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GENERAL STATEMENT

National Institute of Genetics (NIG) was established 49 years ago as a center for genetics research. Major contributions of NIG in the research in genetics, in particular, plant genetics, population genetics and molecular and developmental genetics, have made it one of the distinguished centers with worldwide recognition. In 1984, NIG was reorganized into an Inter-university Research Institute to promote collaborative activities. Together with seven 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 32 graduate students and 10 special research students from other universities, including those from abroad. Eight students obtained Ph.D. this year. In addition, we have the Center Of Excellence (COE) program with which 5 foreign and 8 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 information on DNA sequences. In recent years, 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 established 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 service activities will continue to develop in the coming years. Our institute is uniquely suited for pursuing cooperative work with scientists of various disciplines by sharing the genetic resources.

This year, we have started two new research groups, Divisions of Early Embryogenesis and of Brain Functions. Such complex biological systems have

been new targets of genetics studies. Recent development of various molecular and developmental technologies are now enabling us to genetically dissecting these biological mechanisms. We would like to exploit fundamental and universal nature of genetics to tackle these difficult biological problems. We hope that such new activities will rejuvenate NIG in the 21st century.

In the past year we also saw a number of changes in the staff members of NIG. Prof. Takashi Imamura, Division of Human Genetics, and Prof. Hiroko Okino-Morishima, Division of Agricultural Genetics retired at the end of March. We would like to express our gratitude for their contribution to our institute. Newly appointed as professors are Drs. Hiroyuki Araki, Division of Microbial Genetics and Hiroyuki Sasaki, Division of Human Genetics. Dr. Toshihiko Shiroishi was also promoted as a professor of Mammalian Genetics Laboratory, and Prof. Yuji Kohara moved from Gene Network Laboratory to newly established Genome Biology Laboratory. Promoted as research associates are, Dr. Toshihiko Hosoya, Division of Developmental Genetics, Dr. Yoichiro Kamimura, Division of Microbial Genetics, and Dr. Takashi Tada, Mammalian Development Laboratory. On the other hand, the following members left NIG to take new positions to extend their career. Among them are Prof. Satoshi Horai (Graduate University for Advanced Studies), Drs. Hong-Wei Cai (Japan Forage Seed Association), Hiroshi Hara (Saitama University), Yasuaki Shirayoshi (Tottori University) and Toshihiko Akiba (Ministry of Agriculture, Forestry and Fishery).

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RESEARCH ACTIVITIES IN 1998

A. DEPARTMENT OF MOLECULAR GENETICS

A-a. Division of Molecular Genetics

(1) Mapping of Subunit-subunit Contact surfaces on the β and β' Subunits of *Escherichia coli* RNA Polymerase

Akira KATAYAMA, Tasuku NOMURA, Nobuyuki FUJITA and Akira ISHIHAMA

The assembly of *Escherichia coli* RNA polymerase takes place in a stepwise fashion under the order: $2\alpha \rightarrow \alpha_2 \rightarrow \alpha_2\beta \rightarrow \alpha_2\beta\beta'$(premature core) $\rightarrow E\sigma$ (E represents the core enzyme with the subunit structure $\alpha_2\beta\beta'$). The α subunit plays a key role in the assembly of core enzyme by providing the contact surface for both β and β'subunits. In the assembled core enzyme, one α subunit contacts with β subunit while the other α makes direct contact with β' subunit. The two large subunits, β and β', do not form stable binary complexes under isolated states, but after the core enzyme assembly, do make direct contact, leading to formation of the catalytic site for RNA polymerization. Previously we performed the fine mapping of subunit-subunit contact sites on the α subunit for dimerization and binding of β and β' subunits (Kimura and Ishihama, 1995; 1996). For mapping of the subunit-subunit contact sites on the two large subunits, we employed two approaches: (i) analysis of proteolytic cleavage sites on both β and β' subunits for unassembled free subunits, the intermediate subassembly $\alpha_2\beta$ complex, the core enzyme, and the β'σ^{70}complex; and (ii) analysis of complex formation between β fragments and the α subunit, between β' fragments and the $\alpha_2\beta$ complex, and between β' fragments and σ^{70} subunit.

Results of the β subunit analysis indicated that two regions of the β subunit are involved in the full activity of α binding, *i.e.*, the primary contact site between residues 737 and 904, and the secondary region with assembly con

trol activity downstream from residue 1138. The assembly activity of β fragments was also examined in the presence of both α and β 'subunits. Pseudo-core complexes consisting of α, β fragment and β' were formed for β(1-1318), β(445-1342) and β(737-1342), which all retain nearly the full activity of α binding. The level of pseudo-core complex formation is much lower for β(737-1138) in agreement with its low level activity of α binding. Thus, we conclude that the primary contact site of β' subunit on β is located close to the α-subunit contact sites, indicating that the binding sites for α and β' subunits form a single and the same structural domain (Nomura *et al*., 1999).

For mapping of the subunit-subunit contact sites on the β' subunit of *E. coli* RNA polymerase with the $\alpha_2\beta$ complex or the σ^{70} subunit, we employed two approaches. First, the protease sensitive sites were determined for unassembled free β' subunit (1407 amino acid residues). To identify the subunit-subunit contact sites on the β' subunit, we performed the binding assays of mixtures of proteolytic fragments of β' with either the $\alpha_2\beta$ complex or the σ^{70} subunit. For detailed analysis, we then prepared a series of recombinant β' fragments with or without His_6-tag by expressing the respective cloned gene segments, and tested for their activities of the binding of $\alpha_2\beta$ complex or σ^{70} subunit. Taken these results together we conclude that: (i) the two regions between residues 515-842 and 1141-1407 are involved in binding of the $\alpha_2\beta$ complex, and in addition, the N-terminal proximal region, residues 1-345, has a weak activity of $\alpha_2\beta$ binding; and (ii) the N-terminal proximal region, between residues 201-345, is central for binding of the σ^{70} subunit, and other regions, between residues 515-842 and 1141-1407, have weak activity of σ^{70} subunit binding (Katayama *et al*., 1999). 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.

(2) Search for Novel Transcription Factors Interacting with the β and β' Subunits of *Escherichia coli* RNA Polymerase

Tasuku NOMURA, Akira KATAYAMA, Nobuyuki FUJITA, Claude F. MEARES[1] and Akira ISHIHAMA ([1]Dept. Chem., Univ. Calif., Davis, USA)

RNA polymerase of *Esherichia coli* is composed of the core enzyme with the

subunit structure $\alpha_2 \beta \beta'$ and one of seven species of the sigma subunit. The N-terminal domain of α subunit plays a key role in core enzyme assembly while its C-terminal domain interacts with class-I (α-contact) transcription factors and upstream elements (UP) of promoter DNA. Some of *E. coli* transcription factors, designated as class-II (σ-contact) factors, interact with the σ subunit and regulate its function. Recently, several observations have been reported, which indicate that some *E. coli* transcription factors interact with the β and/or β' subunits. A systematic search for the class-III (β-contact) and class-IV (β'-contact) factors is in progress in this laboratory.

One new approach we employed for detection of protein-protein contacts is the phage display screening. This method is successfully used for mapping of an epitope against an antibody and for search of a peptide ligand against a receptor. One advantage is the large scale of screening (10^8-10^9 phage screening per experiment) and another is the easy amplification of phage clones for DNA sequencing. Filamentous phages used as a library display 15-mer random peptides fused to coat protein pIII (product of phage gene III) on a phage surface. A screening was carried out by mixing the phage library and a tethered ligate on a dish or a resin and followed by washing and elution of bound phages. So far we isolated phage clones which specifically bind to the β subunit of RNA polymerase, which was, after biotinylation, tethered on a surface of polystyrene dish mediated by streptoavidine-biotin bond. Results of the sequencing of 15-mer inserts of the cloned phages indicated that the majority are originated from non-specific binding of phages to the β subunit, the biotin-streptoavidin conjugate or even to plastic dishes, because many clones contained several tryptophan residues in the 15-mer region. Likewise, when Ni^{2+}-NTA agarose resin was used for immobilization of His_6-tagged β subunit, most clones contained several His residues. To exclude false positive clones, the binding assay by using ELISA will be carried out after the isolation of the phage clones bound to the β subunit. A screening against anti-β subunit antibodies will be performed to get a positive control for ELISA. The phage clones thus isolated should contain the epitope seqeuence of the β subunit.

The other approach for a large scale screening of proteins which interact with either β or β' subunit is to employ an affinity chromatography using glutathione S-transferase (GST) gene fusion system. Both β and β' subunits

of *E. coli* RNA polymerase were expressed as GST fused proteins under the control of *tac* promoter in *E. coli* strain W3110 in the absence of inducer IPTG. Cell lysate prepared by sonication was centrifuged to remove ribosomal fractions and the supernatant was applied onto glutathione Sepharose column. After washing the column, bound proteins were eluted with a buffer containing glutathione, and separated by SDS-PAGE. Each protein band was subjected to N-terminal amino acid micro sequencing. After N-terminal amino acid sequencing, we have detected several non-RNA polymerase proteins, including the 110 kDa RapA/HepA protein which has been identified to be associated with the purified RNA polymerase. Likewise, we have identified several proteins associated the His_6-tagged β' subunit.

In collaboration with C.F. Meares and colleagues (UC Davis), search for the class-III (β-contact) and class-IV (β'-contact) transcription factors is also in progress using the novel method of contact-dependent protein cleavage with a chemical protease tethered to known transcription factors. Previously we identified the σ^{70} subunit-contact sites on both β and β' subunits by mapping the cleavage sites generated by FeBABE tethered at various positions along σ^{70} (Owens *et al*., 1998). As an extension of this line studies, we have succeded to identify the contact sites of the NusA, GreA and ω proteins on β and/or β' subunits (Traviglia *et al*., 1999).

(3) Structural requirements for the interdomain linker of 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 structurally independent domains. The N-terminal domain (residues 1 to 235) is required and sufficient for core enzyme assembly, while the C-terminal domain (residues 249 to 329) plays key roles in transcription regulation by directly interacting with either class-I (α-contact) transcription factors or promoter UP elements. The intervening sequence connecting these two domains has high motional flexibility as revealed by the analysis of NMR dynamics (Jeon *et al*., 1997). The flexible nature of interdomain linker is also suggested by the findings that the C-terminal domain can be located along a long

range of the template DNA in different initiation complexes (Murakami *et al*., 1997). To analyze structural requirements, if any, for the interdomain linker, we introduced various mutations in this region and measured *in vitro* transcription activity after reconstitution of the mutant α subunits into respective holoenzymes.

Deletion of 3 amino acids from the interdomain linker exhibited 50% inhibition of the CRP-dependent *lac*P1 transcription. Deletion of 6 or more amino acids completely knocked out the CRP-dependent activity. Insertion of 3 amino acids did not affect the activity whereas insertion of 1, 2, or 4 amino acids showed 40-60% inhibition. Substitution of 10 consecutive glycine residues for almost the entire region of interdomain linker showed nearly 90% inhibition of the CRP-dependent activity. If several conservative amino acid substitutions were introduced so that the linker forms a strong alpha-helix without changing the length and charge distribution, nearly 50% activity was retained. In contrast, if the entire linker sequence was redesigned so as to form an amphiphilic helix, no activation was observed at all. These results altogether suggest that: (i) a sufficient length of the interdomain linker is required for the activation function of C-terminal domain; (ii) the C-terminal domain has a preferred orientation relative to the rest of RNA polymerase and the interdomain linker is not totally free for rotation; and (iii) some amino acid side chains, in addition to the flexibility of the peptide main chain, are important probably through the formation of loose helix-like structure for the formatiion of functional C-terminal domain. The UP element-dependent *rrnB*P1 transcription responded to various linker mutations almost similarly to the CRP-dependent *lac*P1 transcription, supporting our previous proposal that the factor-dependent activation and the UP element-dependent activation share the same structural requirements.

(4) Transcriptional Activation Mediated by the Carboxy-Terminal Domain of RNA Polymerase α Subunit: Multipoint Monitoring with a Fluorescent Probe

Olga N. OZOLINE, Nobuyuki FUJITA and Akira ISHIHAMA

The efficiency of transcription initiation by the RNA polymerase of *Escheri-*

chia coli depends on the nature of the two hexanucleotide sequences, located at -10 and -35 regions, and the third signal, located upstream from the -35 element. The upstream region of promoter *rrnB*P1, generally designated as UP element, contacts directly with the C-terminal domain of RNA polymerase α subunit (αCTD). The αCTD is also responsible for interaction with a set of protein accessory factors (class-I or α-contact factors) regulating transcription efficiency. The most studied regulatory protein from this group factors is cAMP receptor protein (CRP), regulating 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 α CTD for activation. Based on the mutant studies, we proposed a possibility that one and the same surface of αCTD participating in interaction with UP-DNA and class-I transcription factors overlap each other (Murakami *et al*., 1997). For precise identification of the contact sites on αCTD with DNA UP elements and protein factors, some physical approaches are required besides genetic and biochemical studies.

A novel approach employing a fluorescent reporter label positioned at various sites within the carboxy-terminal domain (CTD) of *Escherichia coli* RNA polymerase α subunit was used to monitor conformational changes of the protein upon transcription initiation complex formation. Previously we carried out some spectral measurements using the RNA polymerase containing the α subunit conjugated with a fluorescent probe, fluorescein mercuric acetate (FMMA), only at Cys269 (Ozoline *et al*., 1997). As an extension, we conjugated FMMA at a single Cys residue placed by the site-directed mutagenesis at various positions of αCTD. The FMMA-conjugated α subunits were reconstituted into the respective holoenzymes. Spectral parameters of FMMA were compared between free and promoter-bound forms of the RNA polymerase modified at a single position. The character of local structural changes was then analyzed in spatial dimension. Reliable conformational changes were observed for binary complexes formed with UP element-dependent promoter *rrnB*P1 and ternary complexes formed with cAMP receptor protein (CRP)-dependent promoter *uxuAB* in the presence of cAMP-CRP. Our results confirm the previous data indicating that the helix I of αCTD and loop region

between helices III and IV are involved in these complex formation, and indicate that a similar pathway is used by the enzyme to realize the activation signal accepted from either the UP-DNA or the activator protein CRP.

Some spectral changes were observed when the FMMA-modified RNA polymerase was mixed with cAMP-CRP in the absence of DNA, confirming the direct protein-protein contact. Upon binding to the *uxuAB* promoter in the absence of cAMP-CRP, the different changes were registered, which were similar with those observed for binary complexes with promoters T7*D* and mutant *rrnB*P1 lacking the UP element. These changes were different from those observed for the ternary complex formed on the *uxuAB* promoter in the presence of cAMP-CRP. When the upstream region of T7*D* was substituted by the *rrnB*P1 UP element, we found the changes typical for the latter promoter, suggesting a possibility that αCTD interacts with not only the *rrnB*P1-type UP element but also different types of the promoter upstream sequence using multiple structural elements (Ozoline *et al.*, submitted for publication).

(5) Regulatory Mechanisms of Sigma Subunit Activities of *Escherichia coli* RNA Polymerase

Miki JISHAGE, Hiroto MAEDA[1], Dipak DASGUPTA, Nobuyuki FUJITA and Akira ISHIHAMA ([1]Kagoshima Univ.)

The promoter recognition specificity of *Escherichia coli* RNA polymerase is conferred by one of seven molecular species of the σ subunit. Replacement of the σ subunit on RNA polymerase is an efficient way for switching the transcription pattern. The major σ subunit, σ^{70}, is responsible for transcription of most, if not all, genes expressed during the exponential cell growth. Other six species of the σ subunit are required only during certain growth stages or under specific growth conditions. The levels of these alternative σ subunits vary depending on the cell growth conditions (Jishage *et al.*, 1997; 1998). In addition to the level control, the activity of at least some *E. coli* σ subunits is also subject to control in various ways (Kusano *et al.*, 1997; 1998). For instance, the unused σ subunits are stored in inactive forms by forming complexes with another set of proteins, often designated as anti-σ factors, with the regulatory activity of σ functions. 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 (Jishage *et al.*, 1998). 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*. The expression of a reporter gene, *lacZ*, 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 RNA polymerase σ subunit from σ^{70} to σ^{S} during the transition from exponential growth to stationary phase (Jishage and Ishihama, 1999).

