional AFM. IMF has also

overcome the disadvantage of AFM that the cantilever changes its position when forces are exerted, because IMF system keeps the cantilever position unchanged. Thus, we can measure intermolecular or intramolecular forces exerted on a single molecule as a function of the distance with subpiconewton resolution.

We have refined the IMF system to improve the following points: 1) expansion of force ranges up to 100 - 200 pN, 2) development of simultaneous measurement with single molecule fluorescence imaging, and 3) development of a new horizontal system in which cantilever moves horizontally and horizontal positions are controled by the feedback system.

(4) Single molecule measurement of protein folding using intermolecular force microscopy

Isao SAKANE, Kunihiro KUWAJIMA[1] and Makio TOKUNAGA ([1]Department of Physics, University of Tokyo)

The understanding of the mechanism of protein folding has remarkably progressed during the past decade. Many globular proteins have been found to accumulate intermediate states during refolding from the unfolded state to the native state. Although the molten globule state has been proposed as a major intermediate of protein folding, it has proven difficult to obtain thermodynamic data characterizing this state. Certain globular proteins show complex multiphasic refolding kinetics that are not directly related to the accumulation of the folding intermediates; the complex folding kinetics of these proteins have often been interpreted in terms of the heterogeneity of the unfolded state. Thus, a novel technique is required to study the folding process. Our single molecule technique using intermolecular force microscopy could characterize dynamic structures of intermediate states during folding.

Staphylococcal Nuculease (SNase) is a small globular protein, and is one of the proteins most generally used for folding researches. Its folding kinetics is well characterized, and also its atomic structure is available. Therefore, SNase is thought to be suitable for single molecule measurement. We have prepared

a mutant protein of SNase, which lacks the C-terminal helix and has cysteinyl residues at the both N- and C-terminal end.

Publication

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H-b. Molecular Biomechanism Laboratory

(1) The arrest of *E. coli* RNA polymerase at promoters, and the effect of the Gre factors

Motoki SUSA[1], Tomoko KUBORI[1], Hiroki NAGAI[1], Tamas GAAL[2], V. Georgi MISKELISHIVILI[3] and Nobuo SHIMAMOTO[1] ([1]Structural Biology Center, National Institute of Genetics, [2]Microbiology, Wisconsin-Madison, [3]Institute fms Genetik und Mikrobiologie)

The initiation frequency of transcription is conventionally attributed to the stability of holoenzyme-promoter binary complex, the rate/degree of strand opening, and the rate of promoter clearance. We previously showed that a portion of an otherwise homogeneous holoenzyme preparation following the binding to the λP_R promoter is converted into a complex incapable of synthesizing full-length RNAs but capable of synthesizing abortive transcripts. This non-productive complex, designated moribund complex, is converted into dead-end complex which has lost any activity for elongation, constituting a dead-end branched pathway of transcription initiation (Nuc. Acids Res. 24, 1380-1381 (1996): J. Mol. Biol. 256, 449-457 (1996)).

We kinetically and biochemically isolated the nonproductive complexes at several promoters. But no such complexes are found at T7A1 and *rrnB*P1 promoters, indicating the existence of two classes of promoter. Binary complexes with λP_R-type promoters are resistant to heparin while T7A1 type is not (SEN et al., J. Biol. Chem. 1998).

DNA and protein footprinting analyses showed that the dead-end complex is backtracked from the promoter, lacks the strand opening and has more exposed conserved region 3 of σ^{70} (Sen et al. J. Biol. Chem. in press). This inactivation was mitigated by a σ^{70} factor with S506F mutation in region 3 (SEN et al., J. Biol. Chem. 1998) or the Gre factors (GreA and GreB). These factors introduce reversibility between productive and non-productive pathways so that moribund complex can be converted into productive complex at a high concentration of the initiating nucleotide. This result shows that two branching pathways diverge before binding of the initiating nucleotide and RNA synthesis.

The Gre factors that have been assigned to be elongation factors, therefore, also work in initiation. GreB has stronger activity as an elongation factor than GreA, but GreA turns out to be stronger than GreB as an initiation factor in vitro. We have constructed single and double disruptants of *greA* and *greB*, and the disruption of GreA shows stronger phenotype. This indicates that the Gre factors are initiation factors also in vivo. An analysis of 2-dimensional protein gels and an analysis using gene array indicate 300 candidates for the target of Gre action.

(2) Role of ω subunit of *E. coli* RNA polymerase

Dipankar CHATTERJI[2], Kakoli MUKHERJEE[2], Hiroki NAGAI[1], and Nobuo SHIMAMOTO[1] ([1]Structural Biology Center, National Institute of Genetics, [2]Centre of Cellular and Molecular Biology)

The function of ω subunit of *E. coli* RNA polymerase has not been identified for decades, and a disruption of its gene rpoZ does not have any clear phenotype. We have purified RNA polymerase core enzyme from a *rpoZ* disruptant and found the enzyme is tightly bound to GroEL shaperon. The core enzyme can bind to σ^{70} and active. It is inactivated, however, with a loss of binding activity to σ^{70} if the associated GroEL is removed with a dye-affinity column or denaturation by urea. Reconstitution of activity is greatly stimulated by the presence of ω . These results show ω is structurally required for maturation of core enzyme. The lack of any phenotype of *rpoZ* mutant may be due to the functional supression by *groEL*.

(3) Inactivation of σ^{70} by oligomerization and identification of the role of its spacer region

Hiroki NAGAI[1], Taciana KAUSCIUKOVIC[2], Richard S. HAYWARD[2] Yumiko SATO[1] and Nobuo SHIMAMOTO[1] ([1]Nat. Inst. of Genet./Grad. School of Adv. Stud., [2]Inst. Of Cell and Molec. Biol., Edinburgh Univ., Scotland)

We have found that σ^{70}, the major σ factors of E. coli, forms aggregate in vivo and in vitro at a high temperature within physiological condition. The oligomeric σ^{70} has little transcriptional activity and the oligomerization in vitro showed a sharp temperature dependence. We have constructed a strain with a disrupted rpoD (σ^{70}), and plasmid born σ^{70} supports its growth. We have observed a positive correlation between the intracellular concentration of σ^{70} and upper limit of growing temperature. This raises a possibility that σ^{70} is a molecular thermometer.

The major σ factors of proteobacteria mostly have a big spacer region between the conserved regions 1 and 2 which is not conserved in eubacteria. In *E. coli* σ^{70} this region has extensive acidic patches which may be concerned with the property of σ^{70} to readily interact with nonspecific and specific surfaces. An *rpoD*-disrupted strain was constructed to test the viability of strains expressing plasmid-borne mutated *rpoD* or another sigma factor. *E. coli rpoS* and *M. tubeculosis sigA* failed to complement the disruption, and we are now testing *B. subtilis sigA* and others. The mutant σ^{70} lacking the spacer region of aa130-374 (ΔSR) complemented the disruption at 30 and 25°C, proving that the region is not essential for growth at low temperature. At all tested temperature this protein predominantly exists as aggregate which are in equilibrium with a small fraction of monomer. Therefore, the role of the spacer region is the maintenance of active monomeric form.

(4) Single-Molecule Dynamics of Transcription: Sliding of proteins along DNA

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Mechanical Engineering, Kyoto University, [3]Advance Co., [4]Department of Molecular and Life Science, Daiichi College of Pharmaceutical Science)

We have showed the existence of a sliding motion of protein along DNA through direct visualization of single molecules of *E. coli* RNA polymerase (Science 262, 1561-1563 (1993)). 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.

The most distinct difference between the movements of RNA polymerase and CamR was the pathway of dissociation from their specific sites. RNA polymerase slides out of its specific site into nonspecific sites and then dissociate from non-specific sites into bulk. This two-step dissociation was not observed in the case of CamR. CamR seemed to dissociate directly into bulk and its sliding upon dissociation from the specific site was not long enough to be detected. CamR also slides extensively upon association to the specific site, and thus long non-specific DNA segment flanking the specific site accelerate association but not dissociation, making its affinity for the specific site on longer DNA stronger. Thus long DNA can harvest CamR like an antenna.

There is a long-standing contradiction on *E. coli* TrpR that its specificity is too small to compete binding to its operator against the predominant nonspecific sites with the copy number present in the cell. We challenged to solve this contradiction by introducing the concept of antenna effect by sliding. We found that the affinity of TrpR for *trpO* strongly depends on the length of DNA and is enhanced more than 10,000 -fold. A control experiment showed that this enhancement is not due to the stabilization by an additional interaction with a long DNA. Therefore, antenna effect by sliding is really present *in vivo*. This effect open up several new ways of gene regulation and further proof of antenna effect will be obtained.