Along this line of studies, we carried out the search for a regulatory protein of σ^{S} function. By using the GST-fusion version of the σ^{S} subunit expressed in *E. coli* W3110, we have identified one specific protein associated with the σ^{S} subunit. The identification of this σ^{S}- associated protein is in progress.

(6) Role of the σ^{S} subunit in stationary phase survival of *E. coli*: Search for σ^{S}-dependent promoters and analysis of *rpoS* mutations

P.R. SUBBARAYAN and Akira ISHIHAMA

Seven different molecular species of the σ subunit, the promoter recognition subunit of RNA polymerase, are known to exist in *E. coli*, each recognizing a specific set of promoters. The σ^{38}(σ^{S})subunit encoded by the *rpoS* gene is synthesized in the stationary phase of cell growth (Jishage and Ishihama, 1995; 1996), and is involved in transcription of at least some of the stationary

phase-specific genes. Up to now, however, no consensus sequence has been identified for σ^S-dependent promoters, and instead we found that most of the σ^S-dependent promoters are recognized by the σ^{70} subunit, which is involved in the recognition of growth-related genes in the exponential phase (Tanaka *et al*., 1995; Kolb *et al*., 1995). Moreover, some *E. coli* strains lack the σ^S subunit, but they can survive in the stationary phase (Jishage and Ishihama, 1997). Thus, the physiological role of σ^S subunit remains unclear.

In order to search for promoters under the control of the σ^S subunit, we tried three different systems of *in vivo* screening: (1) search in the stationary phase for antibiotics-resistant *E. coli* W3110 transformants carrying a promoter cloning vector fused to an antibiotic resistance reporter gene; (2) search for H_2O_2-resistant transformants carrying a promoter cloning vector fused to the gene encoding Dps (DNA binding protein from starved cells) which confers protection against the killing effect by H_2O_2 in the stationary phase *E. coli* ; and (3) search in the growing phase for high level β-galactosidase expressing transformants carrying both a promoter cloning vector fused to the *lacZ* reporter gene and an expression vector for the σ^S subunit. All three trials were, however, unsuccessful for large scale screening of the σ^S-dependent promoters.

A group of *E. coli* strains have been suggested to contain a truncated form of the σ^S protein. Sometime ago we found that, although the original *E. coli* W3110 strain contains the full-length complete copy of the σ^S subunit, one lineage W3110 lacks the σ^S protein and another lineage contains a truncated form of the σ^S subunit (Jishage and Ishihama, 1997). To understand the molecular basis of the variation in σ^S subunit composition, we analyzed the nucleotide sequences of the entire *rpoS* gene for all five lineage of the W3110 strain. In parallel, we analyzed the *rpoS* gene sequence of several *E. coli* strains, and found that a group of *E. coli* strains different from *E. coli* W3110 variant also carry the truncated form of σ^S subunit. Taken together with reports from other groups, it appears that a large number of mutations have accumulated in the *rpoS* gene coding for the RNA polymerase σ^S subunit.

(7) Growth Phase-Dependent Variation in the Levels of Twelve Species of the Nucleoid Protein in *Escherichia coli*

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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. For the switching of global pattern of gene transcription among about 4,000 genes on the *E. coli* genome, the understanding of growth-dependent change in the conformation of the nucleoid. In this study, we performed for the first time a systematic determination of the intracellular concentrations of twelve species of the nucloeid protein, 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 Inversion Stimulation), Hfq (Host Factor for phage Q_β replication), H-NS (Histone-like Nucleoid Structuring protein), HU (Heat-Unstable nucleoid protein), IciA (Inhibitor of Chromosome Initiation A), IHF (Integration Host Factor protein), Lrp (Leucine-Responsive regulatory Protein), and StpA (Suppressor of *td*- Phenotype A). For this purpose, we over-expressed all these nucleoid proteins using the respective cloned genes. Previously, we determined the sequence specificity and DNA-binding affinity for these twelve nucleoid proteins (Talukder and Ishihama, 1999). Here we produced specific antibodies in rabbits against each of the twelve nucleoid proteins and using the quantitative Western blot method, we measured the intracellular concentrations of all these nucleoid proteins in *E. coli* strain W3110 growing at different growth phases (Talukder *et al*., 1999).

The level is maximum at the growing phase for nine proteins, CbpB, 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 in growing *E. coli* is: Fis>Hfq>HU>StpA>H-NS>IHF*>CbpB>Dps*>Lrp>DnaA>IciA>CbpA* (stars represent the station-

ary-phase proteins). The major protein components of nucleoid change from Fis, Hfq and HU in the growing phase to Dps and IHF in the stationary phase. The order of abundance in the early stationary-phase is: Dps*>IHF*>HU>Hfq>H-NS>StpA>CbpB>DnaA>Lrp>IciA>CbpA>Fis, while that in the late stationary phase is: Dps*>IHF*>Hfq>HU>CbpA*>StpA>H-NS>CbpB>DnaA>Lrp>IciA>Fis. The curved DNA-binding protein, CbpA, appears only in the late stationary phase. This change in nucleoid protein composition in the stationary phase is accompanied by compaction of the genome DNA and silencing of the genome functions.

(8) Growth Phase-Coupled Changes in the Ribosome Pattern of *Escherichia coli* and Effect of Polyamines on Ribosome Dimerization

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The 70S ribosomes in *Escherichia coli* are converted into 100S ribosome dimers during the growth transition from exponential to stationary phase. A 55 amino acid long basic protein called ribosome modulation factor (RMF) is required for ribosome dimerization. RMF is a stationary phase-specific gene product. Since *E. coli* mutants lacking the *rmf* gene encoding RMF are unable to survive in the stationary-phase, RMF is considered to be an essential gene product for survival in the stationary phase (Ishihama, 1999). The dimeric form of ribosomes is virtually inactive in translation of at least some mRNA templates. This suggests that ribosome dimers are inactive, stored forms of ribosomes in stationary-phase *E. coli*. Overproduction of spermidine results in reduction of the synthesis level of RMF, ultimately leading to cell death in the stationary phase (Apirakaramwong *et al.*, 1998). The addition of Mg^{2+} restores partially the cell death in the absence of RMF production.

In order to further explore the role of RMF-dependent ribosome dimerization in cell viability, we examined the relationship between the formation of ribosome dimers and the fate of *E. coli* cells. For this purpose, we used a set of *E. coli* strains from the ECOR collection, that includes a set of 72 reference *E. coli*

strains isolated from a variety of hosts and geographical locations. Distributions of ribosomes and of ribosomal subunits were studied by zone centrifugation analysis in sucrose density gradients of extracts from overnight cultures of 19 strains from the ECOR collection. These 19 strains were classified into four groups on the basis of differences in their ribosomal sedimentation patterns revealed in extracts from 24-hour cultures. Group-I strains revealed the typical 100S dimers, while ribosomes from group-IV contained as their fastest component the 70S monomer-like peak. Group-II and -III strains yielded ribosomes with components that sedimented at velocities intermediate between 100S dimers and 70S monomers.

Culture time-dependent change in the ribosome pattern was analyzed with extracts from representatives of each group, grown in medium E supplemented with 2% polypeptone. All the test strains showed a common growth phase-coupled change in the sedimentation pattern for ribosomes. This consists of four discrete stages: (i) formation of 100S dimers in the early stationary phase; (ii) transient decrease in the dimer level; (iii) resumption of dimes to the maximum level; and (iv) dissociation of 100S dimers into 70S ribosomes which are quickly disassembled into subassemblies. The total time length for this cycle of ribosome pattern change was, however, different among the four group strains, leading to the apparent difference in the ribosomal distributions when observed at fixed time points. The group I strains retained the complete 100S ribosome dimers at least until day 4 to 5 in the stationary phase under the culture conditions employed, while ribosomes in the group IV strains were disassembled earlier. The number of viable cells decreases concomitantly with the disappearance of ribosome dimers. Therefore, we propose that a close correlation exists between the time of disappearance of ribosome dimers and that of cell death. In effect, it appears that ribosome dimerization is essential for the stationary phase survival of *E. coli* (Wada *et al*., submitted for publication).

(9) Cloning and Identification of Subunit 4 (Rpb4) of RNA Polymerase II from the Fission Yeast *Schizosaccharomyces pombe*

Hitomi SAKURAI, Hiroshi MITSUZAWA, Makoto KIMURA and Akira ISHIHAMA

The RNA polymerase II in eukaryotes is composed of more than ten different polypeptides (Ishihama *et al*., 1998). The genes coding for all 12 putative subunits of the RNA polymerase II have been isolated from the budding yeast *Saccharomyces cerevisiae* and human. Previously we reported that the purified RNA polymerase II from the fission yeast *Schizosaccharomyces pombe* contained at least eleven polypeptides, devoid of the component corresponding to RPB4 of *S. cerevisiae* (Sakurai *et al*., 1998). Recently, the genes coding for the subunit 4 were cloned from human and plant *Arabidopsis thaliana*. We then re-examined whether the purified RNA polymerase II from *S. pombe* contains Rpb4 or not. After search for a sequence containing the conserved sequences of the subunit 4 from the budding yeast, human and plant in the PomBase (the DNA database for *S. pombe*), we identified the putative *rpb4* gene. From the DNA sequence of a cDNA clone that was isolated by PCR using the synthetic primers with the *rpb4* sequence, Rpb4 was found to consist of 135 amino acid residues with a molecular mass of 15,362.

For identification of the protein product, we made antibodies against the over-expressed and purified protein encoded by the putative *rpb4* cDNA, and carried out Western blotting using various step fractions of RNA polymerase II purification from *S. pombe*. The Rpb4 band was found to co-migrate on SDS-PAGE with other three subunits, Rpb8, Rpb9 and Rpb11 [thus, previously, we failed to detect Rpb4] (Sakurai *et al*., 1999). As in the case of the corresponding subunits from higher eukaryotes such as human and plant *A. thaliana*, Rpb4 is smaller in size than RPB4 from the budding yeast *S. cerevisiae*, and lacks several segments, which are present in the *S. cerevisiae* RPB4, including the highly charged sequence in the central portion. The RPB4 subunit of *S. cerevisiae* is not essential for normal cell growth but is required for cell viability under stress conditions. In contrast, *S. pombe* Rpb4 was found to be essential even under normal growth conditions. The fraction of RNA polymerase II containing RPB4 in exponentially growing cells of *S. cerevisiae* is about 20%,

but the *S. pombe* RNA polymerase II contains the stoichiometric amount of Rpb4 even at the growing phase. Rpb4 and Rpb7 formed a stable heterodimer when co-expressed in *Escherichia coli*.

(10) Subunit Assembly of *Schizosaccharomyces pombe* RNA Polymerase II

Makoto KIMURA and Akira ISHIHAMA

RNA polymerase II from the fission yeast *Schizosaccharomyces pombe* consists of twelve species of subunits, Rpb1 to Rpb12 (Sakurai *et al*., 1999). We expressed the eleven subunits, except for Rpb4, simultaneously in the same cultured insect cells using combinations of various recombinant bacculovirus expression vectors (Kimura *et al*., 1999) [When we started this project, the gene for Rpb4 had not yet been cloned, but recently, we have succeded the cloning of *rpb4* gene and cDNA (Sakurai *et al*., 1999)]. For this purpose, we constructed the recombinant bacculoviruses, each carrying one or two subunit cDNAs. Coinfection of up to six species of the recombinant virus attained the coexpression of any combination among all eleven subunits. For isolation of subunit complexes formed in the infected insect cells, a glutathione-S-transferase (GST) sequence was fused to the *rpb3* cDNA as to produce GST-Rpb3 fusion protein and a histidine(His_{10})-tag sequence was inserted into *rpb1* cDNA to produce Rpb1H protein. After glutathione affinity and succeeding Ni^{2+}-affinity chromatographies, a subunit complex consisting of seven subunits, Rpb1H, Rpb2, GST-Rpb3, Rpb5, Rpb7, Rpb8 and Rpb11, was identified. Removal of any one of the six subunits besides GST-Rpb3 did not affect the assembly of other subunits, indicating two possibilities that: (i) the Rpb3 interacts directly with other six subunits; and/or (ii) the subassembly formation depends on the multiple contacts, in which the absence of any single subunit does not affect the assembly of other subunits. Direct interaction between the Rpb3 and other six subunits were detected by pairwise coexpression experiments. Coexpression experiments of various combinations of a few subunits revealed that the Rpb11 enhances the Rpb3-Rpb8 interaction and that Rpb8 does the Rpb1-Rpb3 interaction. Taken together we propose that the multile subunit-subunit contacts are involved in stabilization of the RNA

polymersase II consisting of twelve subunits (Kimura *et al.*, submitted for publication).

(11) Isolation and Characterization of Temperature-sensitive Mutations in the Subunit 3 Gene *(rpb3)* of RNA Polymerase II of the Fission Yeast *Schizosaccharomyces pombe*

Jiro MITOBE, Hiroshi MITSUZAWA, Kiyoshi YASUI and Akira ISHIHAMA

The RNA polymerase II of the fission yeast *Schizosaccharomyces pombe* consists of twelve subunits (Sakurai *et al.*, 1999). The subunit 3 (Rpb3) is homologue of the prokaryotic RNA polymerase α subunit, which plays a key role in subunit assembly of this complex enzyme by providing the contact surfaces for both β and β' subunits. In order to get insight into the physiological role(s) of Rpb3, we have performed mutant studies for the *S. pombe rpb3* gene. A total of nine temperature-sensitive (Ts) and three cold-sensitive (Cs) *S. pombe* mutants have been isolated, each carrying a single mutation(s) in the *rpb3* gene in one of the four regions (A to D) conserved among the eukaryotic subunit 3 homologues. The three Cs mutations were all located in the region A, in agreement with the most important role of the corresponding region in the assembly of prokaryotic RNA polymerase, while the Ts mutations were scattered in all four regions. Among them, seven representative Ts mutants were subjected to further analysis.

Growth curve of these Ts mutants was characteristic for the asembly defective mutant at non-permissive temperatures. Two recessive suppressors were independently isolated from Ts3-154 which produces a mutant RNA polymerase with mutation. After cloning and sequencing, it was found that one suppressor mutation codes for a mutant Pts1, a proteasome component, and the other encodes a mutant version of a homolog of *S. cervisiae* PRS7 (26S protease regulatory subunit 7). These plasmids were not exchangeable each other. Taken these observations together with a high reversion rate we indicate that multiple pathways exist for suppression of the Ts phenotype. Western analysis of seven Ts strains revealed the amounts of mutant Rpb3 proteins were significantly reduced under restrictive temperature. Since the meta-

bolic stability of most Ts mutant Rpb3 proteins were markedly reduced at a non-permissive temperature, these mutant Rpb3 proteins seems defective in the assembly or the mutant RNA polymerases containing the mutant Rpb3 were inactive. In addition to these observation, the Ts phenotype of all seven mutants were suppressed to various extents by the over-expression of Rpb11, the pairing partner subunit in the core subassembly. We conclude that the majority of *rpb3* mutations affects the subunit assembly of Rpb3, even though the extent of influence on the subunit assembly is different depending on the location of mutations (Mitobe *et al*., 1999).

(12) Isolation and Characterization of Temperature-sensitive Mutations in the Genes for Rpb6, Rpb7 and Rpb11 of RNA Polymerase II of the Fission Yeast *Schizosaccharomyces pombe*

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The RNA polymerase II of the fission yeast *Schizosaccharomyces pombe* consists of twelve subunits (Sakurai *et al*., 1999). The two large subunits, Rpb1 and Rpb2, are the eukaryotic homologues of the β and β' subunits, repespectively, of prokaryotic RNA polymerase, and form the catalytic center for RNA polymerase, as observed by photo-affinity cross-linking of nascent RNA products (Wlasoff *et al*., 1999). Both subunits 3 and 11 carry limited sequence homology with the N-terminal assembly domain of prokaryotic α subunit. Little is known about the functions of other eight subunits. For identification of the roles of minor subunits in the assembly and function of RNA polymerase II, mutant studies are in progress. So far we isolated temperature-sensitive (Ts) mutants of *S. pombe* with mutations in the *rpb3, rpb6*, *rpb7* and *rpb11* genes encoding subunits 3, 6, 7 and 11, respectively.

Rpb6 (142 amino acid residues) is one of the common subunits among RNA polymerases I, II and III. From the deletion analysis, the essential region for functions was located within the C-terminal proximal half of *rpb6* (Ishiguro *et al*., 1999). By random mutagenesis by PCR and after replacement of the chromosomal *rpb6* gene by the mutant alleles, we isolated a total of 14 Ts

mutants with mutations in the *rpb6* gene. The mutations, however, clustered along the entire *rpb6* gene including the non-essential N-terminal half, but all located within the conserved sequences among the known Rpb6 homologues. Among the multi-copy suppressors of one *rpb6* mutation, we identified the gene for TFIIS, the elongation factor for RNA polymerase II, indicating the direct protein-protein contact between Rpb6 and TFIIS. In fact, the formation *in vitro* of binary complex was confirmed using the purified preparations of Rpb6 and TFIIS (Ishiguro *et al*., 1999).

In the case of *S. cerevisiae*, Rpb7 is known to be reversibly dissociated, forming a complex with Rpb4, from the RNA polymerase II from growing cells. In contrast, both Rpb4 (135 amino acid residues) and Rpb7 (172 amino acid residues) were found to be the essential subunits in *S. pombe* and are tightly associated with the RNA polymerase II (Sakurai *et al*., 1999). By random mutagenesis of *rpb7* and replacement of the chromosomal *rpb7* gene by the mutant alleles, we isolated three Ts mutants, each carrying a single mutation in the *rpb7* gene. One of the suppressor mutants was identified to carry a second mutation in *rpb7*, indicating the intragenic suppression for the observed phenotypic reversion (Mitsuzawa, unpublished).

Rpb11 (135 amino acid residues), another homologue of prokaryotic α subunit, forms a heterodimer with Rpb3. For isolation of the *rpb11* mutants, we constructed a *rpb11* disruptant of *S. pombe* which can survive in the presence of plasmid carrying the *rpb11* gene. In the absence of this plasmid, the mutant *S. pombe* is unable to survive, indicaing that *rpb11* is an essential gene for cell viability. By random mutagenesis by PCR of the *rpb11* gene on the plasmid and after integration of the mutagenized *rpb11* gene into the chromosome, we isolated five Ts and three Cs (cold-sensitive) mutants, each carrying a single amino acid substitution mutation on the *rpb11* gene. By GST-pull down assays, recombinant mutant Rpb11 protein was found to have reduced activity of Rpb3 binding. The isolation of multicopy suppressors is in progress (Komoto, unpublished).

(13) Identification of the RNA Cap 1-binding Sites on the PB2 Subunit of Influenza Virus RNA Polymerase

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Influenza virus genome is composed of eight RNA segments of negative polarity, which is transcribed into plus-strand translatable RNA by the virus-associated RNA polymerase. The viral RNA polymerase is composed of three viral P proteins, PB1, PB2 and PA. PB1 carries the catalytic site of RNA polymerization while PB2 plays a role in capped RNA recognition for generation of transcription primers. We have been concerned with the mapping of functional sites for each P subunit polypeptide, including the subunit-subunit contact sites. Previously, we identified two sites of photo-affinity crosslinking of radioactive 8-azido GTP (8-N_3 GTP) on the PB1 subunit, *i.e.*, the amino terminal-proximal site I and the carboxy terminal-proximal site II, each being close to that of sequence motif A and motif D, respectively, conserved among RNA-dependent RNA polymerases.

On the other hand, capped RNA with ^{32}P label only at its 5' cap-1 structure was photo-affinity cross-linked to the PB2 subunit. In order to identify the capped RNA-binding sites on PB2, we prepared capped RNA with ^{32}P radioactivity only at the cap-1 structure, and UV-crosslinked it to the isolated RNP (ribonucleoprotein cores consisting of vRNA, the PB1, PB2 and PA subunits of RNA polymerase, and NP). After RNase A treatment, the radio-labeled PB2 was isolated by SDS-PAGE, and digested, together with unlabeled PB2, with V8 protease. Cleavage fragments were separated by SDS-polyacrylamide gel electrophoresis. Fragments with cross-linked cap-1 were blotted on PVDF membranes and subjected to amino acid sequencing. Results indicated that two regions of PB2 are involved in the binding of 5' cap-1 structure of capped RNA, one at the N-terminal proximal region downstream from the PB1-contact site and the other around 500 amino acid residue region overlapping the motif commonly found in cellular cap-binding proteins (Honda *et al.*, 1999).

(14) Search for Host Factor(s) Interacting with Influenza Virus RNA Polymerase

Ayae HONDA, Takuto OKAMOTO and Akira ISHIHAMA

Influenza virus RNA polymerase 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 (Honda *et al*., 1997). 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 are being made to identify host proteins which interact with each of the P proteins using yeast two hybrid screening system. So far several positive clones have been isolated for each P protein, and some of these putative PB1-, PB2- and PA-interacting host factors were found to form complexes *in vitro* by mixing with the respective P proteins. Functional conversion of the RNA polymerase after interaction with each of these proteins is being analyzed.

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

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For the molecular anatomy of the multi-functional RNA polymerase of influenza virus with the activities of both RNA polymerization and RNA degradation, playing in both tanscription and replication, experimental systems are required, for both the purification of large quantities of the RNA polymerase and the construction of mutant RNA polymerases with a specific mutation at a defined site of a specific subunit. The efficiency of enzyme reconstitution

we established was still low and thus the reconstitution system is costly, time-consuming and technically inconvenient. To meet the demand for large amounts of the influenza virus RNA polymerase for analysis the structure-function relationship of each P protein subunit, we succeded to establish a simultaneous expression system of all three P protein subunits in the methylotrophic yeast *Pichia pastoris*, and to purify the template-free functional RNA polymerase.

The methylotrophic yeast *Pichia pastoris* is able to catabolize 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 includes: (i) a strong, tightly-regulated alcohol oxidase promoter, 5'AOX1,is used; (ii) a stable production can be achieved in a large volume fermentor culture; (iii) a secretory pathway allows the product secreted into the medium, separating the foreign protein from most of the native proteins; (iv) the expression system can be easily constructed; and (v) the cost is equally as low as the *Escherichia coli* expression system. For the expression of influenza virus RNA polymerase, the cDNAs for RNA segments 1, 2 and 3 of influenza virus A/PR8, each under independent control of the alcohol oxidase gene promoter, were integrated into the *P. pastoris* chromosome. For the purification purpose of P protein complexes, a hexa-histidine (His_6 tag) was added at N-terminus of the P2 protein. Expression of the P proteins in *P. pastoris* was optimized by varying the concentration of methanol and the induction time after the addition of methanol. Results of the recombinant protein expression indicate that: (i) a reasonable amount of all three P proteins was expressed and recovered in the cell lysate; (ii) all three P proteins formed a complex(es) as detected from the co-elution pattern from Ni^{2+} NTA agarose column; (iii) the P protein complex carried the model RNA-dependent transcription activity *in vitro* ; and (iv) the RNA polymerase recognizes specific sequences for transcription initiation as determined by using various model RNA templates with or without the terminal conserved sequences of both vRNA and cRNA segments. We conclude that the functional influenza virus RNA polymerase can be formed in the methylotrophic yeast *P. pastoris*.

(16) Isolation from TMV-infected Tobacco of the RNA-dependent RNA Polymerase with the Subunit Composition of 126K/183K Heterodimer

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The genome of tobacco mosaic virus (TMV) consists of a single-stranded RNA molecule of about 6,400 nucleotides in length with positive polarity, which encodes at least four polypeptides, the 126 and 183 kDa proteins required for transcription and replication, the 30 kDa protein for cell-to-cell virus movement in infected plants, and the 18 kDa protein for virus coat formation. Three segments of the 183K protein, each containing the sequence motifs of methyltransferase (M), helicase (H) or RNA-dependent RNA polymerase (P), were expressed in *Escherichia coli* as fusion proteins with hexa-histidine (His_6) tag, and domain-specific antibodies were raised in rabbits against the purified His_6-tagged M and P proteins. By immunoaffinity column chromatography, a heterodimer(s) consisting of one molecule each of the 126K (amino terminal-proximal portion of 183K protein) and full-length 183K viral proteins was isolated. To check the RNA synthesis activity for the purified 183K-126K heterodimer, we constructed model RNA templates of short chain length, but containing the 5'- and 3'-terminal sequences of TMV RNA. In the presence of this model RNA template, the 183K-126K heterodimer exhibited the activity of synthesis *in vitro* of template-sized RNA, but the activity was not detected with RNA without the TMV terminal sequences. We propose that the TMV RNA polymerase protomer is composed of one molecule each of the 130K and 180K proteins (Watanabe *et al.*, 1999).

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A-b. Division of Mutagenesis

(1) 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 F-box proteins, have been implicated in Cdc34-dependent ubiquitination in *Saccharomyces cerevisiae*. Grr1, which is 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. Grr1 also interacts in vitro with phosphorylated Cln1 and Cln2. Domain analysis was performed to understand the role of Grr1 in the degradation of Cln2. In addition to the F-box, Grr1 has another motif, leucine-rich repeats (LRR). We found that the LRR is a domain for Cln2 binding. A deletion of half of the LRR abolished the interaction of Grr1 with phosphorylated Cln2 but not with Skp1 in vivo, and a

deletion of the F-box abolished the interaction of Grr1 with Skp1 but not with phosphorylated Cln2 in vivo. Based on these results, we constructed *grr1* mutants that are defective in association with either Skp1 or Cln2. Cln2 was highly stabilized and accumulated with the phosphorylated forms in the mutant cells. Furthermore, Skp1 associated in vivo with phosphorylated Cln2 in a Grr1-dependent manner. These data suggest that Grr1 is required for degradation of Cln2 through linking phosphorylated Cln2 to Skp1 in SCF^{Grr1} complex. See Ref 1 and 2.

(2) Ubiquitin pathway functioning in mitotic regulatory mechanism

Toshimasa TADAKI, Hiroaki SEINO and Fumiaki YAMAO

A ubiquitin-conjugating enzyme, UbcP4, in fission yeast is essential for cell growth. In vivo depletion of the UbcP4 demonstrated that it was necessary for cell cycle progression at two phases, G2/M and metaphase/anaphase transitions. The G2-arrest of UbcP4-depleted cells was dependent upon *chk1* which mediates checkpoint pathway. Arrest at metaphase resulted in duplicated and condensed, but not separated chromosomes. Overexpression of UbcP4 specifically rescued the growth defect of $cut9^{ts}$ cells at a restrictive temperature. *cut9* encodes a component of the anaphase promoting complex (APC) which is required for chromosome segregation at anaphase and moreover is defined as cyclin-specific ubiquitin ligase. Cdc13, a mitotic cyclin in fission yeast, was accumulated in the UbcP4-depleted cells. The phenotypes were also confirmed by using temperature-sensitive mutants of *ubcP4*. These results strongly suggested that UbcP4 is a ubiquitin-conjugating enzyme working in conjunction with APC. To get more genes that could be functioning in or around the UbcP4/APC pathway, two approaches were adopted, screening of the multicopy suppressors of *ubcP4* mutations, and search of conditional mutants suppressible by UbcP4 overproduction. Two types of multicopy suppressors, a kind of transcription factors and a tentative component of nuclear pore complex, have been identified. Multiple essential genes associated with the UbcP4 pathway were found through the latter screening. Characterization of these candidates is now undergoing.

(3) Degradation of Cig2 protein is regulated by ubiquitin-pathway involving UbcP4 and APC complex

Hiroaki SEINO and Fumiaki YAMAO

Ubiquitin-conjugating enzyme UbcP4 in fission yeast works together with anaphase promoting complex (APC), mediating ubiquitin-pathway forward destruction of Cut2 for onset of anaphase as well as of Cdc13, a mitotic cyclin in fission yeast, for exit from mitosis. Another cyclin, Cig2, functions during S-phase in fission yeast, and is destroyed before mitosis. Cig2 has a degradation signal (named destruction box) similar to that of Cdc13. From analysis with temperature-sensitive mutants of *ubcP4* and *cut9* encoding a component of APC, Cig2 was suggested to be another target of the UbcP4/APC pathway. Both temperature-sensitive *ubcP4* and *cut9* mutant cells are hypersensitive to an overproduction of Cig2 protein. Null mutation of *cig2* caused a decrease of restrictive temperature. Expression of the Cig2 lacking the destruction box was deleterious to the cells. These suggested that Cig2 protein is regulated via proteolysis involving UbcP4 and APC. The cyclin boxes of Cig2 and Cdc13 were found exchangeable each other. The phase-dependent destruction of Cig2 and Cdc13, was evidently determined by their structures of N-terminus outside the cyclin 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.

(4) Survey and functional analysis of ubiquitin-conjugating enzymes in *S. pombe*

Joon-Hyun PARK, Hiroaki SEINO and Fumiaki YAMAO

Previous works and completion of genome sequencing revealed 13 families of ubiquitin-conjugating enzyme (Ubc) in *S. cerevisiae*. Phenotype of each Ubc differs each other. Two of them are essential for growth while others are not. Some Ubc's are functionally redundant. To understand the functional differences of Ubc in other species, we have surveyed Ubc's in fission yeast. In our previous works, four Ubc's (UbcP1-P4) was identified through screening by

their enzymatic activities. By homology search of the fission yeast genome data base, other five Ubc's (UbcP5-P9) have been found. Now the functional characterization of the Ubc's are undergoing. Some genes were found essential for the cell growth while its ortholog in the budding yeast were not (i.e. UBC11/ubcP4, UBC4/ubcP1). Furthermore, there exist phenotypic differences between the fission yeast Ubc's and its structural homologues in the budding yeast. Thus, the whole spectrum of functional diversity in Ubc does not seems to be identical between the two yeast lines.

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A-c. Division of Nucleic Acid Chemistry

(1) The roles of Mre11 of budding yeast in recombination

Jun-ichi TOMIZAWA

In yeast *Saccharomyces cerevisiae*, the biochemical process of meiotic recombination contains two temporally coupled processes, formation and processing of double-strand breaks (DSBs). About ten genes are involved in these processes. Among them the *mre11* gene plays pivotal roles in both processes. Mre11 forms a complex with Rad50 and Xrs2, acting as the binding core and participates in DSB processing. The C-terminal region of Mre11 is required only for DSB formation and binds to some meiotic proteins. Although these and other proteins are involved in DSB formation, Mre11 is not necessarily holding them. The N-terminal half of Mre11 specifies nuclease activities that are collectively required for DSB processing. Mre11 has a DNA binding site for DSB formation and another site for DSB processing. It has two regions to bind to Rad50. In addition, Mre11 repairs methyl methanesulfonate-induced DSBs by reactions that require the nuclease activities and those do not.

This work was performed by collaboration with Tomoko Ogawa, Tsutomu Ohta, and Hiroyuki Oshiumi of the Department of Cell Genetics of this institute and Hideyuki Ogawa and Takehiko Usui of the Department of Biology of Osaka University. For more detail, see the report from the Department of Cytogenetics.

(2) A new congenital muscular dystrophy with mitochondrial structural abnormalities

Ichizo NISHINO[1],Yuichi GOTO[1], Satoshi HORAI and Ikyuya NONAKA[1] ([1]Department of Ultrastructural Research, National Institute of Neuroscience, National Center of Neurology and Psychiatry)

We report a new form of congenital muscular dystrophy (CMD) in 4 patients from three unrelated families with probable autosomal-recessive inheritance. All patients had the clinical characteristics of merosin-positive congenital muscular dystrophy, but had marked mental retardation. The disease was

slowly progressive and 1 patient died from dilated cardiomyopathy at the age of 13 years. In addition to dystrophic changes with necrosis and regeneration in muscle, the most striking finding was mitochondrial depletion in the center of the sarcoplasm. Mitochondria at the periphery of fibers were markedly enlarged ("megaconial" appearance) with complicated cristae, and contained a normal amount of mitochondrial DNA by in situ hybridization. Mitochondrial enlargement may represent functional compensation for mitochondrial depletion in the central sarcoplasm, where myofibrillar degeneration occurred. For details, see Ref. 1.