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H-c. Multicellular Organization Laboratory

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

Akane OISHI, Takeshi ISHIHARA and Isao KATSURA

Fluoride-resistant (*flr*) mutations of *C.elegans* are recessive and grouped into two categories: class 1 mutations, which map in *flr-1*, *flr-3* and *flr-4*, and class 2 mutations, which map in *flr-2* and *flr-5* (Katsura, I. *et al.*: Genetics **136**, 145-154, 1994). Class 1 *flr* mutations show many phenotypes: slow growth, short defecation cycle periods, frequent skip of the expulsion step of defecation, and synthetic abnormality in dauer formation (See (2) below), besides strong resistance to fluoride ion. The *flr-1* gene encodes an ion channel belonging to the DEG/ENaC (*C. elegans* degenerins and mammalian amiloride-sensitive epithelial sodium channels) superfamily, while *flr-4* and *flr-3* code for a novel Ser/Thr protein kinase and a kinase-like molecule, respectively, both having a hydrophobic domain on the carboxyl-terminal side. A functional *flr-1*::GFP fusion gene is expressed only in the intestinal cells from the comma stage of embryos to the adult stage, while *flr-4*::GFP is expressed in the intestinal cells from the 1.5-fold stage, in the isthmus of the pharynx from the 3-fold stage and in a pair of head neurons called AUA from L1 larvae to adults. Moreover, the expression of *flr-3*::*lacZ* is detected mainly in the intestine. We therefore think that class 1 *flr* genes constitute a regulatory system that acts in the intestine and that controls many food-related functions. Class 2 *flr* mutations, which confer weak resistance to fluoride ion, suppress the slow growth and dauer formation abnormality but not the defecation abnormality

and strong fluoride-resistance of class 1 *flr* mutations. Hence, it seems that the regulatory pathway bifurcates after class 1 genes, and that class 2 genes control only one of the two branches. Of the class 2 genes, *flr-2* encodes a secreted protein belonging to the gremlin/DAN/cerberous family, most of which are TGF-β antagonists.

We obtained the following results in 1999.

(i) Measuring the probability of climbing the petri dish of plate cultures, we found that class 1 *flr* mutants have lower tendency to stay on bacterial lawn. This phenotype is suppressed by class 2 mutations. We are now investigating the relationship between this phenotype and the phenotype of dauer formation abnormality, which also suggests abnormality in chemoreception or its regulation.

(ii) The four mutant alleles of *flr-4* have a mutation at a splice-acceptor site (*ut3*), a missense mutation in the C-terminal hydrophobic domain (*ut7*), and missense mutations in the protein kinase domain (*n2259, sa201*), respectively. While the former two mutants grow slowly, the latter two mutants grow at a normal rate, which is exceptional for class 1 *flr* mutants. Of the heterozygotes of these alleles, *ut7/n2259* and *ut7/sa201* unexpectedly grew at a reduced rate, while *ut3/n2259* and *ut3/sa201* grew at a normal rate, as expected. It is known that the heterozygotes *n2259/+* and *sa201/+* grow at a normal rate, i. e., *n2259* and *sa201* are not haplo-insufficient. Rather, it seems that *ut7* acts as a dominant-negative allele to *n2259* and *sa201*. The result suggests that the FLR-4 protein may act as an oligomer or that the FLR-4(*ut7*) mutant protein may deprive FLR-4(*n2259*) and FLR-4(*sa201*) of the substrate.

(iii) We carried out temperature-shift experiments concerning the phenotype of defecation cycle periods, using the temperature-sensitive allele *n2259*. The results showed that the active FLR-4 is required at the time of the defecation cycle assay. This suggests that *flr-4* plays a role in a cell function that controls defecation cycle periods, rather than in the generation or differentiation of cells that are required for the normal defecation cycle period.

(iv) FLR-2 has homology to TGF-β antagonists. We therefore introduced each of the four TGF-β genes in *C.elegans* (*daf-7, dbl-1/cet-1, unc-129*, F39G3.8) into the *flr-4(ut7)* mutant as an extra chromosomal array. The transformants

grew as slowly as the *flr-4* mutant. The results were the same when a heat shock promoter was used for driving the expression of the TGF-β genes. Thus, there is so far no evidence showing that FLR-2 acts as a TGF-β antagonist.

(v) A functional *flr-2*::GFP fusion gene was expressed in a small number of neurons in the pharynx, head and tail. We are identifying these neurons.

(2) Analysis of Synthetic Dauer-constitutive Mutations

Kouji MIYAHARA, Kiyotaka OHKURA, Takeshi ISHIHARA and Isao KATSURA

If newly hatched larvae of *C. elegans* are in a crowded state and given a limited food supply, they sense the environmental signal of pheromone and food with head sensory organs called amphids and deviate from the normal life cycle, ending in non-feeding larvae called dauer larvae. 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 mutations in more than 50 known genes show synthetic dauer-constitutive (Sdf-c) phenotypes, i.e.,they develop to dauer larvae in a certain mutant background, regardless of the environmental conditions. The synthetic nature of the phenotype, we think, is based on the structure of the neural circuit, in which neural signals are transmitted through parallel routes consisting of different types of neurons so that two mutations may be required to block the signals. We are studying the combinations of mutations for the Sdf-c phenotype and the pattern of suppression of the Sdf-c phenotype by various suppressor mutations. In this way we hope to determine the functional neural network for dauer regulatory signals and the function of relevant genes in the network. Furthermore, we isolated and mapped 44 new mutations that show the Sdf-c phenotype in combination with the *unc-31(e169)* mutation. Many of them are expected to cause defects in ASI neurons, since the *unc-31(e169)* mutant shows dauer-constitutive phenotype, if ASI neurons are killed. Eight of the mutations mapped in 4 known genes (*tax-2*, *osm-6*, *che-11*, and *aex-3*), but most of the remaining 36 mutations, which map in at least 13 genes, seem to be alleles of novel genes. Of these mutants, that in *sdf-1* gene (1 allele) avoids benzaldehyde, isoamyl alcohol,

butanone. NaCl, and lysine, which are attractants of wild-type animals. It behaved as thermophilic in thermotaxis. Mutants in *sdf-13* gene (2 alleles) showed defects in adaptation of chemotaxis to benzaldehyde, isoamyl alcohol and butanone, although they showed normal chemotaxis to these attractants. This gene encodes a homolog of mammalian Tbx2 and *Drosophila* Omb, a transcription factor containing the T-box domain.

We obtained the following results in 1999.

(i) The two *sdf-13* alleles (*utl80, utl92*) had amino acid substitutions for the same lysine residue in the T-box domain: glutamic acid in one allele and arginine in the other. For expression studies, GFP cDNA was inserted at the beginning and the end of the *sdf-13* coding region, respectively, and *sdf-13* mutant animals were transformed with the DNA constructs which also contained a 1.5-kb 5' upstream sequence. While the former transformant showed mutant phenotypes, the latter transformant showed wild type phenotypes. Both GFP fusion genes were expressed in M2 and I5 neurons in the pharynx. We are now making antibodies against SDF-13 to confirm the expression pattern and to test if SDF-13 acts in pharyngeal neurons or amphid sensory neurons.

(ii) Of the genes identified by the new synthetic dauer-constitutive mutations, *sdf-9* has the largest number (five) of alleles. Mutants in *sdf-9* produce many dauer larvae in the presence of a chromosomally integrated array of a *daf-7*::GFP transcriptional fusion gene, where *daf-7* encodes a TGF-β protein that represses dauer formation. Interestingly, these dauer larvae recover spontaneously and grow to adults.

(3) Analysis of Mutants That Show Abnormality in the Selection between Two Behaviors

Takeshi ISHIHARA, Yuichi IINO[1] and Isao KATSURA ([1] Molecular Genetics Laboratory, University of Tokyo)

The nematode *C.elegans* shows avoidance of copper ion and chemotaxis to odorants by perceiving them with different sensory neurons in the head. We devised a behavioral assay method for the interaction between the two responses to learn a possible role of interneurons. Wild-type animals change

their preference between the responses, depending on the relative concentration of copper ion and odorants. This suggests that the two sensory signals interact with each other in a neural circuit consisting of about 10 neurons, on the basis of the neural circuitry of *C.elegans* and the identity of the sensory neurons that act in these behaviors.

While these experiments were performed with well-fed animals, animals starved for 5 hours showed a stronger preference for odorants, because they responded more weakly to copper ion. This starvation effect was not detected in the presence of serotonin, which is considered to induce a well-fed state. The behavioral change upon starvation seems appropriate, because starved worms can look for food over a wider area.

To elucidate the mechanism of the complex behaviors mentioned above, we are isolating and analyzing mutants that show abnormality in these behaviors. The mutant *ut236* had a tendency to choose avoidance of copper ion rather than chemotaxis to odorants, although it showed no abnormality in each behavior in case only one of the stimuli was given. The result shows that *ut236* is abnormal in the interaction between the two behaviors. Furthermore, *ut236* seems to be abnormal in the conditioning (associative learning) by the paired presentation of starvation and NaCl. Thus, it probably has abnormality in many aspects of sensory signal processing. We identified the gene for *ut236* by positional cloning. It encoded a novel secretory protein having an LDL receptor ligand-binding domain. A functional GFP fusion gene was expressed in a small number of sensory neurons and interneurons. We are investigating the expression of *ut236* by antibodies against its recombinant protein.

The mutant *ut235* lacked the effect of starvation in the above assay, but was essentially normal in the increase of locomotion speed by starvation and in various responses to food. Namely, this mutant has defects only in part of the behavioral responses to starvation. The double-mutant *ut235; ut236* showed a preference to avoidance of copper ion, regardless of starvation. We are now trying to identify the gene responsible for the *ut235* mutation by positional cloning.

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H-d. Biomolecular Structure Laboratory

(1) Crystallographic Study of F1-ATPase:Structural Analysis of Supramolecule

Yasuo SHIRAKIHARA and Aya SHIRATORI

F1-ATPase, with a subunit composition of $\alpha 3\beta 3\gamma\delta\varepsilon$, is a catalytic sector of the membrane bound ATP synthase. The ATP synthase 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. We previously solved the structure of the $\alpha 3\beta 3$ sub-assembly of F1-ATPase from a thermophilic bacterium *Bacillus* PS3. We have been extending the structural study to a nucleotide-bound form of the $\alpha 3\beta 3$ sub-assembly and to $\alpha 3\beta 3\gamma$ and $\alpha 3\beta 3\gamma\varepsilon$ sub-assemblies.

We previously attempted to look at the structure of the nucleotide-bound form of the $\alpha 3\beta 3$ sub-assembly by analyzing x-ray diffraction data from the crystals, originally grown in absence of nucleotide but later soaked in nucleotide-containing media. We could trap a state where the over-all structure is

very similar to that of the nucleotide-free state, but where phosphate binding to the β-subunit gets weaker than in the nucleotide-free state. In order to look at other states distinctly different from the above, we have tried to crystallize the sub-assembly in presence of non-hydrolyzable nucleotide analog AMPPNP, and got crystals. We plan to examine a structure the crystals will present.

In recent years, we have made considerable efforts to get good crystals of the $\alpha 3 \beta 3 \gamma$ sub-assembly, as the sub-assembly exhibits kinetic properties very similar to those of F1, and a number of interesting mutant sub-assemblies have been engineered. Both preparation method and crystallization conditions have been examined extensively in this year as well. It has now been established that treatments to the crude extract such as heat treatment and ammonium sulfate fractionation are very effective. A different combination of chromatographic columns, like gel filtration (1st) and weak hydrophobic (2nd) columns gave performance slightly better than the usual combination of hydrophobic and ion exchange columns. Inclusion of sodium sulfate, which is thought to stabilize the sub-assembly, in the chromatographic solution had a good effect on crystallization, but inclusion of ATP, assumed to play a similar role of sodium sulfate, was not effective. A single most important point in improving crystallization found this year was inclusion of non-ionic detergent like LAO and Decyl-trio-maltoside in the mother liquor. When they were included, the crystals grown in those conditions were much larger, at least in two directions, and better-shaped, that is, like plates or columns rather than petals which were usually seen in the previous crystallization experiments. It took some time to be able to record diffraction pattern from these crystals, because, as found later, the crystals were sensitive to changes of environment and seemed to have very weak diffraction capability (to 20 Å resolution). It is being planned to do diffraction experiment in SPRING8 with very brilliant x-ray source on these crystals, and, for that purpose, search for the cooling conditions for these crystals is in progress.

Eighteen mutant $\alpha 3 \beta 3 \gamma$ sub-assemblies were subjected to crystallization study this year. The mutations were in the β-subunit and may be classified into 3 groups: 1) a hinge region of the subunit was changed to interfere with the hinge bending motion of the subunit, 2) the DELSEED region which inter-

act with the γ-subunit was modified, and 3) cross link between two β-subunits could be under control. Mutants belonging group1 gave somewhat larger crystals than the wild type.

The $\alpha 3\beta 3\gamma\varepsilon$ sub-assembly preparation got available due to overproduction system of this multi-subunit assembly established by Dr S. Suzuki at Tokyo Institute of technology. The preparation crystallized fortunately at the conditions for the $\alpha 3\beta 3\gamma$ sub-assembly. Further purification with a weak hydrophobic column produced better preparation which could be crystallized into much larger bi-pyramids with sizes of about 0.3mm. The hydrophobic column removed contaminating $\alpha 3\beta 3$ sub-assembly which could have interfered with crystallization of the $\alpha 3\beta 3\gamma\varepsilon$ sub-assembly. Further efforts are being made to tune up the crystallization conditions.

These structure studies were done in collaboration with Masasuke Yoshida, Toyoki Amano, Eiro Muneyuki, Tomoko Masaike, Kiyotaka Hara, Shunji Suzuki and Satoshi Tsunoda at Research Laboratory of Resource Utilization, Tokyo Institute of technology.

(2) Crystallization of ATPsynthase

Kazuyasu SHINDO and Yasuo SHIRAKIHARA

ATPsynthase may be described as a combination of soluble F1 and a membrane-embedded Fo (with subunit composition of ab2c8-12), and is therefore a membrane protein, a challenging target for crystallization. We started to examine purification method for the ATPsynthase extracted with Dodecylmaltoside from the PS3 membrane. Also we started to screen crystallization conditions for an assembly comprising F1 and a soluble portion of the b subunit (b'), of which preparation were provided by Dr S. Suzuki at Tokyo Institute of technology.

The structure study were done in collaboration with Yoshida, Shunji Suzuki and Dirk Bald at Research Laboratory of Resource Utilization, Tokyo Institute of technology.

(3) Crystallization of cytoplasmic domain of CD40 receptor in B-cell

Yasuo SHIRAKIHARA

CD40 signalings play crucial roles in B-cell function. Signals through CD40 rescue B cells from apoptosis induced by cross-linking of the surface immunoglobulin complex and also induce B cells to differentiate. TRAF5 and TRAF6, signal transducers, interact with a cytoplasmic tail (about 70 amino acid residue) of CD40. We tried to crystallize the cytoplasmic tail to understand the mechanism of signal transduction using that part. The cytoplasmic portion (aa 216-277)was expressed in *E.coli* as GST fusion protein, and was purified with Glutathione-Sepharose. It was anticipated that the tendency of GST part to crystallize could be utilized to crystallize the fusion protein, as in the case of Mre11fragment-GST fusion protein reported last year. The crystallization condition search was extensive, however, ended up in finding only dendlytes formation conditions (ammonium sulfate at pH5).

The crystallization study were done in collaboration with Jun-ichiro Inoue, Asuka Naitoh at the Institute of medical science, The university of Tokyo.

(4) Crystallization of D-aminoacylase from *Alcaligenes*

Yasuo SHIRAKIHARA

D-aminoacylase catalyzes hydrolysis of N-acylderivatives of various neutral D-amino acids to D-amino acids and fatty acids. The enzyme has not only industrial relevance with potential to produce large amount of D-amino acids, but also possibly unique active site structure that distinguishes L-amino acids that are abundant in nature. The enzyme from *Alcaligenes xylosoxydans subsp. xylosoxydans A-6* was purified from cell extract of overproducing *E.coli* with DEAE ion exchange and butyl Toyopearl columns. The crystallization condition search was done with grid screen and crystal screen from Hampton Research. Small crystals grew from MPD or isopropanol solutions, however, they were not reproducibly obtained. Purity of preparations was found to be variable, and therefore efforts have been made to establish a solid prepara-

tion procedure.

The crystallization study was done in collaboration with Mituaki Moriguchi, Akiko Sato, at Oita University, and Mamoru Wakayama, at Ritsumeikan University.

(5) Crystallization of Na^+-translocating ATPase

Yasuo SHIRAKIHARA

Na^+-translocating ATPase from *Enterococcus hirae* is classified as a Vacuolar-type ATPase (V-type), and is expected to have a structure similar to F1-ATPase (F-type ATPase) from amino acid sequence comparison. Na^+-translocating ATPase was highly purified and was subjected to crystallization experiment. Plate shaped crystals with largest dimension of 0.2mm was produced from polyethylene glycol solution, and was found to diffract to 6 Å resolution. Further diffraction studies conducted this year allowed us to record weak diffraction spots at 3.6 Å resolution. Preparation methods are still being improved to get better crystals. This work has been done in collaboration with Toshiyuki Meguro and Ichiro Yamato, Science University of Tokyo.