(3) Peopling of Japan inferred from mitochondrial DNA polymorphisms in East Asians

Satoshi HORAI and Keiichi OMOTO[1] ([1]International Research Center for Japanese Studies)

Nucleotide sequences of the major noncoding (D-loop) region of human mitochondrial DNA from five East Asian populations including mainland Japanese, Ainu, Ryukyuans, Koreans and Chinese were analyzed. Based on a comparison of 482 base pair sequences in 293 East Asians, 207 different sequence types were observed. Of these, 189 were unique to their respective populations, whereas18 were shared between two or three populations. Among the shared types, eight were found in common between the mainland Japanese and Koreans, which is the largest number in the comparison. Phylogenetic analysis revealed that East Asian lineages were classified into at least 18 monophyletic clusters, though lineages from the five populations were completely intermingled in the phylogenetic tree. However, we assigned 14 out of the 18 clusters for their specificity based on the population from which the maximum number of individuals in each cluster was derived. Of note is the finding that 50% of the mainland Japanese had continental specificity in which Chinese or Koreans were dominant, while less than 20% of either Rynkyuans or Ainu possessed continental specificity. Phylogenetic analysis of the entire human population revealed the closest genetic affinity between the mainland Japanese and Koreans. Thus, the results of this study are compatible with the hybridization model on the origin of modern Japanese. It is suggested

that about 65% of the gene pool in mainland Japanese was derived from the continental gene flow after the Yayoi Age. For details, see Ref. 2.

(4) Confirmation that a T-to-C mutation at 9176 in mitochondrial DNA is an additional candidate mutation for Leigh's syndrome

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Among 80 patients with the clinical and brain imaging characteristics of Leigh's syndrome, 11 patients had a well-known mutation at nucleotide position (nt) 8993 in mitochondrial DNA. In addition, three patients had a T-to-C mutation at nt 9176 which had been described previously in only two brothers with bilateral striatal necrosis and one patient with Leigh's syndrome. In our three patients, one had the typical clinical characteristics of Leigh's syndrome from early infancy, and two had the later onset of neurological deficits. All had a slowly progressive course and basal ganglia abnormalities by neuroimaging. As nt 8993 and 9176 are located in the ATPase 6 coding region, altered ATPase function may be one of the enzyme abnormalities in Leigh's syndrome and other similar conditions with bilateral striatal necrosis. For details, see Ref. 3.

(5) Phylogenetic analysis of mitochondrial DNA in Japanese pedigrees of sensorineural hearing loss associated with the A1555G mutation

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Thirteen Japanese families (ten of which were from the northern part of Japan), with sensorineural hearing loss associated with the 1555 A to G (A1555G) mitochondrial mutation, a known cause of non-syndromic hearing loss, were phylogenetically analysed using data obtained by restriction frag-

ment length polymorphism (RFLP) and D-loop sequencing of mitochondrial DNA (mtDNA). Various types of mtDNA polymorphism were detected by restriction enzymes and D-loop sequence. No common polymorphic pattern throughout the 13 families was found, though three families exhibited the same restriction patterns and the same sequence substitution in the D-loop. To find where each of the 13 families are situated in the phylogenetic tree, the 482-bp of D-loop sequence were compared with those of 62 normal Japanese subjects. Despite the three families mentioned above appearing to be clustered, the remaining 10 families were scattered along the phylogenetic tree. This indicates that there was no common ancestor for the 13 Japanese families bearing the A1555G mutation except three families, and that the A1555G mutation occurred sporadically and multiplied through evolution of the mtDNA in Japan. The present results showed that the common pathogenicity (hearing loss associated with the A1555G mutation) can occur sporadically in families which have different genetic backgrounds, even in the Japanese population. For details, see Ref.4.

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B. DEPARTMENT OF CELL GENETICS

B-a. Division of Cytogenetics

(1) Mechanisms of Yeast Genetic Recombination

Tomoko OGAWA, Tsutomu OHTA, Hiroyuki OSHIUMI and Shimako MATSUDA

Most recombination appears to begin by forming double-strand breaks (DSBs) in the DNA. Then, one of the strands at the break site is processed, producing a tail of single-stranded extension. The tail is used to search for the complementary sequence in a partner and to promote pairing with it. This outline of the basic mechanisms of homologous recombination is conserved in prokaryotes and in eukaryotes. Studies on yeast, as one of the simplest eukaryotes, should provide important information on the chromosome mechanisms of genetic recombination. We have studied two subjects. One is the versatility of yeast Mre11 protein that plays pivotal roles in various reactions in the early phase of meiotic recombination and in repair of DNA damage in vegetatively growing cells. The other is functions of Rad52 and RPA in Rad51-mediated recombination reaction.

(a) Complex Formation and Functional Versatility of Mre11 of Budding Yeast in Recombination

Meiotic recombination of *Saccharomyces cerevisiae* consists temporally coupled two processes, formation of double-strand breaks (DSBs) and the processing of their ends. Mre11 plays pivotal roles in both processes. The C-terminal region that is specifically required for DSB formation binds to meiotic proteins. The N-terminal region specifies ssDNA endonuclease, 3' to 5' ssDNA exonuclease and 3' to 5' dsDNA exonuclease which are collectively required for DSB processing. Cleavage by the endonuclease near the 5'-end of a strand may yield a 3'-overhanging structure and simultaneously eliminate Spo11. Mre11 has two sites to bind to DNA, the central site for DSB processing and the C-terminal site for DSB formation. It has two regions to

bind to Rad50. These functional duality would permit binding to two DNA molecules simultaneously or to the same DNA segment differently to help transition of the complex for DSB formation to that for DSB processing. Mre11 participates in repair of methyl methanesulfonate-induced DSBs by reactions that require nuclease activities and those do not. See Ref. 1

(b) Stimulation of Yeast Rad51-mediated Recombination by Rad52

Rad51 catalyzes strand exchange between circular single-stranded DNA (ssDNA) and homologous linear double-stranded DNA (dsDNA) to form joint molecules (JM), and open circular duplex DNA molecules (OC). However, Rad51 is much less active than RecA protein in this reaction, even in the presence of Replication protein-A (RPA), suggesting that Rad51 might be either inherently inefficient or weak due to a missing cofactor(s).

We found that both Rad52 and RPA proteins stimulate Rad51-mediated reaction. In the enhancement, the bindings of the Rad52 to both Rad51 and RPA proteins are necessary. The stimulation by RPA after formation of nucleoprotein filaments has been suggested to be due to its relaxation of the secondary structures of ssDNA. However, prior access of RPA to ssDNA inhibits the Rad51 reactions and Rad52 alleviates this inhibition. Both stimulation of the Rad51 reaction and alleviation of the inhibition by Rad52 are specific to the Rad51 reaction, and this specificity resides in the specificity of its interaction with Rad51. We conclude that the binding plays a crucial role in the recombination, by facilitating the formation of Rad51 nucleoprotein filaments.

Although recombination is a complex process involving other gene products, the Rad51 reactions assisted by Rad52 are likely to be central to the molecular process of homologous recombination in eukaryotes. See Ref. 2 and 3.

(2) The hepatitis B virus X protein is a co-activator of activated transcription that modulates the transcription machinery and distal binding activators

Tsutomu OHTA

Hepatitis B virus X protein (HBx) transactivates viral and cellular genes through a wide variety of cis-elements, but the mechanism has not been well elucidated. Evidence for nuclear events in HBx transactivation has been reported. Here we examine the role of HBx in modulation of transcription with a transient transfection system and an in vitro transcription assay. Reporters bearing Gal4-binding sites were applied to avoid the effects of endogenous transcription factors with or without signaling processes. The Gal4-DNA binding domain fused form of HBx exhibited no effect on Gal4-responsive reporters. However, HBx augmented activated transcription by transcriptional activators, suggesting HBx retains a co-activator but not a transcriptional activator function. The functional domain for co-activation was the same as that for HBx transactivation, and the transcription factor IIB- and RNA polymerase II subunit 5-interacting sites of HBx, which were critical for HBx transactivation, were shown to be crucial for the co-activation function. Importantly, HBx stimulated transcription on templates bearing the X responsive elements in vitro with endogenous activators. These results imply that HBx acts as a co-activator that modulates transcriptional machinery and distal-binding activators, which may explain one of the mechanisms of transactivation by HBx when localized in nuclei. See Ref 4.

(3) Analysis of the role of TFIIE in transcriptional regulation through structure-function studies of the TFIIE β subunit

Tsutomu OHTA

The general transcription factor TFIIE plays important roles at two distinct but sequential steps in transcription as follows: preinitiation complex formation and activation (open complex formation), and the transition from initiation to elongation. The large subunit of human TFIIE(TFIIEalpha) binds to

and facilitates the enzymatic functions of TFIIH, but TFIIE also functions independently from TFIIH. To determine functional roles of the small subunit of human TFIIE (TFIIEbeta), deletion mutations were systematically introduced into putative structural motifs and characteristic sequences. Here we show that all of these structures that lie within the central 227-amino acid region of TFIIEbeta are necessary and sufficient for both basal and activated transcription. We further demonstrate that two C-terminal basic regions are essential for physical interaction with both TFIIEalpha and single-stranded DNA, as well as with other transcription factors including the Drosophila transcriptional regulator Kruppel. In addition, we analyzed the effects of the TFIIEbeta deletion mutations on TFIIH-dependent phosphorylation of the C-terminal domain of RNA polymerase II and on wild type TFIIEbeta-driven basal transcription. Both responsible regions also mapped within the essential 227-amino acid region. Our results suggest that TFIIE engages in communication with both transcription factors and promoter DNA via the TFIIEbeta subunit. See Ref. 5.

(4) DNA supercoiling factor localizes to puffs on polytene chromosomes in *Drosophila melanogaster*

Tsutomu OHTA

DNA supercoiling factor (SCF) was first identified in silkworm as a protein that generates negative supercoils in DNA in conjunction with eukaryotic topoisomerase II. To analyze the in vivo role of the factor, we cloned a cDNA encoding *Drosophila melanogaster* SCF. Northern analysis revealed 1.6- and 1.8-kb mRNAs throughout development. The longer mRNA contains an open reading frame that shares homology with mouse reticulocalbin whereas the shorter one encodes a truncated version lacking the N-terminal signal peptide-like sequence. An antibody against SCF detected a 45-kDa protein in the cytoplasmic fraction and a 30-kDa protein in the nuclear fraction of embryonic extracts. Immunoprecipitation suggests that the 30-kDa protein interacts with topoisomerase II in the nucleus, and hence that it is a functional form of SCF. Immunostaining of blastoderm embryos showed that SCF is present in nuclei during interphase but is excluded from mitotic chromosomes.

In larvae, the antibody stained the nuclei of several tissues including a posterior part of the salivary gland. This latter staining was associated with natural or ecdysteroid-induced puffs on polytene chromosomes. Upon heat treatment of larvae, the staining on the endogenous puffs disappeared, and strong staining appeared on heat shock puffs. These results implicate SCF in gene expression. See Ref.6.

(5) Biochemical basis of hyper-recombinogenic activity of *Pseudomonas aeruginosa* RecA protein in *Escherichia coli* cells

Andrei ALEXEEV

The replacement of *Escherichia coli* recA gene (recA[Ec]) with the *Pseudomonas aeruginosa* recA(Pa) gene in *Escherichia coli* cells results in constitutive hyper-recombination (high frequency of recombination exchanges per unit length of DNA) in the absence of constitutive SOS response. To understand the biochemical basis of this unusual in vivo phenotype, we compared *in vitro* the recombination properties of RecA (Pa) protein with those of RecA (Ec) protein. Consistent with hyper-recombination activity, RecA (Pa) protein appeared to be more proficient both in joint molecule formation, producing extensive DNA networks in strand exchange reaction, and in competition with single-stranded DNA binding (SSB) protein for single-stranded DNA (ssDNA) binding sites. The RecA (Pa) protein showed *in vitro* a normal ability for cleavage of the *E. coli* LexA repressor (a basic step in SOS regulon derepression) both in the absence and in the presence (i.e. even under suboptimal conditions for RecA (Ec) protein) of SSB protein. However, unlike other hyper-recombinogenic proteins, such as RecA441 and RecA730, RecA (Pa) protein displaced insufficient SSB protein from ssDNA at low magnesium concentration to induce the SOS response constitutively. In searching for particular characteristics of RecA (Pa) in comparison with RecA (Ec), RecA441 and RecA803 proteins, RecA (Pa) showed unusually high abilities: to be resistant to the displacement by SSB protein from poly(dT); to stabilize a ternary complex RecA-ATP-ssDNA to high salt concentrations; and to be much more rapid in both the nucleation of double-stranded DNA (dsDNA) and the steady-state rate of dsDNA-depen-

dent ATP hydrolysis at pH7.5. We hypothesized that the high affinity of RecA (Pa) protein for ssDNA, and especially dsDNA, is the factor that directs the ternary complex to bind secondary DNA to initiate additional acts of recombination instead of to bind LexA repressor to induce constitutive SOS response. See Ref. 7

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B-b. Division of Microbial Genetics

(1) Dpb11 controls association of DNA polymerases with replication origins in *Saccharomyces cerevisiae*

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. By using a cross-linker, we detected a complex of Pol ε and Dpb11 and this complex existed in S phase.

Dpb11 associated with replication origins at the same timing with Pol ε in S phase by chromatin immunoprecipitation assay. Association of Dpb11 with origins depended on functions of Mcm5, a component of the pre-RC. Conversely, Pol α - primase complex and Pol ε could not associate with origins in temperature sensitive *dpb11-1* mutant cells at the restrictive temperature. Therefore, Dpb11 functions for association of DNA polymerases with origins.

Hydroxyurea inhibits late origin firing in *S. cerevisiae* and checkpoint genes, and checkpoint genes *RAD53* and *MEC1*, are involved in this control. In *dpb11-1* cells, DNA polymerases associated with early and late origins in the presence of hydroxyurea at permissive temperature whereas they associated only

with early origins in wild-type cells. Thus, Dpb11 functions for association of DNA polymerases with origins in DNA replication and for the control of late-firing origins in the S-phase checkpoint.

(2) Sld2, which interacts with Dpb11 in *Saccharomyces cerevisiae*, is required for chromosomal DNA replication

Yoichiro KAMIMURA and Hiroyuki ARAKI

To identify factors interacting with Dpb11, we have isolated 10 *sld* (synthetic lethal with *dpb11-1*) mutations which fall into 6 complementation groups (*sld1 - 6*). So far, we have cloned *SLD1, 2, 3, 4* and *5* and found that *SLD1* is identical to *DPB3* encoding the third largest subunit of Pol ε and *SLD4* is identical to *CDC45* required for the initiation of chromosomal DNA replication. The *SLD2, 3* and *5* genes were new genes essential for the cell growth. We first characterized *SLD2* encoding an essential 52 kDa protein.

High copy *SLD2* suppressed the thermosensitive growth defect of *dpb11-1*. Conversely, high copy *DPB11* suppressed the temperature-sensitive growth defect of *sld2-6*. The interaction between Sld2 and Dpb11 was detected in two hybrid assay. This interaction was evident at 25℃ but not at 34℃ when Sld2-6 or Dpb11-1 replaced its wild type protein. No interaction could be detected between Sld2-6 and Dpb11-1 even at 25℃. Immunoprecipitation experiments confirmed that Dpb11 physically interacts with Sld2.

Sld2-6 cells were defective in DNA replication at the restrictive temperature as were *dpb11-1* cells. Further, in *dpb11-1* and *sld2-6* cells, the bubble shaped replication intermediates formed in the ARS region reduced quickly after temperature shift. These results strongly suggest the involvement of the Dpb11/Sld2 complex in a step close to the initiation of DNA replication.

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

Yuko TAKAYAMA, Yoichiro KAMIMURA and Hiroyuki ARAKI

The *SLD5* gene encodes an essential 34 kDa protein. We first isolated thermosensitive alleles of the *SLD5* gene by the plasmid shuffling method to know its function. At the restrictive temperature, a thermosensitive *sld5-12* mutant cells arrested with a dumbbell shape which is the typical terminal morphology for mutants defective in DNA replication. FACS analysis showed that the mutant cells arrest at the S-phase. Thus, Sld5 seems to be required for chromosomal DNA replication.

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. Moreover, the recombinant Sld5 and Psf1 proteins purified from *Escherichia coli* cells made a complex, indicating that Psf1 directly interacts with Sld5. 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. These results suggest that the Sld5/Psf1 complex is required for DNA replication.

(4) Is there an additional monofunctional glycosyltransferase for cell wall peptidoglycan biosynthesis in *Escherichia coli* ?

Hiroshi HARA (Present address: Department of Biochemistry and Molecular Biology, Saitama University)

The last stage of cell wall peptidoglycan biosynthesis in bacteria involves two enzymatic activities, glycosyltransferase (GTase) that polymerizes glycan chains from lipid-linked disaccharide-peptide precursors and transpeptidase (TPase) that cross-links adjacent glycan chains via short peptide chains, the latter being sensitive to penicillins. In *Escherichia coli*, penicillin-binding proteins (PBPs) 1a and 1b were shown to carry not only TPase but also GTase activity. In addition to these bifunctional enzymes, we found a monofunctional

GTase activity in an ion-chromatographic fraction (CM-fraction) containing no detectable PBP, purified the enzyme, and characterized it (Hara, H. and Suzuki, H., FEBS Lett. 168, 155-160, 1984). Recently, a gene (*mtgA*) whose 242-residue product's sequence showed significant homology to the well-conserved fingerprints found in the N-terminal GTase domain, but not to those in the C-terminal TPase domain, of bifunctional PBPs was identified. Overexpression of the gene led to increased peptidoglycan-synthesizing activity (Di Bernardino, M. et al., FEBS Lett. 392, 184-188, 1996).

In this study, the chromosomal *mtgA* gene was destroyed by inserting Ω interposon into a position corresponding to the 51st codon. The resultant null mutant was viable, and the CM-fraction prepared from it was not impaired for the glycan-polymerizing activity. These results indicate that *mtgA* is not responsible for the monofunctional GTase we previously identified and that the gene is dispensable for growth. MtgA protein has a molecular weight of about 27 kDa, a little smaller than that of the enzyme purified from the CM-fraction (34 kDa). The purified enzyme required Ca^{2+} or Mn^{2+} but not Mg^{2+} for its full activity, whereas such requirement was not confirmed for the GTase activity detected in the solubilized membrane protein fraction of *mtgA*-overexpressing cells. There seems to be two species (or more) of monofunctional GTases in *E. coli*, although search of the whole genome sequence did not reveal an additional gene other than *mtgA* that could code for a protein with homology to the GTase fingerprints.

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B-c. Division of Cytoplasmic Genetics

(1) Studies on behavioral disorders of Fyn tyrosine kinase knockout mice

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

In a series of experiments we found that our Fyn-deficient mice showed increased fearfulness in a variety of tests for fear response and enhanced seizure susceptibility induced by intense sound and convulsive drugs. In 1997, we found that Fyn-deficient mice were hyper-sensitive to the hypnotic effect of ethanol. In these Fyn-deficient mice, up-regulation of tyrosine phosphorylation of NMDAR in the hippocampus by ethanol administration (usually noted in the control mice) was not observed. Acute tolerance to ethanol inhibition of NMDAR-mediated EPSPs in the hippocampal slices developed in control mice but not in Fyn-deficient mice. In 1998 we examined aggressiveness of Fyn-deficient mice, and found that they showed decreased offenssive aggression (intruder-evoked attack) and increased defensive aggression (restraint-induced target biting).

Concerning the pharmacological correlates of increased fearfulness of our Fyn-deficient mice, we conducted a receptor-binding experiment in collaboration with Dr. Yoshii at Tokyo Institute of Psychiatry. In 1998 we found that Fyn-deficient mice have a lower density of central benzodiazepine receptor binding sites in brain tissues.

As Fyn is strongly expressed in the olfactory bulb, we examined LTP in the olfactory bulb in collaboration with Prof. Kawai at Jichi Medical School. In

1998 we found an impairment in LTP of the olfactory bulb along with abnormality of GABA A function in Fyn-deficient mice (Kitazawa, H. et al, 1998).

(2) Studies on behavioral disorders of NMDAR subunit knockout mice

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

Concerning the studies on mice lacking NMDAR subunit epsilon 1 or epsilon 4, we obtained the following results in 1998. Mice lacking epsilon 1 were less susceptible to audiogenic seizure, but they did not differ from wild-type mice in auditory startle response. On the other hand, mice lacking epsilon 4 showed augmentation in startle response, but they showed no difference in audiogenic seizure susceptibility.

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C. DEPARTMENT OF DEVELOPMENTAL GENETICS

C-a. Division of Developmental Genetics

(1) Regulation of ras signaling by Sprouty and EDL ensures constant outcome of induction

Masataka OKABE, Takuma YAMADA, Susanne KRAMER[1] and Yasushi HIROMI ([1]Institut fur Genetik, Universität Wurzburg)

Photoreceptor neurons in the *Drosophila* compound eye and chordotonal organs in the embryo are both formed by induction by preexisting neurons or their precursors. In both systems, prespecified cells induce surrounding cells to assume fates similar to its own. The inducing molecule is a TGFα-like factor Spitz, which activates EGF receptor and the downstream ras/MAPK signaling pathway. While there are a large number of cells that are competent to be induced, the outcome of induction is remarkably constant; each ommatidium always contain eight photoreceptor neurons, and each hemisegment contains precisely eight chordotonal organs. This constancy is achieved by the precise control of the inducing ability and its responsiveness.

Sprouty is a novel protein with an evolutionarily conserved Cystein rich motif. Sprouty acts as an antagonist of receptor tyrosine kinase pathways that are triggered by the extracellular signaling molecules, such as Spitz EGF and Branchless FGF. In the absence of *sprouty* function, supernumerary photoreceptor neurons and chordotonal organs form, due to hyperactivation of ras signaling. We find that the expression of Sprouty is induced by the ras signal. While cells that are close to the signaling center and receive high concentration of the signal can still respond and assume neuronal fate, those that are further away cannot cope with the signal antagonism by Sprouty and fail to respond. Thus the role of Sprouty is to limit the field of induction by restricting the number of responding cells to those receiving the highest concentration of the inducing signal.

A nuclear target of the ras/MAPK signaling pathway is the Ets transcription

factor Pointed P2 protein. Phosphorylation of Pointed P2 by MAPK activates the transcriptional activation function of Pointed P2, which in turn is required for the specification of photoreceptor neurons and chordotonal organs that are formed by induction. We found that hyperactivation of *pointed* function in cells that normally induce other photoreceptor neurons or chordotonal organs results in a decrease in the inducing ability. The inhibition of inducing ability by *pointed* is likely to operate in the induced cells, thereby preventing unlimited spread of induction. EDL is a novel Ets protein that is specifically expressed in the cells with inducing ability. EDL contains the Ets-specific Pointed domain, but does not contain the Ets DNA-binding domain. It binds to the Pointed domain of Pointed P2, blocks the transcriptional activation function of Pointed P2, thereby allowing cells to express their inducing ability. For details, see publications 1 and 2.

(2) A screen for proteins that interact with the ligand binding domain of a nuclear receptor, Seven-up

Hiroyuki KOSE, Steve WEST[1], Emiko SUZUKI and Yasushi HIROMI ([1] Princeton University)

Nuclear receptors are a class of transcription factors that share two conserved domains, the DNA-binding domain and the ligand-binding domain. While some of the nuclear receptors act as true “receptors”by responding to the signals presented by their ligands, the vast majority of nuclear receptors have no known ligands, even though they share a domain similar to the ligand binding domain of ligand-responsive nuclear receptors. Seven-up is one such nuclear receptor for which ligand has not been identified. It is one of the receptors with the most ancient origin, and is by far the most evolutionarily conserved nuclear receptor. During compound eye development in *Drosophila*, *seven-up* is expressed in a subset of photoreceptor neurons and specifies their identity as a particular neuronal subtype. Genetic evidence suggests that the function of *seven-up* is mediated by protein(s) that act through the ligand-binding domain of Seven-up.

We have carried out a yeast two-hybrid screen for molecules that interact with the ligand-binding domain of Seven-up. One of the proteins identified is

a novel protein that contains the SAM domain, a protein-protein interaction motif found in some receptor kinases, cytoplasmic scaffold and adaptor proteins, and members of the Polycomb group chromatin proteins. We have identified strains harboring transposon insertions in the vicinity of the open reading frame, and are carrying out the genetic analysis of the gene.

(3) Relationships between organogenesis and positional information in Drosophila imaginal discs

Nao NIWA, Masataka OKABE and Yasushi HIROMI

Recently, *Drosophila* genes have been identified whose misexpression induces ectopic organs, such as eyes and wings. Such genes and has been regarded as the master control genes for development of *Drosophila* organs. Ectopic organs, however, does not form anywhere in the body, but appears to respond to the prepattern present in the ectopic location. Thus there are positions in the body that are capable of responding to the expression of master control genes and initiating organogenesis, in addition to those where organogenesis normally occurs. We are investigating the relationships between the types of induced organs and the positional information surrounding ectopic organ in the imaginal discs.

As a first step, we chose as a model the ectopic eye formed by *eyeless* (*ey*) gene expression. To identify the conditions required for the ectopic eye formation, *ey* was ubiquitously expressed by UAS/heat shock GAL4 system in all imaginal discs during larval stages, and we have defined the timing of *ey* expression that allows ectopic eye formation, and the positions of ectopic eyes in imaginal discs. We found that the ectopic eye formation was most frequent when *ey* was expressed by heat shock for an hour during 78-85h after egg laying, and the positions of ectopic eyes were restricted to several domains near the anterior-posterior compartment borders in antenna, leg and wing imaginal discs. Thus the responsiveness to ectopic *ey* was clearly restricted, both spatially and temporally.

We are currently investigating the conditions for timing and domains of the master control gene expression with respect to formation of other organs, such as ocelli and chordotonal organ, and comparing those with the conditions found

for ectopic eye formation.

(4) Molecular mechanisms of Neuron-glia developmental decision

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

The nervous system is composed of two major types of cells, neurons and glia. Despite their distinct functions, neurons and glia are derived from a common precursor in both vertebrate and invertebrate nervous systems. In the Drosophila central nervous system (CNS), both neurons and glia are derived from common precursor cells called neuroblasts. The *gcm* gene is expressed transiently in early glial lineage. The mutation of *gcm* causes presumptive glial cells to differentiate into cells with neuron-like characteristics, whereas *gcm* ectopic expression forces virtually all CNS cells to become glial cells. Thus the *gcm* gene governs glial vs. neuronal developmental decision by turning on the glial fate while inhibiting the default neuronal fate of the neuroblasts and their progeny.

In order to further clarify the neuron-glia decision mechanisms, the cell lineage and gcm expression was examined in detail. NB6-4 in the thoracic segment (NB6-4T) is a neuroglioblast, although the corresponding cell in the abdominal segment (NB6-4A) produces only glia. The first cell division of NB6-4T occurred in the medial-lateral orientation, and was found to bifurcate the glial and neuronal lineage. After division, the medial daughter cell expressed GCM protein to produce three glial cells, while the lateral daughter cell with no GCM expression produced ganglion mother cells, secondary precursors of neurons. Although *gcm* mRNA was present evenly in the cytoplasm of NB6-4T before the first cell division, it became detected asymmetrically in the cell during mitosis and eventually only in the medial daughter cell. In contrast, NB6-4A showed a symmetrical distribution of *gcm* mRNA and GCM protein through division. Our observations suggest that mechanisms regulating *gcm* mRNA expression and its translation play an important role in glia and neuron (Akiyama-Oda et al., 1999).

The regulation of gcm expression was examined in the peripheral nervous

system (PNS). In the Dorsal bipolar dendritic lineage, the precursor cell divides asymmetrically to generate two progeny. The gcm gene is expressed in one of the progeny, then inhibit neuronal differentiation while promoting glial differentiation. In this lineage, activation of the Notch signaling pathway was shown to be essential for gcm transcription, suggesting that Notch activation induces gcm expression, which may be the first step for glial fate induction.

(5) Axon guidance mechanisms

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

Glial functions in axonal pathfindings was investigated by examining axonal behavior in gcm mutants, which lack glial cells. In the earliest stage of normal axon bundle formation, a small number of " pioneer " neurons extend the first axons. These axons become associated with glial cells, on which a large number of "follower" axons elongate. In the gcm mutants, the pioneer neurons behave normally, but the follower neurons show severe phenotypes. Axon bundle formation is disrupted and abnormal migration of glia leads to misrouting of axons. These results indicate that although glial cells appear to be dispensable for the early pathfindings of the pioneer neurons, they have essential supportive functions in the correct tract formation by a large number of follower axons (Takizawa, 1999).

The role of Netrin signaling in axon guidance was also investigated. Netrin (NetA and NetB in Drosophila) is a ligand molecule that functions as a component of positional information for growth cones. Frazzled have been characterized as Netrin receptors. In the developing CNS, Netrin molecule is concentrated on the Frazzled positive region. This Netrin localization disappears in the *frazzled* mutants, whereas misexpression of manipulated Frazzled localizes Netrin ectopically, suggesting that Frazzled have a novel function to localize Netrin. Two pioneer neurons, MP1 and dMP2, track the boundary of Netrin positive region in the wild type. Their growth cones respond to ectopic Netrin positive region created by misexpression of either Netrin itself or manipulated Frazzled. Thus it is proposed that Frazzled captures and translo-

cates Netrin molecules to form barriers for MP1 and dMP2 growth cones, thereby controlling the direction of extension (Hiramoto, 1999).

In order to isolate novel genes that might be involved in the formation of axon tracts, an enhancer-trap screening was performed using the tau-lacZ gene, a marker gene that can visualize axons. Three strains that show defects in the longitudinal axon pathway have been isolated. A candidate gene, whose mutation might be responsible for the phenotype of one strain, encodes a novel kinase expressed in the nervous system from the early stages. Because the mutant strain shows defects in the formation of neurons and glia, this kinase may be involved in neurogenesis (Shinza, 1999).

(6) The gcm transcriptional regulator family

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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 that controls glial vs. neuronal decision by controlling expression of cell-type specific genes. The gcm-family is most likely 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. Since gcm is also expressed in glia and hemocytes, these two gene may interact in the development of those cell types (Higashi, 1999). Two gcm genes have been isolated from Mouse, which show tissue specific expression. Gene disruption of these mouse genes are currently underway.

(7) Hydra Peptide Project: A Novel Neurotransmitter that Controls Muscle Contraction in *Hydra*

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

During a course of systematic screening of peptide signaling molecules in *Hydra*, we have identified a novel neuropeptide, Hym-176, which evokes ectodermal muscle contraction of the *Hydra* body colum. The peptide consists of 10 amino acids with an amidated C-terminus. Since the exogenously added Hym-176 can induce contraction of epithelial hydra which are soley made of epithelial cells, the peptide acts directly on epithelial cells. We have cloned a cDNA encoding the peptide. Within the coding sequence, a signal sequence at the N-terminal region and one copy of Hym-176 precursor sequence was found. In addition, another precursor sequence of Hym-357 was found. Hym-357 had been sequenced but its function is unknown. The expression of *Hym-176* gene was examined by whole mount in situ hybridization. The strong signal was observed in the neurons in the peduncle region. A weak signal was also observed in neurons scattered sparsely along the body column. No signal was detected in basal disk and head including tentacles. Immunohistochemistry using antisera which specifically recognize the C-terminal half of Hym-176 confirmed the in situ hybridization result. These results together suggest that Hym-176 is a neurotransmitter which acts at the neuro-muscular junctions and evokes muscle contraction in the peduncle region.