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I. CENTER FOR INFORMATION BIOLOGY

I-a. Laboratory for DNA Data Analysis

(1) Reconstruction of the Ancestral Genome and Evolutionary Study of Genome Structures by Comparison of Complete Genome Sequences

Takeshi ITOH and Takashi GOJOBORI

A number of complete genome sequences have been determined in various species such as microbes. These sequence data gave us a unique opportunity to investigate the evolution of the whole genome structures. By comparison of orthologous gene orders, we elucidated that gene orders had been frequently shuffled during long-term evolution, and that operon structures, which are thought to be functionally important, had been evolutionarily unstable.

In this study, in order to effectively detect orthologous gene pairs between distantly related species, we improved our method for ortholog-finding in the following two points: (1) we employed log-normalized Smith-Waterman scores for evaluation of sequence similarity significance, (2) gene fusion events were considered. By comparing gene orders of orthologs defined by this method, we reconstructed the ancestral genome structure between eubacteria and archaebacteria, and estimated the degrees of genome stability. As a result, we found that the degrees of genome instability were positively correlated with the occurrence frequencies of insertion sequences (ISs). This implies that ISs strongly affect genome stability. Moreover, it was revealed that frequent rearrangements of genomes diminished strand-specific nucleotide biases such as GC-skew.

In a comparison of gene fusion between eukaryotes and prokaryotes, it was strongly suggested that eukaryotes possess more fused genes than prokaryotes, and almost all of fused genes in eukaryotes appeared to be derived from prokaryotic operons. These observations indicate that the prokaryotic genome structure is ancestral, and eukaryotes have lost the structure but partly re-

tained them as forms of fused genes. Drastic changes of the genome structure and gene regulation may have happened in the early evolution of the eukaryotic ancestor.

(2) Different evolutionary histories in two subgenomic regions of the major histocompatibility complex

Silvana GAUDIERI, Jerzy K. KULSKI[1], Roger L. DAWKINS[1] and Takashi GOJYOBORI ([1]Center for Molecular Immunology and Instrumentation, University of Western Australia)

Two subgenomic regions within the major histocompatibility complex, the alpha and beta blocks, contain members of the multicopy gene families HLA class I, human endogenous retroviral sequence (HERV-16; previously known as P5 and PERB3), hemochromatosis candidate genes (HCG) (II, IV, VIII, IX), 3.8-1, and MIC (PERB11). In this study we show that the two blocks consist of imperfect duplicated segments, which contain linked members of the different gene families. The duplication and truncation sites of the segments are associated with retroelements. The retroelement sites appear to generate the imperfect duplications, insertions/deletions, and rearrangements, most likely via homologous recombination. Although the two blocks share several characteristics, they differ in the number and orientation of the duplicated segments. On the 62.1 haplotype, the alpha block consists of at least 10 duplicated segments that predominantly contain pseudogenes and gene fragments of the HLA class I and MIC (PERB11) gene families. In contrast, the beta block has two major duplications containing the genes HLA-B and HLA-C, and MICA (PERB11.1) and MICB (PERB11.2). Given the common origin between the blocks, we reconstructed the duplication history of the segments to understand the processes involved in producing the different organization in the two blocks. We then found that the beta block contains four distinct duplications from two separate events, whereas the alpha block is characterized by multisegment duplications. We discussed these results in relation to the genetic content of the two blocks. See Gaudieri *et al.* (Genome Res 9:541-549, 1999) for details.

(3) Identification of a ribonuclease H gene in both *Mycoplasma genitalium* and *Mycoplasma pneumoniae* by a new method for exhaustive identification of ORFs in the complete genome sequences

Matthew I. BELLGARD and Takashi GOJOBORI

Exhaustive identification of open reading frames in complete genome sequences is a difficult task. It is possible that important genes are missed. In our efforts to reanalyze the intergenic regions of *Mycoplasma genitalium* and *Mycoplasma pneumoniae*, we have newly identified a number of new open reading frames (ORFs) in both *M. genitalium* and *M. pneumoniae*. The most significant identification was that of a ribonuclease H enzyme in both species which until now has not been identified or assumed absent and interpreted as such. We discusses the biological importance of RNase H and its evolutionary implication. We also stressed the usefulness of our method for identifying new ORFs by reanalyzing intergenic regions of existing ORFs in complete genome sequences. See Bellgard and Gojobori (FEBS Letters 445:6-8, 1999) for details.

(4) Significant differences between the G+C content of synonymous codons in orthologous genes and the genomic G+C content

Matthew I. BELLGARD and Takashi GOJOBORI

The relationship between the overall G+C content of the genome (GC) and the GC content at the third codon positions (GC3) of genes, which we refer to as a GC3-plot, was examined using 15 currently available complete genome sequences. A remarkably linear relationship was found between these two quantities, confirming previous observations of a strong positive correlation in the GC3-plot. In order to conduct a more detailed analysis of the GC3-plot, we examined the GC3 content by separating orthologous codons into three categories: synonymously different codons (namely identical amino acids, IA), different amino acids (DA), and identical codons (IC), for a pairwise compari-

son of two closely related species. When we took pairwise species comparisons between *Mycoplasma genitalium* (Mg) and *Mycoplasma pneumoniae* (Mp) and between *Mycobacterium tuberculosis* (Mt) and *Mycobacterium leprae* (Ml) as examples, we found that for Mp and Ml, the GC3 for IA deviated the most from the linear expectation in the GC3-plot, whereas for Mg and Mt the deviation was minimal. These findings suggest that the major changes of GC content took place in Mp and Ml, but not in Mg and Mt. This analysis also enables us to predict the future direction of the evolutionary changes of the genomic GC content. See Bellgard and Gojobori (Gene 238:33-37, 1999; Trends in Genetics 15:254-256, 1999) for details.

(5) MITEs, interspersed nuclear elements, in the plant genomes and their significance in the plant evolution

Katsuhiko MINETA, Kazuho IKEO, Yasunari OGIHARA[1] and Takashi GOJOBORI
([1]Yokohama City University)

The chloroplast is the organelle where photosynthesis takes place, and the chloroplast genome is highly suitable for molecular evolutionary analyses of plants. Focusing on the chloroplast genome in higher plants, we looked for evidence of genomic rearrangements in the evolutionary history of this organelle. We compared the complete chloroplast genomes of five species. We found one large inversion evident between the genomes of monocotyledons and dicotyledons. We also found that the physical ordering of orthologous genes was strongly conserved. To investigate possible mechanisms of genome rearrangement in the chloroplast genome, we searched for the transposable elements. Consequently, we found a putative MITE sequence, that seems to have been inserted following the emergence of monocotyledons. This discovery of MITE sequences in the chloroplast genome of monocotyledons is the first of its find. However, transposable elements in organella genomes are extremely uncommon, and may not play a role in rearrangement of these genomes. Alternatively, a mechanism may exist whereby transposable elements are rapidly eliminated from the chloroplast genome after rearrangements have taken place.

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I-b. Laboratory for Gene-Product Informatics

(1) Cooperative approach for the protein fold recognition

Motonori OTA, Takeshi KAWABATA, Akira R. KINJO and Ken NISHIKAWA

We, four independent predictors, organized a team and tackled blind protein structure predictions using fold recognition methods. We tried to assign the homologous or analogous folds in the protein structure database for a number of target sequences that showed no apparent sequence homology to the proteins of known folds. After primary analyses by conventional softwares, these sequences were threaded through the structural library using three different programs developed by ourselves, which employed different compatibility functions. Collecting the results of our individual analyses, and the available biological knowledge about the target, we held meetings and discussed all plausible structures for the target. For 25 target sequences, we submitted 56 models including NONE: This was the first time the fold was determined. At the time of the meeting (CASP3), 19 protein structures (21 domains) categorized as the threading targets were available. We succeeded in predicting eight out of 18 targets (20 domains) that we submitted; however, alignment accuracies were not satisfactory for some of the models. We often obtained correct answers even if some of us missed the right prediction; therefore it would appear that our threaders compensated each other. When all the information is managed effectively, the prediction gains more accuracy. See Ref. 2 for the details.

(2) Strong hydrophobic nature of cysteine residues in proteins

Nozomi NAGANO[1], Motonori OTA, Ken NISHIKAWA ([1]Biochemistry and Molecular Biology Department, University College London)

The differences between disulfide-bonding cystine (Cys_SS) and free cysteine (Cys_SH) residues were examined by analyzing the statistical distribution of both types of residue in proteins of known structure. Surprisingly, Cys_

SH residues display strong hydrophobicity than Cys_SS residues. A detailed survey of atoms which come into contact with the sulfhydryl group (sulfur atom) of Cys_SH revealed that contacting atoms are essentially the same in number and variety as those of the methyl group of isoleucine, but are quite different to those of the hydroxyl group of serine. Moreover, the relationships among amino acids were also determined using the 3D-profile table of known protein structures. Cys_SH was located in the hydrophobic cluster, along with residues such as Met, Trp and Tyr, and was clearly separated from Ser and Thr in the polar cluster. These results imply that free cysteines behave as strongly hydrophobic, and not hydrophilic, residues in proteins. See Ref. 3 for the details.