(8) Feeding and wounding response of hydra: hydra nervous system is more advanced than a diffuse system

Hiroshi SHIMIZU

Freshwater coelenterate hydra has been considered the best example of an organism having diffuse nervous system. By examining physiological response of hydra, we obtained a piece of evidence that hydra's nervous system is more advanced than a simple diffuse nervous system. Feeding, touching and wounding stimuli were applied to tissue in tentacles or body column, and response of

the tissue to the stimulii was examined under a dissecting microscope. Response obtained in the tentacles was of most interest. There, proximal part of tentacle to the prey attachment site or wounding site contracted enormously whereas distal part of it contracted only slightly or did not contract at all. To examine if nervous system is involved in the polarized responses, a similar analysis was carried out in epithelial tentacles which have no nerve cells. It was found that the wounding stimulus provoked contraction of tissue only in the vicinity of the wounding site thus suggesting that nerve cells are responsible for the polarized response in wounding in the normal tentacles. It was also found that polarized response in feeding occurred in distal to proximal orientation in the normal tentacles. Despite an extensive search in the body column and peduncle regions, no polarized response was found by wounding stimulus. From these observations, I conclude that hydra's nervous system in the tentacles has a mechanism which enables polarized responses to external stimulii and that this nervous system is more advanced than a simple diffuse system. I speculate that this polarization could have been a trigger to evolve to centralized nervous system.

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C-b. Division of Gene Expression

(1) Chromatin remodeling and transcription

Susumu HIROSE

The fundamental building unit of chromatin is the nucleosome, in which 146 base pairs of DNA wind around an octamer of core histones. The chromatin structure is essential not only for the compact packaging of the eukaryotic genome but also for regulation of transcription. *In vivo* evidence for a role of chromatin in the global repression of transcription has been obtained by placing a histone gene under the control of an inducible promoter in the yeast *Saccharomyces cerevisiae*. When expression of core histone H4 or H2B is repressed, certain genes including *PHO5*, *CUP1* and *HIS3*, that are normally inactive, become activated. Subsequent studies have led to a consensus that transcriptional activation and repression are achieved through chromatin acetylation and deacetylation, respectively.

Genetic analyses of transcriptional regulation in yeast have led to the identification of a number of *SWI* and *SNF* genes (*SWI* for mating type switching, *SNF* for sucrose non-fermenting). These genes are required for proper expression of certain inducible promoters including *PHO5*, *CUP1*, and *HIS3*. Among *SWI/SNF* gene products, SWI1, SWI2/SNF2, SWI3, SNF5, and SNF6 are

functionally interdependent, suggesting that they exert their function as a complex. Yeast SWI/SNF complex has been purified and found to possess a chromatin remodeling activity. In *Drosophila*, three distinct chromatin remodeling factors, NURF, ACF and CHRAC, have been identified in addition to the SWI/SNF (BRAHMA) complex. All of these chromatin remodeling complexes contain a SWI2/SNF2-related subunit harboring an ATP-dependent DNA helicase domain. It has been postulated that the SWI2/SNF2-related proteins may achieve ATP-driven DNA translocation to disrupt histone-DNA interactions. Interestingly, recent data demonstrate that chromatin remodeling in the promoter region is necessary for transcription. For details, see Ref. 1.

(2) Chromatin remodeling mediated by *Drosophila* GAGA factor and ISWI activates *fushi tarazu* gene transcription *in vitro*

Masahiro OKADA and Susumu HIROSE

GAGA factor is known to remodel the chromatin structure in concert with the nucleosome remodeling factor NURF in an embryonic S150 extract of *Drosophila*. The promoter region of the *Drosophila fushi tarazu* (*ftz*) gene carries several binding sites for GAGA factor. Both the GAGA factor-binding sites and GAGA factor *per se* are necessary for the proper expression of *ftz in vivo*. We observed transcriptional activation of the *ftz* gene when a preassembled chromatin template was incubated with GAGA factor and the S150 extract. The chromatin structure within the *ftz* promoter was specifically disrupted by incubation of the preassembled chromatin with GAGA factor and the S150 extract. Both transcriptional activation and chromatin disruption were blocked by an antiserum raised against ISWI or by base substitutions in the GAGA factor-binding sites in the *ftz* promoter region. These results demonstrate that GAGA factor and ISWI-mediated disruption of the chromatin structure within the promoter region of *ftz* activates transcription on the chromatin template. For details, see Ref. 2.

(3) DNA supercoiling factor localizes to puffs on polytene chromosomes in *Drosophila melanogaster*

Masatomo KOBAYASHI[1], Noriko AITA[1], Shigeo HAYASHI, Kohichi OKADA, Tsutomu OHTA and Susumu HIROSE ([1]These authors contributed equally to this work)

DNA supercoiling factor (SCF) was first identified in silkworm as a protein that generates negative supercoils into DNA in conjunction with eukaryotic topoisomerase II. To analyze the *in vivo* role of the factor, we cloned a cDNA encoding *Drosophila* SCF. Northern analysis revealed 1.6 and 1.8 kb mRNAs throughout development. The longer mRNA contains an open reading frame that shares homology with mouse reticulocalbin whereas the shorter one encodes a truncated version lacking the N-terminal signal peptide-like sequence. An antibody against SCF detected a 45 kDa protein in the cytoplasmic fraction and a 30 kDa protein in the nuclear fraction of embryonic extracts. Immunoprecipitation suggests that the 30 kDa protein interacts with topoisomerase II in the nucleus, and hence it is a functional form of SCF. Immunostaining of blastoderm embryos showed that SCF is present in nuclei during interphase but excluded from mitotic chromosomes. In larvae, the antibody stained nuclei of several tissues including a posterior part of the salivary gland. This latter staining was associated with natural or ecdysteroid-induced puffs on polytene chromosomes. Upon heat treatment of larvae, the staining on the endogenous puffs disappeared and strong staining appeared on heat shock puffs. These results implicate SCF role in gene expression. For details, see Ref. 3.

(4) Yeast coactivator MBF1 mediates GCN4-dependent transcriptional activation

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Transcriptional coactivators play a crucial role in gene expression by communicating between regulatory factors and the basal transcription machinery. The coactivator MBF1 (multiprotein bridging factor 1) was originally identi-

fied as a bridging molecule that connects the *Drosophila* nuclear receptor FTZ-F1 and TATA-binding protein TBP. The MBF1 sequence is highly conserved across species from yeast to human. Here we provide *in vitro* and *in vivo* evidence that yeast MBF1 mediates GCN4-dependent transcriptional activation by bridging the DNA-binding region of GCN4 and TBP. These findings indicate that the coactivator MBF1 functions by recruiting TBP to promoters where DNA-binding regulators are bound. For details, see Ref. 4.

(5) Comparison of sequences of a transcriptional coactivator MBF2 from three Lepidopteran species *Bombyx mori*, *Bombyx mandarina* and *Samia cynthia*

Qing-Xin LIU, Hitoshi UEDA and Susumu HIROSE

MBF2 was first isolated from the silkworm *Bombyx mori* as a positive cofactor that activates transcription through its interaction with TFIIA. To identify conserved domain(s) within the MBF2 molecule, we isolated cDNAs encoding MBF2 homologues from other silkworms *Bombyx mandarina* and *Samia cynthia*. Bacterially expressed and purified MBF2 of *B. mandarina* and *S. cynthia* activated transcription *in vitro*. The predicted amino acid sequences of MBF2 from two *Bombyx* species share 97% homology. When we compared between *B. mori* and *S. cynthia* factors, the homology reduced to 50%. Four regions in MBF2 are conserved among these three species. Two of them are present in the middle region of MBF2 that is essential for the transcriptional activation. For details, see Ref. 5.

(6) DSIF, a novel transcription elongation factor that regulates RNA polymerase II processivity, is composed of human Spt4 and Spt5 homologs

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We report the identification of a transcription elongation factor from HeLa cell nuclear extracts that causes pausing of RNA polymerase II (Pol II) in conjunction with the transcription inhibitor 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole (DRB). This factor, termed DRB sensitivity-inducing factor (DSIF), is also required for transcription inhibition by H8. DSIF has been purified and is composed of 160-kD (p160) and 14-kD (p14) subunits. Isolation of a cDNA encoding DSIF p160 shows it to be a homolog of the *Saccharomyces cerevisiae* transcription factor Spt5. Recombinant Spt4 protein, the human homolog of yeast Spt4, is functionally equivalent to DSIF p14, indicating that DSIF is composed of the human homologs of Spt4 and Spt5. In addition to its negative role in elongation, DSIF is able to stimulate the rate of elongation by RNA Pol II in a reaction containing limiting concentrations of ribonucleoside triphosphates. A role for DSIF in transcription elongation is further supported by the fact that p160 has a region homologous to the bacterial elongation factor NusG. The combination of biochemical studies on DSIF and genetic analysis of Spt4 and Spt5 in yeast indicates that DSIF associates with RNA Pol II and regulates its processivity *in vitro* and *in vivo*. For details, see Ref. 6.

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C-c. Division of Physiological Genetics

(1) NELF, a multisubunit complex containing RD, cooperates with DSIF to repress RNA polymerase II elongation

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The purine nucleotide analog 5,6-dichloro-1-β - ribofuranosylbenzimidazole (DRB) is a classic inhibitor of transcription elongation by RNA polymerase II (Pol II). Since DRB generally affects class II genes, factors involved in this process must play fundamental roles in pol II elongation. Two elongation factors essential for DRB action were identified, namely DSIF (Wada et al., Genes Dev., 12, 343-356, 1998) and P-TEFb (Marshall and Price, J. Biol. Chem., 270, 12335-12338, 1995). Recently, we have identified and purified a third protein factor required for DRB-sensitive transcription from HeLa cell nuclear extracts. This factor, termed *negative elongation factor* (NELF), cooperates with DSIF and strongly represses pol II elongation. This repression is reversed by P-TEFb-dependent phosphorylation of the pol II C-terminal domain. NELF is composed of five peptides, the smallest of which is identical to RD, a putative RNA-binding protein of unknown function (Yamaguchi et al., Cell, 97, 41-51, 1999). We have shown a molecular mechanism for DRB action and a regulatory network of positive and negative elonagtion factors.

(2) Gene expression level and species-specific diversity of codon usage based on multivariate analysis

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We examined codon usage in *Bacillus subtilis* genes by multivariate analysis, quantified its cellular levels of individual tRNAs, and found a clear constraint of tRNA contents on synonymous codon choice. Individual tRNA levels were proportional to the copy number of the respective tRNA genes. This indicates that the tRNA gene copy number is an important factor to determine in cellular tRNA levels, which is common with *Escherichia coli* and yeast *Saccharomyces cerevisiae*. Codon usage in 18 unicellular organisms whose genomes have been sequenced completely was analyzed and compared with the composition of tRNA genes. The 18 organisms are as follows: yeast *S. cerevisiae, Aquifex aeolicus, Archaeoglobus fulgidus, B. subtilis, Borrelia burgdorferi, Chlamydia trachomatis, E. coli, Haemophilus influenzae, Helicobacter pylori, Methanococcus jannaschii, Methanobacterium thermoautotrophicum, Mycobacterium tuberculosis, Mycoplasma genitalium, Mycoplasma pneumoniae, Pyrococcus horikoshii, Rickettsia prowazekii, Synechocystis sp.,* and *Treponema pallidum*. Codons preferred in highly expressed genes were related to the codons optimal for the translation process, which were predicted by the composition of isoaccepting tRNA genes. Genes with specific codon usage are discussed in connection with their evolutionary origins and functions. The origin and terminus of replication could be predicted on the basis of codon usage when the usage was analyzed relative to the transcription direction of individual genes.

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D. DEPARTMENT OF POPULATION GENETICS

D-a. Division of Population Genetics

(1) Local recombination- and mutation-effects on molecular evolution in Drosophila

Toshiyuki TAKANO

Deleterious mutations clearly arise at a much higher rate than advantageous and compensatory mutations, indicating the importance of effective elimination of deleterious mutations from a population. Linkage associations between selective loci reduce the efficacy of natural selection (the so-called Hill-Robertson effect), and thus recombination rates affect accumulation rates of deleterious and advantageous mutations. I previously showed the significant difference in the synonymous-substitution pattern found in the *achaete-scute complex (AS-C)* genes in two Drosophila lineages, higher codon-bias in *D. yakuba*, and lower bias in *D. melanogaster* (for detail, see Ref. 1). Besides these genes, the functionally unrelated *yellow* (*y*)gene showed the same substitution pattern, suggesting a phenomenon specific to the *X*-chromosome telomere region (the *y* and *AS-C* genes are all in the tip of the X chromosome). Because the numbers of A/T→G/C substitutions were not significantly different from those of G/C→A/T in the *y* noncoding regions of these species, a AT/GC mutational bias could not thoroughly account for the synonymous-substitution biases. By contrast, we did find about a 14-fold difference in recombination rates in the *X*-chromosome telomere regions between the two species, suggesting that the reduction of recombination rates in this region resulted in the reduction of the efficacy of selection in *D. melanogaster.*

The *y* locus also revealed a very high GC-bias of the substitutions in the *D. orena* branch, which increased the G+C content at silent sites by about 5% since the divergence from *D. erecta*. The ratio of the number of A/T → G/C substitutions to the number of the reverse in the *orena* branch was significantly different from those at the *Adh* and *Amy* loci. The ratio was also sig-

nificantly different from those at the *y* in the *D. erecta* branch. This suggests that the local mutation pressure changed in the *orena* branch specifically at the *y* locus. This change, in turn, probably led to the higher substitution rate in the *orena* branch compared with the *erecta*.

In conclusion, this study provided evidence that local changes in recombination rates and mutational pressure are contributing to the irregular synonymous-substitution patterns in Drosophila.

(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

With the aim of revealing genetic variation accumulated among closely related species during the course of evolution, this study focuses on loss of macrochaetae on the notum as one of the developmental anomalies seen in inter-specific hybrids between *D. melanogaster* and its closely related species. I previously presented evidence that bristle loss in inter-specific hybrids is found between *D. melanogaster* and *D. simulans*, but not between pairs of *D. melanogaster* on one hand, and *D. mauritiana* and *D. sechellia* on the other. Genetic analysis showed that the *D. simulans* X chromosome confers a large effect on hybrid bristle loss, although X-autosome interaction is presumably involved. Use of cell-type markers suggests that the defect does not lie in cell fate decisions during bristle development, but in the maintenance of neural fate and/or differentiation of the descendants of sensory mother cells. For detail, see Ref. 2.

To isolate genes responsible for the bristle loss of hybrids, deficiency screening and QTL (quantitative trait loci) mapping based on the within-species variation of *D. simulans* were performed. The latter analysis revealed that a single major effect locus (or cluster of loci) on the X chromosome accounted for about two-thirds of the parental difference. I have screened this region for duplications that affect the hybrid bristle phenotypes. One duplication rescued hybrids from the bristle loss to a great degree. Subsequently, deficiencies were induced on this duplication with 4000 rad of gamma-ray

irradiation. In a screen of 13000 mutagenized chromosomes, 31 deletions were recovered. Physical mapping of the deletion-induced duplications and other non-rescue duplications allowed us to localize the locus to a small region in polytene interval 14 A-B. Molecular mapping at fine scale is now under way.

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D-b. Division of Evolutionary Genetics

(1) Non-B DNA structures spatially and sequence-specifically associated with individual centromeres in the human interphase nucleus

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Chromosome bands are associated with various genome characteristics such as DNA replication timing and long-range GC% mosaic structures (i.e. isochores), and therefore their boundaries are thought to correspond to switching regions for replication timing and isochore borders. We previously found a boundary for both the Mb-sized GC% mosaic domains and the replication-timing zones at the junction between human MHC classes II and III. Within this junction region, there is a 209-bp polypurine/polypyrimidine sequences (Pur/Pyr tract) with triplex-forming potential. Triplex formation occurs readily in Pur/Pyr tract leaving single-strand DNAs that can hybridize with other single-strand DNAs and RNAs. Distantly spaced DNAs may also form transmolecular triplexes organizing themselves into an ordered array. Therefore, triplex formation is thought important as a molecular mechanism determining spatial organization of genome DNA in the nuclei. To investigate

biological significance of non-B forming DNAs, unusual DNA structures such as triplexes were searched in the human interphase nucleus with a modified fluorescence in situ hybridization method; "nondenaturing" hybridization. Various Pur/Pyr tracts present in the human genome were found to have triplex-forming potential in the interphase nucleus. The triplexes visualized by DNA probes and antitriplex antibodies were characteristically spatially associated with centromeres. We hypothesize that different triplex-forming DNAs associate, sequence-specifically, with distinct centromeres in the interphase nucleus. For details, Chromosomes Today 13, in press, 1999.