(3) Design and synthesis of a globin fold

Yasuhiro ISOGAI[1], Motonori OTA, Tetsuro FUJISAWA[2], Hiroyuki IZUNO[3], Masahiro MUKAI[1], Hiro NAKAMURA[1], Tetsutaro IIZUKA[1] and Ken NISHIKAWA ([1]The Institute of Physical and Chemical Research (RIKEN), [2]RIKEN Harima Institute, [3]Gakushuin University)

We propose a simple method to find an amino acid sequence that is foldable into a globular protein with a desired structure based on a knowledge-based 3D-1D compatibility function. An asymmetric α-helical single-domain structure of sperm whale myoglobin consisting of 153 amino acid residues was chosen for the design target. The optimal sequence to fit the main-chain framework has been searched by recursive generation of the protein 3D profile. The heme-binding site was designed by fixing His64 and His93 at the distal and proximal positions, respectively, and by penalizing residues that protrude into the space with a repulsive function. The apparent bumps among side chains in the computer model of the converged, self-consistent sequence were removed by replacing some of the bumping residues with smaller ones according to the final 3D profile. The finally obtained sequence shares 26% of sequence with the natural myoglobin. The designed globin-1 (DG1) with the artificial sequence was obtained by expression of the synthetic gene in *Escherichia coli*. Analyses using size-exclusion chromatography, circular dichroism spec-

troscopy, and solution X-ray scattering showed that DG1 folds into a monomeric, compact, highly helical, and globular form with an overall molecular shape similar to the target structure in an aqueous solution. Furthermore, it binds a single heme per protein molecule, which exhibited well-defined spectroscopic properties. The radius of gyration of DG1 was determined to be 20.6 Å, slightly larger than that of natural apoMb, and decreased to 19.5 Å upon heme binding based on X-ray scattering analysis. However, the heme-bound DG1 did not stably bind molecular oxygen as natural globins do, possibly due to high conformational diversity of side-chain structures observed in the NMR and denaturation experiments. These results give insight into the relationship between the sequence selection and the structural uniqueness of natural proteins to achieve biological functions. See Ref. 4 for the details.

(4) Experimental verification of the 'stability profile of mutant protein' (SPMP) data using mutant human lysozymes

Kazufumi TAKANO[1], Motonori OTA, Kyoko OGASAHARA[1], Yuriko YAMAGATA[2], Ken NISHIKAWA and Katsuhide YUTANI[1] ([1]Institute for Protein Research, Osaka University, [2]Graduate School of Pharmaceutical Science, Osaka University)

The stability profile of mutant protein (SPMP) (Ota, M., Kanaya, S. and Nishikawa, K., 1995, *J.Mol.Biol.*, 248, 733-738) estimates the changes in conformational stability due to single amino acid substitutions using a pseudo-energy potential developed for evaluating structure-sequence compatibility in the structure prediction method, the 3D-1D compatibility evaluation. Nine mutant human lysozymes expected to significantly increase in stability from SPMP were constructed, in order to experimentally verify the reliability of SPMP. The thermodynamic parameters for denaturation and crystal structures of these mutant proteins were determined. One mutant protein was stabilized as expected, compared with the wild-type protein. However, the others were not stabilized even though the structural changes were subtle, indicating that SPMP overestimates the increase in stability or underestimates negative effects due to substitution. The stability changes in the other mutant human lysozymes previously reported were also analyzed by

SPMP. The correlation of the stability changes between the experiment and prediction depended on the types of substitution: there were some correlations for proline mutants and cavity-creating mutants, but no correlation for mutants related to side-chain hydrogen bonds. The present results may indicate some additional factors that should be considered in the calculation of SPMP, suggesting that SPMP can be refined further. See Ref. 5 for the details.

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I-c. Laboratory of Gene Function

(1) DNA Data Bank of Japan dealing with large-scale data submission

Hideaki SUGAWARA, Satoru MIYAZAKI, Takashi GOJOBORI and Yoshio TATENO

The DNA Data Bank of Japan (DDBJ) has developed a software system for mass submission to cope with a recent expansion of EST and genome data submissions. The system is composed of four parts, the WWW data submission, large-scale submission, submission management and storing. Using this system one can submit data on a large number of sequences or a very long

sequence while checking the consistency between the annotation and sequence without much effort. DDBJ has received large-scale data of *Homo sapiens, Arabidopsis* and *Pyrococcus* from Japanese researchers who made full use of the new submission system. See Ref. 1 for details.

(2) A phosphate-induced gene which promotes *Penicillium* mediated bioconversion of cis-propenylphosphonic acid to Fosfomycin. Applied and Environmental Microbiology

M.WATANABE[1], N. SUMIDA[1], S. MURAKAMI[1], H. ANZAI[1], C.J. THOMPSON[2], Y. TATENO and T. MURAKAMI[1] ([1]Meiji Seika Kaisha, Ltd., [2]University of Basel)

Penicillium decumbens is able to epoxidize cis-propenylphosphonic acid (cPA) to produce the antibiotic fosfomycin [FOM; also referred to as phosphonomycin and (-)-cis-1,2-epoxypropylphosphonic acid], a bioconversion of considerable commercial significance. We sought to improve the efficiency of the process by overexpression of the genes involved. A conventional approach of isolating the presumed epoxidase and its corresponding gene was not possible since cPA epoxidation could not be achieved with protein extracts. As an alternative approach, proteins induced by cPA were detected by two-dimensional gel electrophoresis. The observation that a 31-kDa protein (EpoA) was both cPA induced and overaccumulated in a strain which more efficiently converted cPA suggested that it might take part in the bioconversion. EpoA was purified, its amino acid sequence was partially determined, and the corresponding gene was isolated from cosmid and cDNA libraries with oligonucleotide probes. The DNA sequence for this gene (epoA) contained two introns and an open reading frame encoding a peptide of 277 amino acids having some similarity to oxygenases. When the gene was subcloned into *P. decumbens*, a fourfold increase in epoxidation activity was achieved. epoA-disruption mutants which were obtained by homologous recombination could not convert cPA to FOM. To investigate the regulation of the epoA promoter, the bialaphos resistance gene (bar, encoding phosphinothricin acetyltransferase) was used to replace the epoA-coding region. In *P. decumbens*, expression of the bar reporter gene was induced by cPA, FOM, and phosphorous acid but not by phosphoric acid. See Ref. 2 for details.

(3) Genomic organization around the centromeric end of the HLA class I region: Large-scale sequence analysis

Masa-aki YAMAZAKI[1,2], Yoshio TATENO and Hidetoshi INOKO[2] ([1]Fujiya Co. Ltd., [2]Tokai University)

We previously sequenced two regions around the centromeric end of HLA class I and the boundary between class I and class III. We analyzed the two regions of about 385 kb and confirmed, giving a new line of evidence, that the following two pairs of the genomic segments were duplicated in evolution: (i) a 43-kb genomic segment including the HLA-B gene showing the highest polymorphism among the classical HLA class I loci (class Ia) and a 40-kb segment including the HLA-C locus showing the lowest polymorphism and (ii) a 52-kb segment including the MIC (MHC class I chain related gene) B and a 35-kb segment including MICA. We also found that repetitive elements such as SINEs, LINEs, and LTRs occupy as much as 47% of nucleotides in this 385-kb region. This unusually high content of repetitive elements indicates that repeat-mediated rearrangements have frequently occurred in the evolutionary history of the HLA class Ia region. Analysis of LINE compositions within the two pairs of duplicated segments revealed that (i) LINEs in these regions had been dispersed prior to both the duplication of the HLA-B and -C loci and the duplication of the MICB and MICA loci, and (ii) the divergence of the HLA-B and -C loci occurred prior to the duplication of the MICA and MICB loci. To find novel genes responsible for HLA class I-associated or other diseases, we performed computer analysis applying GenScan and GRAIL to GenBank's dbEST. As a result, at least five as yet uncharacterized genes were newly mapped on the HLA class I centromeric region studied. These novel genes should be analyzed further to determine their relationships to diseases associated with this region. See Ref. 3 for details.

(4) Domain dislocation: a change of core structure in periplasmic binding proteins in their evolutionary history

Kaoru FUKAMI-KOBAYASHI, Yoshio TATENO and Ken NISHIKAWA

Periplasmic binding proteins (PBPs) serve as receptors for various water-soluble ligands in ATP-binding cassette (ABC) transport systems, and form one of the largest protein families in eubacterial and archaebacterial genomes. They are considered to be derived from a common ancestor, judging from their similarities of three-dimensional structure, their mechanism of ligand binding and the operon structure of their genes. Nevertheless, there are two types of topological arrangements of the central beta-sheets in their core structures. It follows that there must have been differentiation in the core structure, which we call "domain dislocation", in the course of evolution of the PBP family. To find a clue as to when the domain dislocation occurred, we constructed phylogenetic trees for PBPs based on their amino acid sequences and three-dimensional structures, respectively. The trees show that the proteins of each type clearly cluster together, strongly indicating that the change in the core structure occurred only once in the evolution of PBPs. We also constructed a phylogenetic tree for the ABC proteins that are encoded by the same operon of their partner PBP, and obtained the same result. Based on the phylogenetic relationship and comparison of the topological arrangements of PBPs, we obtained a reasonable genealogical chart of structural changes in the PBP family. The present analysis shows that the unidirectional change of protein evolution is clearly deduced at the level of protein three-dimensional structure rather than the level of amino acid sequence. See Ref. 4 for details.

(5) Evolutionary pattern of influenza B viruses based on the HA and NS genes during 1940 to 1999: origin of the NS genes after 1977

C. LUO[1], T. MORISHITA[2], K. SATOU[2], Y. TATENO, K. NAKAJIMA[1], K. and E. NOBUSAWA[1]
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Phylogenetic analysis was carried out for genes encoding hemagglutinin (HA) (24 new and 25 previously reported sequences) and nonstructural proteins (NS) (22 new and 14 previously reported sequences) of influenza B virus isolates obtained from 1940 to 1999. Two antigenically and genetically distant

HA lineages are presently known to exist. Divergence into these two lineages was estimated to have occurred around 1969. Phylogenetic analysis of NS genes revealed that their phylogenetic relationships were not linked to the two HA lineages but suggested that reassortment of viral genes between the viruses of two HA lineages had occurred. In addition two distinct NS lineages which were not linked the two HA lineages were observed. Viruses isolated after 1997 formed their own lineage in combination with B/Houston/84 while other virus isolates obtained from 1973 to 1995 comprised the other NS lineage. See Ref. 5 for details.

Publications

1. SUGAHAWA, H., MIYAZAKI, S., GOJOBORI, T. and TATENO, Y.: DNA Data Bank of Japan dealing with large-scale data submission. Nucleic Acids Res. **27**: 25-28, 1999.
2. WATANABE, M., SUMIDA, N., MURAKAMI, S., ANZAI, H., THOMPSON, C.J., TATENO Y. and MURAKAMI, T.: A phosphate-induced gene which promotes *Penicillium* mediated bioconversion of cis-propenylphosphonic acid to Fosfomycin. Applied and Environmental Microbiology. **65**: 1036-1044, 1999.
3. YAMAZAKI, M., TATENO, Y. and INOKO, H.: Genomic organization around the centromeric end of the HLA class I region: Large-scale sequence analysis. J. Mol. Evol. **48**: 317-327, 1999.
4. FUKAMI-KOBAYASHI, K. TATENO, Y. and NISHIKAWA, K.: Domain dislocation: a change of core structure in periplasmic binding proteins in their evolutionary history. J. Mol. Biol. **286**: 279-290, 1999.
5. LUO, C., MORISHITA, T., SATOU, K., TATENO, Y., NAKAJIMA, K. and NOBUSAWA, E.: Evolutionary pattern of influenza B viruses based on the HA and NS genes during 1940 to 1999: origin of the NS genes after 1977. Arch. Virol. **144**: 1881-1891, 1999.

I-d. Laboratory for Molecular Classification

(1) Data processing system for DDBJ

Hideaki SUGAWARA, Satoru MIYAZAKI

We enhanced the core system of the data processing in DDBJ to capture, review, accumulate and disseminate massive data produced by genome projects

and biodiversity study in collaboration with three laboratories in the Center for Information Biology.

(2) WFCC-MIRCEN World Data Centre for Microorganisms (WDCM)

Hideaki SUGAWARA, Satoru MIYAZAKI and Yumi FUJISAWA

We construct the Web site of WDCM for the World Federation for Culture Collections (WFCC) and the Microbial Resources Centers Network (MIRCEN). We provide our original databases of CCINFO and STRAIN and also a search engine named AHMII. The site was highly evaludated by the journal HELIX and the American Society for Microbiology and the number of accesses increased by 50% this year.

(3) Workbench for the microbial systematics

Hideaki SUGAWARA, Satoru MIYAZAKI and Hiroyuki OGAWA

Biodiversity is one of key subjects in biology in the 21st century. It is also an important issue on socioeconomic aspects such as sustainable use and equitable benefit sharing. However, we have only a limited knowledge on organisms on the planet. In the case of microorganisms, for example, it is said that we know less than 10% of all the strains that are alive in the environment and in other organisms. Therefore, taxonomy of known strains and identification of unknown strains is the infrastructure of the study and socioeconomic judgement about biodiversity. Without the systematics, we can not make a right decision on any biodiversity issues. We aimed at the development of an information system that would improve the systematics by the integration of analysis based on phenotypic characteristics (physiology, biochemistry, genetics and etc) and phylogenetic analysis based on sequence data. The final product provides such functions as: the integration of data sources distributed on the Internet; the integration of numerical taxonomy and phylogenetic analysis; the integration of probabilistic identification and the identification by a decision tree.

We actually sequenced 16sRNA of some fungi and did identification and classification in the course of the system development to put the system to practical use. We could also find the diversity of insertion of introns at genus level and species level.

For the novel function of the workbench, we define and study properties of the new measure based on Shannon entropy to evaluate the significance of the partial region on multiple aligned sequences for the reconstruction of phylogentic tree.

Publications

1. SUGAWARA, H., MIYAZAKI, S., GOJOBORI, T. and TATENO, Y.: DNA Data Bank of Japan dealing with large-scale data submission. Nuc Acids Res, **1**, 24-26, 1999.
2. SUGAWARA, H.: Culture Collections and Data Activities. Protein, Nucleic Acid and Enzyme. **44**, 175-180, 1999.
3. SUGAWARA, H: Proposal of Information Biology. Iden **53**, 39-43, 1999.
4. MIYAZAKI, S. and SUGAWARA, H.: Reconstruction of large-scale phylogenetic trees: problems and solutions,Amino Acids Vol.**17**, No.1, pp119-120.

Oral presentations

1. SUGAWARA, H. and MIYAZAKI, S.: An information system for data integration and polyphasic analysis of microbes. The Proceedings of the 99th General Meeting of the American Society for Microbiology, Chicago, 1999/5/30-6/3
2. MIYAZAKI, S. and SUGAWARA, H.: From linking to integration of biological databases, The International Joint Workshop for Studies on BIODIVERSITY (Species 2000, CODATA and Global Environment Tsukuba), Tsukuba, 1999/7/14-16
3. SUGAWARA, H.: Links between microbial resources and gene sequences, The proceedings of IXth International Congress of Bacteriology & Applied Microbiology,Sydney,1999/8/16-20
4. SUGAWARA, H.: Bio-resources are strategic information resources, the proceedings of Bio Resource Network Symposium, pp2-10, Tokyo, 1999/12/17
5. MIYAZAKIi, S. and SUGAWARA, H.: Development of a prototype system for sharing and analysing data of microbial cultures, the proceedings of Bio Resource Network Symposium, pp36-45, Tokyo, 1999/12/17

J. EXPERIMENTAL FARM

(1) Development, propagation and distribution of the genetic stocks of rice

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

We have conducted development, reproduction, reevaluation and distribution of genetic stocks of wild and cultivated strains of rice. About last two years, we have also been introducing new projects to propagate genetically engineered and transposon-tagged rice strains for utilizing them as genetic materials for functional analyses of a large number of rice genes. Resources we are generating are enhancer trap lines of Ac/Ds system and insertional mutant lines tagged by the endugenous transposon Tos17. Another trial to analyze the composition of the centromeric heterochromatin in rice chromosomes is also progressed. These projects are cooperatively conducted with the members of the plant genetics laboratory. For detail information, see the reports of plant genetics laboratory.

Publications

1. NONOMURA, K.I. and KURATA, N.: Organization of 1.9-kb repeat unit RCE1 in the centromeric region of rice chromosomes. Mol. Gen. Genet., **261**, 1-10, 1999.
2. MIYOSHI, K., NAKATA, E., NAGATO, Y. and HATTORI, T.: Differential *in situ* expression of three ABA-responsive genes of rice, *Rab16A*, *REG2* and *OSBZ8*, during seed development. Plant Cell Physiol., **40**, 443-447, 1999.
3. ITO, Y., EIGUCHI, M. and KURATA, N.: Expression of novel homeobox genes in early embryogenesis in rice. Biochim. Biophys. Acta, **1444**, 445-450, 1999.
4. ASHIKAWA, I., KURATA, N., SAJI, S., UMEHARA, Y. and SASAKI, T.: Application of restriction fingerprinting with a rice microsatellite sequence to assembling rice YAC clones. Genome, **42**, 330-337, 1999.
5. SENTOKU, N., SATO, Y., KURATA, N. ITO, Y., KITANO, H. and MATSUOKA, M.: Regional expression of the rice *KN1*-type homeobox gene family during embryo, shoot and flower development. The Plant Cell, **11**, 1651-1664, 1999.
6. MIYOSHI, K and KURATA, N.: Cloning of two cDNAs encoding the HAP3 subunit protein

from developing rice seeds. Rice Genetics Newsletter, **16**, 103-104, 1999.