(2) Studies on codon usages and tRNA genes of 18 unicellar organisms and quantification of *Bacillus subtilis tRNAs*: Gene expression level and species-specific diversity of codon usage based on multivariate analysis

Shigehiko KANAYA and Toshimichi IKEMURA

We examined codon usages in *Bacillus subtilis* genes by multivariate analysis, and also quantified cellular contents of individual tRNAs of the species. Clear constraint of tRNA contents on synonymous codon choice was observed. Contents of individual tRNAs were proportional to copy number of the respective tRNA genes, in common with *Escherichia coli* and yeast *Saccharomyces cerevisiae*. This shows that the tRNA gene number is an important factor to determine cellular tRNA content. Codon usages of eighteen unicellular organisms whose genomes have been completely sequenced, were similarily analyzed and compared with compositions of tRNA genes. The eighteen organisms are as follows; yeast *S. cerevisiae, Aquifex aeolicus, Archaeoglobus fulgidus, B. subtilis, Borrelia burgdorferi, Chlamydia trachomatis, E. coli, Haemophilus influenzae, Helicobacter pylori, Methanococcus jannaschii, Methanobacterium thermoautotrophicum, Mycobacterium tuberculosis, Mycoplasma genitarium, Mycoplasma pneumoniae, Pyrococcus horikoshii, Rickettsia prowazekii, Synechocystis* sp., and *Treponema pallidum*. Codon usage in highly expressed genes were related with the codons optimal for translation predicted by composition of isoacceptor tRNA genes. Genes with specific codon usage were also discussed in connection with these specific functions as well

as their specific evolutionary origin; e.g., the evolutionary processes that establish chloroplast and mitochondria genomes were discussed in view of codon usage of cyanobacteria and proteobacterium genes, respectively. The origin and terminus of replication could be predicted for a few species using the species-specific codon heterogeneity. For details, Gene, in press, 1999.

(3) Triplex-forming DNAs in the Human Interphase Nucleus Visualized *In Situ* by Polypurine/Polypyrimidine DNA Probes and Antitriplex Antibodies. Triplex-forming DNAs Are Spatially Associated with Centromeres

Mizuki OHNO[1], Toyoaki TENZEN, Hiroyuki SASAKI[1], Jeremy S. LEE[2] and Toshimichi IKEMURA ([1]Inst. Genetic Information, Kyushu Univ., [2]Department of Biochemistry, University of Saskatchewan, Canada)

Triplex formation occurs readily in polypurine/polypyrimidine tracts under physiological conditions leaving the unpaired DNAs available for hybridization with other single-stranded DNAs. Single-strand formation and transmolecular triplex formation could enable sequences spaced distantly along the genome to associate with each other and organize nuclear DNA into ordered structures. Multiple copies of polypurine/polypyrimidine sequences are present in the human genome, and their locations are scattered among and within chromosomes. Triplex-forming DNAs in the human interphase nucleus were investigated using a modified fluorescence *in situ* hybridization method that employed polypurine/polypyrimidine probes. This technique, which is used to detect RNA, will detect single-stranded DNAs in nondenatured nuclei. Polypurine/polypyrimidine probes such as (GA/TC)n and (GAA/TTC)n gave sequence-specific, foci-type signals that overlapped with or were closely associated with triplexes immunolocalized by known antitriplex antibodies. The triplexes visualized differentially with distinct polypurine/polypyrimidine probes were associated spatially with centromeres in the interphase nucleus in a sequence-specific manner. Several trinucleotide repeats that can form non-B structures, including those associated with trinucleotide-expansion diseases, gave foci-type signals, suggesting that several non-B structures could form in the nucleus.

(4) Early and late replication zone borders are present in and around the human X-inactivation center (XIC): correlation with chromosome bands

Yoshihisa WATANABE, Toyoaki TENZEN and Toshimichi IKEMURA

The human genome is composed of long-range G+C% mosaic structures, which are thought to be related to chromosome bands. Replication timing during S phase is associated with chromosome bands; thus, band boundaries are thought to correspond to regions where replication timing switches. The proximal limit of the human X-inactivation center (XIC) has been localized cytologically to the junction zone between Xq13.1 and Xq13.2. Using PCR-based quantification of newly replicated DNA from cell-cycle fractionated THP-1 cells, the precise replication timing in and around XIC was determined at the molecular level. We found two regions where replication timing changes from early to late. One is near the XIST locus, and the other is located near a large inverted duplication proximal to XIC. This identified the 1-Mb late replication zone (from XIST to the large inverted duplication) as G-band Xq13.2. We found several common characteristics between the XIST region and the MHC class II-III junction which was previously defined as a chromosome band boundary. These characteristics included differential, dense clustering of *Alu* and LINE repeats, and the presence of polypurine/polypirimidine tracts, MER41A, MER57 and MER58B.

(5) Compartmentalized DNA-replication timing and GC% levels in and around the human MHC region

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Genomes of warm-blooded vertebrates have long-range GC% mosaic structures (isochores), which are thought to be related to chromosome bands. DNA replication timing, gene density, and repeat sequence density also are related to chromosome bands. Basing on these genome characteristics connected

with chromosome bands, we may define their boundaries at the molecular level. We previously reported a concordant transition of GC% level and replication timing in the junction region of the human MHC classes II and III and proposed the junction to be a band boundary at a high resolution level. In this work, we determined DNA replication timing in and around the MHC region and again found the concordant transition of DNA replication timing and GC% levels. The compartmentalized structures is the following. Non-MHC region on the centromeric-side (very GC-rich; very early replication): class II (AT-rich; relatively late replication): class III (very GC-rich; very early replication): class I (intermediate GC%; early replication): telomeric non-MHC region (AT-rich; late replication). Several characteristic structure including polypurine/polypyrimidine tract were found in the transition regions. The human genome contain a large number of polypurine / polypyrimidine tracts. Polypurine / polypyrimidine sequences have the potential to form triplex and are sites where DNA polymerases pause *in vitro*. We have shown the 209-bp tract in the class II and III junction to form triplex *in vitro* and in the human interphase nucleus.

(6) Conserved evolution of the Rh50 gene compared to its homologous Rh blood group gene

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We have sequenced the complete coding region of the Rh blood group gene for mouse and rat and that of Rh-related 50kD glycoprotein (Rh50) for mouse, rat, and crab-eating macaque. Phylogenetic analyses of Rh and Rh50 amino acid sequences indicate that the Rh50 gene has been evolving about two times more slowly than the Rh blood group gene both in primates and rodents. This conservative nature of the Rh50 gene suggests its relative importance to the Rh blood group gene. The time of gene duplication that produced the Rh and Rh50 genes was estimated to be about 240-310 million years ago. We also conducted window analyses of synonymous and nonsynonymous nucleotide substitutions for those two genes. Some peaks where nonsynonymous substitutions are higher than synonymous ones were located on outer membrane

regions. This suggests the existence of positive Darwinian selection on Rh and Rh50 genes through host-parasite interactions. For details, see Ref. 9.

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D-c. Division of Theoretical Genetics

(1) Evolution by nearly-neutral mutations

Tomoko OHTA

Genetic systems are complex and interact at various levels; among amino acids or nucleotide sites, among gene products, and among regulatory regions and proteins. Patterns of synonymous and nonsynonymous substitutions of mammalian genes indicate that nonsynonymous substitutions are nearly neutral, coming from interactions among amino acids. As an interactive system, the NK model of Kauffman was analysed. This model assumes that each amino acid makes a fitness contribution that depends upon the amino acid and upon K other amino acids among the N that make the protein. Through simulations, it was found that there are numerous nearly-neutral mutations in this model, and that evolution is rapid in small populations and slow in large ones. The system moves on the rugged fitness landscape by mutation, random genetic drift and selection. Small populations have more chance to attain novel genetic systems than large ones because of larger effects of random drift, but the chance of extinction becomes greater. For details, see Genetica 102/103, 83-90.

(2) On the pattern of polymorphisms at major histocompatibility complex loci

Tomoko OHTA

The pattern of polymorphisms at major histocompatibility complex loci was studied by computer simulations and by DNA sequence analysis. Two types of selection overdominance plus short-term selection and maternal-fetal in-

compatibility, were simulated for a gene family with intra- and interlocus gene conversion. Both types of selection were found to be consistent with the observed patterns of polymorphisms. It was also found that the more interlocus conversion occurs, the higher the divergence becomes at both nonsynonymous and synonymous sites. The ratio of nonsynonymous-to-synonymous divergence among alleles decreases as the interlocus conversion rate increases. These results agree with the interpretation that the rate of interlocus conversion is lower in human genes than in genes of other nonprimate mammals. This is because, in the latter, synonymous divergence at the ARS (antigen recognition site) is often higher than that at the non-ARS, whereas in the former, this is not so. Also, the ratio of nonsynonymous to synonymous substitutions at the ARS tends to be higher in human genes than in other mammalian genes. The main difference between overdominance plus short-term selection and maternal-fetal interaction is that the number of alleles and heterozygosity per locus are higher in the latter than in the former under the presumed selection intensities. However, the average divergence among alleles tends to be lower in the latter than in the former under similar conditions. For details, see Jour. Mol. Evol., 46, 633-638.

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1. OHTA, T.: Evolution by nearly-neutral mutations. Genetica 103, 83-90, 1998.
2. OHTA, T.: On the pattern of polymorphisms at major histocompatibility complex loci. Journal of Molecular Evolution 46, 633-638, 1998.

Oral Presentations

1. OHTA, T.: Nearly neutral mutations and the molecular clock. Aug. 12, Beijin, China.
2. OHTA, T.: Nearly neutral theory of molecular evolution. Aug. 23 Fukuoka.
3. OHTA, T.: Nearly neutral theory. Oct. 6, Workshop, Towards a Comprehensive Dynamics of Evolution, Santa Fe, U. S. A.

E. DEPARTMENT OF INTEGRATED GENETICS

E-a. Division of Human Genetics

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

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Our main research subject is genomic imprinting, which causes parental-origin-specific monoallelic expression of a subset of mammalian genes. Imprinted genes tend to form clusters in specific regions of the genome. This 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 study an imprinted domain in mouse chromosome band 7F4/F5. This region is syntenic to human 11p15.5, which contains 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. YAC, BAC, and cosmid contigs covering the 1-Mb region were constructed and a physical map of the region was produced. 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. Since most imprinted genes are associated with a CpG island, we also developed a quick and simple method to identify CpG islands (Ref. 4), which can be used to isolate new imprinted genes.

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

Hiroyuki SASAKI, Ko ISHIHARA[1], Reiko KATO[1], Hiroyasu FURUUMI[1], Mizuki OHNO[1], Mohamad ZUBAIR[1], Naoya HATANO[1] and Tohru IWAKI[2] ([1]Institute of Genetic Information, Kyushu University, [2]Department of Neuropathology, Neurological Institute, Faculty of Medicine, Kyushu University)

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 parental-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 the tissues other than endoderm, we sequenced a 40-kb region containing *H19* in both mouse and human. A comparison of the sequences from the two species revealed a total of ten evolutionarily conserved non-coding segments, two of which precisely coincided to 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 were tissue-specific enhancers (three mesoderm-specific and two ectoderm-specific enhancers). More detailed studies on the mechanism of *Igf2/H19* imprinting is now being conducted.

(3) Cloning of a family of novel mammalian methyltransferases

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In genomic imprinting, 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 code 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) Human genome resources and their application for the analysis of chromosome21

Asao FUJIYAMA, Ayuko MOTOYAMA[1] and Hong-Seog PARK[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, detailed analyses on human 21qcen and 21qter regions are in progress.

(5) Whole genome analysis of signal-transduction pathways in fission yeast

Yong-Sik BONG, Inaho DANJO[1] and Asao FUJIYAMA ([1]Max Planck Institute, Munich)

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.

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E-b. Division of Applied Genetics

(1) Expression pattern of the α-amylase gene during seed germination in rice and barley

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In situ hybridization analysis shows some similarities in the expression pattern between rice and barley α-amylase genes, *RAmy1A* and *Amy1*: i) the expression of these genes initiates in the scutellar epithelium and continues in the aleurone layer, ii) the gene expression in the aleurone layer initiates from the region close to the embryo. However, we also identified some differences between these species; i.e., i) expression of the rice α-amylase gene initiates in the terminal regions of scutellar epithelium, whereas middle-basal region is the initial expression site of the barley gene, ii) the progress of the gene expression in rice aleurone layer is faster than that in barley one. Additionally, analyses using embryo mutants indicate that the abolishment of the scutellum results in no α-amylase gene expression. For details, see Ref.1.

(2) *panicle phytomer 1* mutations affect the panicle architecture of rice

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We have characterized three *panicle phytomer 1 (pap1)* mutations from the viewpoint of phytomer. In *pap1* mutants, rachis phytomers were strongly affected: severe reduction of rachis internode length and the increase in the number of rachis internodes. In addition, bracts were frequently overdeveloped. On the contrary, *pap1* differently affected primary branch phytomers: reduction in both the number and length of internodes. Rudimentary and empty glumes were frequently elongated. Floral organs were mostly normal. However, a double

mutant between *pap1* and *fon1* inducates that *PAP1* has a distinct role in the regulation of floral organ number. Thus *PAP1* participates in the regulation of panicle phytomers. For details, see Ref.2.

(3) A recessive heterochronic mutation, *plastochron 1*, shortens the plastochron and elongates the vegetative phase in rice

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We identified two recessive alleles of a rice heterochronic gene, *plastochron1-1* (*pla1-1*) and *pla1-2* , that reduce the length of the plastochron to about half that of the wild type. The number of leaves produced in the vegetative phase was nearly twice that in the wild type. Panicle development was severely disturbed in *pla1* mutants; primary rachis branch primordia were converted into vegetative shoots. Thus, vegetative and reproductive programs are simultaneously expressed during the reproductive phase of *pla1*. Accordingly, *pla1* is a heterochronic mutation that extends the vegetative period. The *PLA1* gene is considered to regulate the duration of the vegetative phase via controlling the rate of leaf production in the meristem. For details, see Ref.3.

(4) Isolation and characterization of a rice homeobox gene, *OSH15*

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We isolated a cDNA designated *OSH15* that encodes a KNOTTED-type homeodomain protein. Transgenic tobacco plants showed a dramatically altered morphology. In early embryogenesis, *OSH15* is expressed at a region where shoot apical meristem would develop later. Thereafter, *OSH15* expression in the shoot apical meristem ceased but was detected in a ring-shaped pattern at the boundary of some embryonic organs. This expression pattern was also observed in the vegetative and reproductive shoots as if *OSH15* dis-

tinguishes each phytomer near the shoot apex. *OSH15* may play roles in morphogenetic events around the shoot apical meristem. For details, see Ref.4.

(5) Molecular mechanisms of hereditary neurodegenerative diseases

Shoji TSUJI (Niigata University)

The research is focused to elucidate molecular mechanisms of human neurological diseases and eventually to develop therapeutic measures. Among the neurological diseases, the research is focused on diseases caused by expansions of CAG repeats encoding polyglutamine stretches, which include hereditary spinocerebellar degenerations. We have demonstrated that the prevalence rates of individual spinocerebellar degenerations substantially vary among ethnic populations. We have recently found that the frequencies of intermediate alleles, i.e. alleles with large CAG repeats within the normal range, show good correlations with the prevalence rates of the corresponding spinocerebellar degenerations, suggesting that the intermediate alleles are the source for the mutant alleles. We have recently succeeded in creating transgenic mice showing instability of the CAG repeats similar to those observed in human diseases, which should be a good mouse model for investigating the molecular mechanisms of the instability of the CAG repeats. With regard to the mechanisms of neurodegeneration caused by CAG repeat expansions, we have demonstrated that truncated mutant proteins with expanded polyglutamine stretches result in aggregate formation and lead to apoptosis.