7. AHN, B.O., MIYOSHI, K., ITOH, J-I., NAGATO, Y. and KURATA, N.: Mapping of a rice heterochronic gene, *Pla1*, regulating the plastochron and the duration of vegetative phase. Rice Genetics Newsletter, **16**, 36-37, 1999.

ABSTRACTS OF DIARY FOR 1999

Biological Symposium

Jan. 6 Circadian clock gene regulation in silkworm differs from Drosophila (Ivo Sauman)

How does the extra-nuclear transportations of protein and RNA occur ? (Mutsuhito Ohno)

Jan. 8 Histone acetylase complex and transcriptional control (Reiko Ohba)

Jan. 11 The Roles of PKI in the formation of a bilateral axis and mesodermal somite differentiation of chick embryo (Minoru Kawakami)

Jan. 18 Metabolic type glutamic acid receptor can be also activated by extracellular Ca^{2+} ! (Yoshihiro Kubo)

Feb. 1 Intracellular Traffic Control (Shahid S. Siddiqui)

Feb. 8 Polymerization control of actin filaments in the process of forming myofibrils: investigations on actin-binding protein using *C. elegans* as a model (Ichiro Onomasa)

Feb. 9 Finding gene regulatory signals in vertebrates using the compact genome of Fugu (Sydney Brenner)

Feb. 15 Analysis on the interaction between protein and DNA using atomic force microscope: visualization of nucleosome (Kunio Takeyasu)

Feb. 22 Effect of Demethylation on Imprinted X-inactivation (Takashi Sado)

Feb. 23 Effect of low concentration of guanidine hydrochloride upon the transcriptional activity of T7 RNA polymerase (Dipak Dasgupta)

Molecular Anatomy of *rrnB* P1: Transcription activation and regulation without factors besides RNA polymerase and promoter (Tamas Gaal)

Transcriptional activation mediated by the carboxy-terminal domain of RNA polymerase α subunit: Multipoint monitoring

with a fluorescent probe (Olga N. Ozoline)

Feb. 24 Biomathematical approaches on developmental phenomena (Yoh Iwasa)

Mar. 1 Chromosome Pairing and Synapsis in Yeast (Beth Rockmill)

Mar. 5 Olfactory Conditioning in Drosophila (Obaid Siddiqi)

Mar. 15 Site-Directed Mutagenesis of Active Site of Yeast Replicative DNA Polymerases δ and ε (Youri Pavlov)

Patterns of Single Nucleotide Polymorphisms in Human Genes (Aravinda Chakravarti)

New Ca indicator Chameleon using GFP energy transfer (Atsushi Miyawaki)

Molecular mechanism of protein transfer between nucleus and cytoplasm (Naoko Imamoto)

Mechanism of regulating JAK/STAT pathway by CIS/JAB: cytokine signal transmission (Akihiko Yoshimura)

Mar. 24 Ploughing a lonely furrow: the curious case of the *E.coli proU* P1 promoter (J. Gowrishankar)

Physiological roles of aggregation of sigma 70 in *E.coli* (Hiroki Nagai)

GroEL is involved in maturation of *E.coli* RNA polymerase devoid of omega subunit (Dipankar Chatterji)

Gene Express: intelligent system on gene expression regulation (Nikolay A. Kolchanov)

Mar. 31 Cytoplasmic signaling in neuronal growth cone guidance (Muming Poo)

Apr. 5 Protein Machines Directing Myosin Assembly (Henry F. Epstein)

Applications of XML in the replacement and searching for of information on about XML genome sequence of XML (Hideo Matsuda)

Apr. 20 Activity control of Ras family GTP binding protein Rap1: What is the meaning of diverse control mechanisms ? (Michiyuki Matsuda)

May 10 Generation time of HIV-1 (Yun-Xin Fu)

May 19 The Molecular Mechanisms of the Organelle Transport in Cells:

Identification and Characterization of New Motor Proteins, KIFs (Nobutaka Hirokawa)

May 21 Neuronal mechanism of molecular smell discrimination (Kensaku Mori)

May 28 Assembly and disassembly of protein complexes in homologous recombination (Akira Shinohara)

June 4 A new type of eukaryotic protein phosphatase required for dephosphorylation of the CTD and transcription by RNA PolymeraseII in *Saccharomyces cerevisiae* (Jack Greenblatt)

June 7 Structure of RecA protein-DNA complex (Katsumi Morimatsu)

June 9 Control mechanism of asymmetrical division in yeast (Kenji Irie)

June 10 Binding of "Sonic hedgehog" to "Patched" and patterning activity of neural tube (Naoyuki Fuse).

June 16 Survival signaling pathways in neuronal cells (Yukiko Goto)

June 24 Development of HIV vector and gene therapy (Hiroyuki Miyoshi)
Towards Mining Gene Expression Database (Shinichi Morisita)

June 28 Regulation of cytoskeleton and adhesion by intracellular signal (Kozo Kaibuchi).

July 1 p53 family genes: how to explore the biological functions (Yoji Igawa)

July 12 Transcriptional control mechanism in eucaryotic cell: with particular emphasis on TFIID, histone acetylase (Yoshihiro Nakatani)

July 13 Design of animal skin and Turing pattern (means and the limits of mathematical analyseis in biological science) (Shigeru Kondo)

July 14 Conserved Mechanism of Induced Mutagenesis: Newly Emerging Mutagneic DNA polymerases (Oomori Haruo)

July 27 Genetic Analysis of TGF- β Signaling - the long and short of it(Richard W. Padgett)

July 28 Multifunctional membrane molecule Neuropirin-1 that acts on the formations of neuronal circuits and blood vessels (Takahiko Kawasaki)

July 29 Asymmetric Cell Divisions and the Generation of Neuronal Diversity (William Chia)

Aug. 2	Artificial life and development of an evolutionary computer (Katsunori Shimohara)
Aug. 6	Downstream factor "ebi" of EGF receptor regulates "Notch" signals (Reo Tsuda)
Aug. 9	Studies on Learning and memory, and Activity-dependent Development of the Visual System with Genetically Engineered Mice: Multilevel Analysis of Hippocampus-dependent Memory (Susumu Tonegawa)
Aug. 19	Making a difference: the asymmetric division of stem cells in the germline (Haifan Lin)
Aug. 26	Control mechanism of neuronal system with JMK, p38MAP kinase cascade employing nematode *C. elegans* as model systems (Kunihiro Matsumoto)
Aug. 30	Structure and function of human killer cell immunoglobulin receptors (Katsumi Maenaka)
Sep. 1	Regulation of cytoskeleton with phosphorylation of myosin II (Shinichiro Kojima)
Sep. 2	Midline signaling in Zebrafish development (Atsushi Kawakami)
Sep. 13	Directional cues that guide axons in the zebrafish brain (John Y. Kuwada)
Oct. 4	How Cdc20 regulates cell cycle progression in *Saccharomyces cerevisiae* (Masaki Shirayama) Control mechanisms in of microtubular dynamics with Xenopus MAP 4 and identification and analysis of on factor that accumulates on centromere (Nobuyuki Shiina)
Oct. 5	Establishment of large-scale in-situ hybridization system and search for genes being expressed in small intestine and orchis of mouse in a site- or cell-specific manner through the use of such a system (Tohru Komiya)
Oct. 15	Brain mechanisms of behavior (Masakazu Konishi)
Oct. 20	Intracerebral mechanism of attention (Tadashi Isa) The extent to which language acquisition is modularized (Nobuo Masataka)
Nov. 1	TGF-beta signaling in vertebrate development: Insights from

zebrafish (Alexander Schier)
Genetic analysis of forebrain development in zebrafish (Stephen Wilson)

Nov. 8 Computer simulation of cell: follow-up report and future prospects for of E-CELL project (Masaru Tomita)

Nov. 12 Steps Common to Genetic Recombination in Eukaryotes, Prokaryotes, Archaea, and Phage (Stephen Kowalczykowski)

Nov. 15 The Multiple Genomic Origins of the Mitochondrial Proteome (C.G.Kurland)
DNA DAMAGE CHECKPOINTS AND DNA REPAIR IN BUDDING YEAST(Giovanna Lucchini)

Nov. 16 Discovery of new transcriptional control stage (Tadashi Wada)

Nov. 18 DNA replication-, transcription-, and translation-coupled biases from intra-strand Parity Rule2 (A=T and G=C) do not explain inter-specific variations of DNA G+C content(Noboru Sueoka)

Nov. 22 Transcription factories and the path of transcripts to the cytoplasm (Peter R. Cook)

Nov. 24 Strategies for analyzing large DNA clones: A Transposon-mediated insertion technique for analyzing the murine Hoxb Cluster (Timothy M. Jinks)

Nov. 29 Intracerebellar model and evolution of communication (Mitsuo Kawahito)

Dec. 13 REGULATING CHROMOSOMAL DNA REPLICATION IN EUKARYOTIC CELLS(John F.X. Diffley)
Role of *de novo* methyltransferases *Dnmt3a* and *Dnmt3b* in development and disease (En Li)
Structural and Kinetic Mechanisms of T7 RNA Polymerase Regulation (Rui J. Sousa)
Biochemical and Physiological/Genomic Studies of Nitrogen Signalling in Enteric Bacteria (Sydney Kustu)
Integration of Regulatory Signals at Bacterial Promoters (as revealed by the study of CRP- and FNR-dependent promoters) (Steve Busby)

Dec. 14 New gene that controls bilaterally axis (Yuji Yokouchi)

Dec. 15 Mechanism of preserving chromatin structure associated with DNA replication (Yasukazu Shibahara)

Dec. 16 Genetic analysis of on RNAi and inhibition of transposon transfer in nematode (Hiroaki Tahara).

Dec. 17 Study on gene-transfer recombinant molecular mechanism (Kiyoshi Mizuuchi)

FOREIGN VISITORS IN 1999

Jan. 6-7	Ivo Sauman, Institute of Entomology, Czech Academy of Sciences, Czech Republic
Jan. 8	Reiko Ohba, University of Virginia, Health Science Center, U.S.A.
Jan. 11-12	Minoru Kawakami, Harvard Medical School, U.S.A.
Jan. 21-Mar. 25	Thomas Daniel Andrews, Australian National University, Australia
Feb. 8-9	Sydney Brenner, Molecular Sciences Research Institute Inc., U.S.A.
Feb. 9-23	Lim Sun-Hee Univ. of Dong-a Department of Biology, Korea
Feb. 18-Mar. 24	Richard Hayward, University of Edinburgh, U.K.
Mar. 5-6	Obaid Siddiqi, Tata Institute of Fundamental Research, India
Mar. 6-23	Jean Thierry-Mieg, CNRS Laboratoire de Physique Mathematique, University MontpellierII, France
Mar. 6-23	Danielle Thierry-Mieg, CNRS Laboratoire de Physique Mathematique, University MontpellierII, France
Mar. 14-15	Youri Pavlov, Sainkt-Petersburg State University, Russia
Mar. 15-30	Nikolay A. Kolchanov, Russian Academy of Science, Russia
Mar. 24	Sandro J. de Souza, Ludwig Institute for Cancer Research, Brazil
Mar. 30-31	Mu-ming Poo, University of California San Diego, U.S.A.
Apr. 5-6	Henry F. Epstein, Baylor College of Medicine, U.S.A.
June 10-11	Naoyuki Fuse, Johns Hopkins University, U.S.A.
July 27-28	Richard W. Padgett, Waksman Institute Rutgers University, U.S.A.
July 29-Aug. 4	William Chia, National University of Singapore, Singapore
Aug. 2-13	Giorgio Bernardi, Stazione Zoologica Anton Dohrn, Italy
Aug. 6-7	Leo Tsuda, University of California Los Angeles, U.S.A.

Aug. 9-10	Susumu Tonegawa, Massachusetts Institute of Technology, U.S.A.
Aug. 19-20	Haifan Lin, Duke University Medical School, U.S.A.
Sep. 12-13	John Y. Kuwada, University of Michigan, U.S.A.
Oct. 3-4	Masaki Shirayama, Institute of Molecular Pathology, Austria
Oct. 14-15	Masakazu Konishi, California Institute of Technology, U.S.A.
Nov. 13-20	Giovanna Lucchini, Universita delgi Studi di Milano, Italy
Nov. 14-16	Charles G. Kurland, Evolutionary Biology Centre, University of Uppsala, Sweden
Nov. 21-22	Peter R. Cook, The Sir William Dunn School of Pathology The University of Oxford, U.K.
Nov. 24-25	Timothy M. Jinks, National Institute for Medical Research, U.K.
Dec. 4-14	John F.X. Diffley, ICRF Clare Hall Laboratories, U.K.
Dec. 13-14	Chun-Fang Wu, University of Iowa, U.S.A.
Dec. 13-14	En Li, Massachusetts General Hospital, U.S.A.
Dec. 15	Keiichi Shibahara, Cold Spring Harbor Laboratry, U.S.A.
Dec. 15-18	Hiroaki Tabara, University of Massachusetts, U.S.A.
Dec. 15-19	Kiyoshi Mizuuchi, NIH, U.S.A

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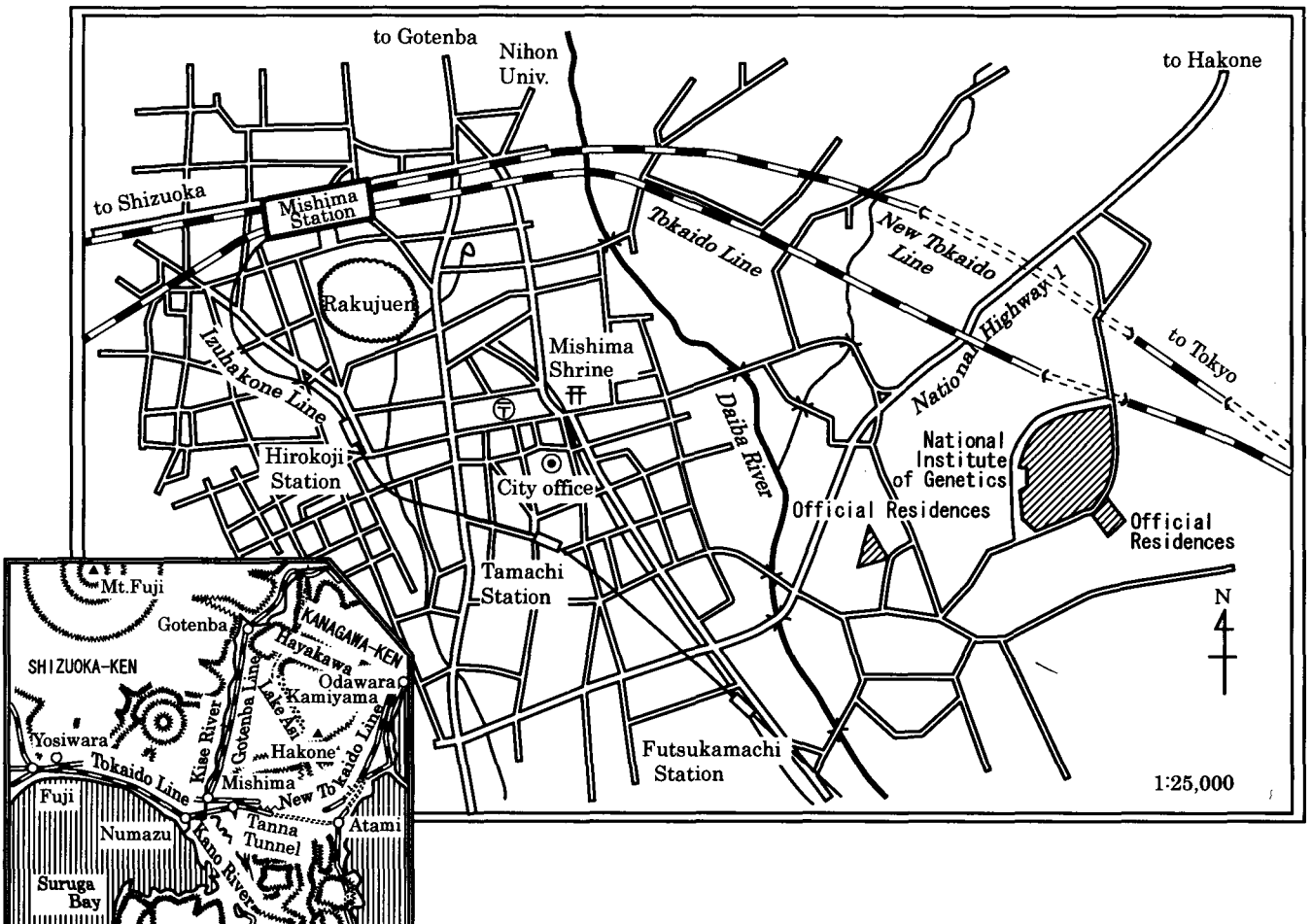
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