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F. GENETIC STRAINS RESEARCH CENTER

F-a. Mammalian Genetics Laboratory

(1) Analysis of the genetic structure of *Pb* hotspot in the classII of MHC

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In the mouse major histocompatibility complex (MHC) class II region, the breakpoints of meiotic recombination are not distributed at random, but are clustered in specific sites, called hotspots. All of the hotspots are located downstream or inside of genes in the MHC. Thus far, four hotspots have been identified in this region. Among four, a hotspot in the vicinity of *Pb* gene (hereafter we called *Pb* hotspot) has been poorly characterized.

In the present study, we constructed a fine restriction map of the 15kbp of DNA fragment around the *Pb* hotspot. Molecular mapping revealed that five recombinants were confined to a 2.4kb DNA region located to the 3'end of *Pb* gene. Comparing the molecular structure of the four hotspots including *Pb* hotspot, we found that all hotspots were located in introns or 3' end of genes, but not at 5' end of genes. We could not detect any sign of the transcription in the 15kbp DNA fragment encompassing the *Pb* hotspot by Northern blot hybridization analysis using male meiotic cells. This contrasts to hotspots identified in budding yeast, in which most of hotspots are located at 5' end of genes overlapping with transcription initiation sites. We sequenced the 2.4kb DNA fragment including the *Pb* hotspot from recombinants and their parental strains. As the result, recombinational breakpoints of at least five recombinants were confined to a 341bp of DNA segment located in the intron of the *Pb* gene. The other recombinant had a breakpoint in the 2.9kb region distal to the intron. The sequence of the hotspot was searched in the database to identify known. We found that the sequence has MT-middle repeti-

tive family, a sequence homologous to the transmembrane domain of rat *Pb* gene and the motif of rat tumor necrosis factor (TNF).

In addition, we have studied higher chromatin structure around the hotspots in meiotic prophase by FISH analysis with DNA probes for the hotspots and the other regions in the MHC class II, being followed by immunostaining with anti-synaptonemal complex (SC) proteins antibody. This study indicated that the DNA region including the *Lmp2* hotspot was randomly placed in relation to the SC.

(2) Mapping of modifier gene suggests interaction of two preaxial polydactylous mutant genes, *Rim4* and *Ist*, in mouse

Hiroshi MASUYA, Tomoko SAGAI and Toshihiko SHIROISHI

The anteroposterior axis patterning in vertebral limb morphogenesis is established by the formation of the zone of polarizing activity (ZPA) at the posterior margin of limb buds. The Sonic hedgehog (*Shh*) gene is expressed exclusively at the posterior margin of limb buds, and it mediates the polarizing activity. A preaxial polydactyly mutant *Rim4* that arose spontaneously in the C57BL/10 background shows ectopic expression of *Shh* at the anterior margin of the limb bud and results in mirror-image duplication of the digits. Crossing the *Rim4* heterozygotes with an inbred strain, MSM, completely suppresses the *Rim4* phenotype, while crossing the same mice with the NZB strain demonstrates almost a full penetrance of the phenotype. In this study, we carried out mapping of the modifier gene for *Rim4*, based on the polymorphism between the MSM and NZB strains. As a result, a major modifier gene was mapped close to Aristaless-like4 (*Alx4*), which is the causative gene of another preaxial polydactyly mutation, Strong's luxoid (*lst*), on Chromosome 2. Double heterozygotes of *Rim4* and *lst*^{J} showed a more severe polydactyly phenotype than that of the heterozygotes of either single mutation. We found three amino acid substitutions in the *Alx4* gene between the MSM and NZB alleles. All these results suggest the possibility that genetic interaction between *Rim4* and *Alx4* genes plays a key role in establishing anteroposterior axis formation in limb development.

(3) Phenotype characterization and fine mapping of a preaxial polydactyly mutant, luxate (*lx*)

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luxate (*lx*) is a spontaneous mouse mutant and mapped to the proximal region of Chromosome 5. *lx* heterozygotes have preaxial polydactyly including hyperphalangy of the first digit only on the hindlimb. The homozygotes show missing of the first digit, hemimelia with the shortened tibia and sacralization of the 26th vertebra.

In addition to several skeletal anomalies, various kidney defects such as horseshoe kidney, polycystic kidney and hydronephrosis were often observed in the mutant. These kidney defects seemed to be influenced by the genetic backgrounds. Only 10 percent of *lx* homozygotes on the NZB background exhibited kidney defect, while as many as 55 percent exhibited the same phenotype on the C57BL/6 background. The *lx* phenotypes were completely suppressed when the mutant was mated with an inbred strain MSM that was established from Japanese wild mouse. In order to carry out the linkage analysis of *lx* mutation, we have mated *lx* homozygotes with the consomic strain, B6.MSM-Chr5, in which the whole Chr5 is introduced from MSM strain into the genetic background of C57BL/6 strain. By using this consomic strain, *lx* exhibited full penetrance of the phenotypes. Thus, we took an advantage to use high frequency of polymorphism between MSM and C57BL/6 in the gene mapping of *lx* mutation. Based on 112 backcross progeny from the above cross, *lx* was mapped to an interval between *D5Mit77* and *D5Mit108*, and was tightly linked to the markers *D5Mit267*, *D5Mit150* and *D5Mit130*.

(4) Physical mapping of polysynductylous mutation, hemimelic extra toes (*Hx*)

Tomoko SAGAI, Hiroshi MASUYA and Toshihiko SHIROISHI

Two limb deformity mutations, Hemimelic extra toes (*Hx*) and hammer-toe (*Hm*) are located on the proximal region of Chr 5. *Hx* shows preaxial polydactyly on all four feet associated with hemimelia, shortening of the radius, tibia

and talus in the original C57BL/10 background. On the otherhand, *Hm* mutation shows interdigital webbing regression, which is probably due to an impairment of apoptosis process in limb development. *Hx* and *Hm* are tightly linked and only one recombinant has been reported in the1,664 offspring from the backcross of these two mutants. In the syntenic region of human chromosome 7q36, a congenital limb deformity with polysyndactyly has been mapped. The causative gene(s) has not been identified yet.

Toward positional cloning of the genes involved in limb morphogenesis in this region, we have carried out the precise genetic and physical mapping of *Hx* mutation. A linkage analysis using 1,570 backcross progeny with MSM strain showed that *Hx* mutation was localized to a 0.6cM interval between sonic hedgehog (*shh*) gene which mediates antero-posterior signaling in limb development and interleukin 6 (*IL6*) gene. Presence of one recombinant excluded a possibility that the coding sequence of *shh* is altered in *Hx* mutation. However a possibility that cis regulatory element of *shh* is affected in *Hx* still remain, since *Hx* is very close to *shh* and ectopic expression of *shh* is observed in the anterior margin of the limb bud of *Hx* mutant.

We constructed YAC and BAC contigs from the *shh* coding region to the *Hx* locus. The result showed that *Hx* and the *shh* coding region are colocalized within a 1.5 Mb single YAC clone, and *Hx* is included in a 600 kb single YAC clone. As a next step for the more precise mapping of *Hx*, we carried out shotgun sequencing of a 150kb BAC clone contained in the 600 kb YAC clone. We generated new STS markers from the BAC clone. At the same time, in order to have new recombinants, we crossed *Hx* with three inbred strains, P/J, MAL and BLG2 and obtained 730 backcross progeny. By the linkage analysis of them using the new STS markers, we obtained five new recombinants in 600 kb DNA fragment of *Hx* critical region. No recombination has occurred in this region among previous 1,570 progeny from cross with MSM. Thus, the result indicated that use of different crosses with plural strains should be very useful for the isolation of recombinants in given regions. Finally the new recombinants mapped *Hx* mutation to a 150 kb single BAC clone. We have finished construction of cosmid contig covering the 150kb DNA fragment. Now we are trying cDNA selection to isolate the transcripts in this regions.

(5) 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 previously mapped to the telomeric region of chromosome 11. *Ts* is likely to be a mouse model for a human skeletal dysmorphology known as Meckel syndrome (MES;OMIM2400), since MES has phenotypes similar to *Ts* and has been mapped to human syntenic region, 17q21-24.

To elucidate the function of the *Ts* gene in mouse embryogenesis and to verify whether it is a model for MES, we are trying to clone the gene by means of positional cloning. First, we employed a fine genetic mapping of this gene based on a large scale intersubspecific backcross between the mutant stock TsJ/Le-*Ts*/+ and Japanese wild mouse-derived MSM strain. *Ts* gene was mapped to a 0.16 cM region between two microsatellite markers, *D11Mit128* and *D11Mit256*. Subsequently, we screened mouse YAC and BAC libraries with the microsatellite markers tightly linked to the *Ts* locus and obtained YAC and BAC clones. The ends of the isolated BAC clones were sequenced to develop new STSs for further screening of clones and for securing the overlaps between the clones. Further chromosome walking with the isolated clones allowed us to construct a complete BAC contig covering the *Ts* causative gene. The critical *Ts* region was narrowed down to a DNA fragment between two new STSs, *D11Rin56* and *D11Nig17*. This contig consists of 3 BAC clones, which spans a 250 kb DNA fragment. In order to search candidate genes for *Ts*, we have isolated several cDNA clones from the critical region by directed cDNA selection using the corresponding BAC clones. We have also obtained

several cDNA clones which is derived from the mouse embryonic cDNA library. We sequenced all cDNA clones mapped to the corresponding BAC contig, and we searched related sequences on the database using BLAST and GRAIL. Three ESTs were isolated from the BAC clones in the critical *Ts* region. The first EST is expressed in mouse myotube, the second in mouse embryo (E14.5) and the last EST is ubiquitously expressed. Now we are characterizing these ESTs to identify the *Ts* causative gene.

(6) Polymorphism at Tail-short (*Ts*) locus among standard inbred strains affects the viability of *Ts* heterozygotes

Junko ISHIJIMA, Akihiko MITA, Kikue UCHIDA, Toshihiko SHIROISHI

Tail-short (*Ts*) heterozygotes have variable types of malformation including kinky tail, vertebral homeotic transformations, developmental retardation and neural tube defects. Expression of the phenotype depends on the mouse strain to which the mutant is crossed. The variation in the *Ts* phenotypes observed in crosses with different inbred strains ranges from viable to dominant full lethal. Detailed linkage analysis indicated that a single chromosomal region, genetically inseparable from the *Ts* locus, is responsible for the difference. This result suggests that there exists functional polymorphism at *Ts* locus among inbred strains, and the different manners of the interactions between the *Ts* mutant gene and polymorphic allele of the inbred strain may affect the viability of the *Ts* heterozygotes. We investigated the phenotype of the *Ts* mutation in crosses with the two groups of strains, which give viable and lethal *Ts* embryos respectively. *Ts* heterozygous embryos derived from the lethal cross exhibited more severe defects than those from the viable cross. Morphological anomalies of the *Ts* heterozygotes in the lethal cross were observed in as early as neural plate stage. The embryonic region was poorly developed compared with the extraembryonic region due to formation of excess mesoderm in the extraembryonic region. In the later developmental stage, the umbilical vein does not develop properly, which may be the cause of the lethality of the *Ts* heterozygotes around 12.5 dpc.

(7) Cloning of a responsible gene for mouse mutant *Rim3*

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Rim3 is a mouse mutant characterized by abnormalities in ectoderm-derived epithelium. Macroscopically it shows alopecia, coarse feature of the entire skin and turbidity of the cornea. Microscopical observation shows hyperkeratosis, acanthosis and few hair follicles in the skin, hyperkeratosis, acanthosis, hypergranulosis and flat-shaped basal cells in corneal epithelium. The phenotype significantly resembles that of previously reported mutant *Rex denuded* (*Re^{Den}*). Since *Rim3* is mapped to the distal portion of chromosome 11, where *Re^{Den}* is reported to localize, it is highly likely that both mutations are alleles of the same gene that functions in development of ectoderm-derived epithelium.

In the process of drawing up a detail physical map of the region in which *Rim3* is localized, it was disclosed that just just 3 clones of bacterial artificial chromosome (BAC) cover the entire region. Using these BAC clones, we have attempted to isolate the responsible gene by a method of direct cDNA selection. We have already isolated 3 fragments of partial cDNAs that share sequences of expression sequence tag (EST) expressed in mouse skin. An investigation to characterize the feature of these cDNAs is in progress.

(8) Genetic and physical mapping of *Idd4* that controls onset of IDDM in mice

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The development of IDDM in the NOD mouse is under the control of multiple insulin dependent diabetes (*Idd*) genes. One of them, *Idd4*, was mapped to mouse chromosome 11 and defined as a broad peak in a linkage analysis based on a cross between NOD and B10.H-2^{g7} strains (Todd J.A.*et.al*, 1991,1993). For fine mapping of the *Idd4* gene without any influence of other susceptibility genes, we have established a congenic strain for *Idd4* by intro-

ducing the chromosomal segment of the MSM strain, which spans from *Acrb* to *Mpo* on chromosome 11, into the NOD genetic background. The incidence of diabetes at early onset (~20wks) in female NOD/Shi.*Idd4* $^{msm/msm}$ mice was somewhat higher than that in NOD mice or NOD/Shi.*Idd4* $^{nod/msm}$ congenic mice. This result indicated that an *Idd4* allele from the non-diabetic MSM is more susceptible to early onset of diabetes than that of NOD mice. In this study, we produced a series of eight recombinant strains that carry various segments of the MSM-derived chromosome in the congenic region containing *Idd4.* By comparing the incidence of early onset diabetes among these recombinant strains, we mapped *Idd4* within a short segment less than approximately 0.8 cM on chromosome 11. To construct a physical map across the *Idd4* region, YAC and BAC libraries were screened with the DNA markers on the region. Finally, a contig of the three YAC and twelve BAC clones was obtained, which completely covered the *Idd4* region.

(9) Behavioral study of inbred strains established from wild mice. II. Learning and memory

Tsuyoshi KOIDE, Kazuo MORIWAKI[1], and Toshihiko SHIROISHI ([1]Grad. Univ. Adv. Stud.)

Behavior of mouse is studied from many aspects using only small numbers of laboratory strains. However, those laboratory mice have been domesticated in the course of establishing laboratory strains, and their characteristic behavior has been lost by the domestication. Another problem we will confront during the analyses of behavior of mice is that the variation of behavior is limited because the most of the laboratory strains has been established from relatively small mouse colony and the genetic diversity between them is small. In this point of view, use of inbred strains originated from variety of wild mice in different countries, which belong to different mouse subspecies, will provide a good chance to find novel behavioral patterns. We have started a new project in that behavioral pattern of variety of inbred strains are studied and compared. Currently, we use eight wild-derive strains, MSM, NJL, BLG2, BFM/2, SWN, KJR, HMI and CAST/Ei, as well as JF1 established from Japanese fancy mouse and two laboratory strains, C57BL/6 and DBA/1.

First, we studied ability for the learning and memory in mice by using a passive avoidance test. As a result, we found a wide range of difference in the performance among the strains used in this test. It has been reported that C57BL/6 has the good ability in the passive avoidance test, while DBA/1 has the poor ability. We obtained essentially same result for the C57BL/6 and DBA/1 in our study. In addition to this, we noticed that although BFM, KJR, SWN, and MSM have the good performance similar to that of C57BL/6 strain, CAST/Ei and BLG2 has less ability than DBA/1 strain. There is significant difference in the ability in the passive avoidance test between the good performers that include BFM/2, KJR, SWN, MSM and C57BL/6 strains, and the poor performers that include CAST/Ei and BLG2. This difference would allow us to conduct a genetic analysis for the learning and memory ability using this system. Currently, we are conducting a genetic cross between strains of the good and poor performers to map gene(s) responsible for the difference in the performance in the passive avoidance test.

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F-b. Mammalian Development Laboratory

(1) Analysis of Cellular and Molecular Mechanisms in the Development of Mouse Fetal Germ Cells and Sex Differentiation of Gonads

Norio NAKATSUJI, Masaru TAMURA, Shinichiro CHUMA, Yasuhiko KANNO, Takayuki SAKURAI, Zhenyong HUANG, Tetsuichiro SAITO, and Takashi TADA

We have been studying proliferation and growth regulation of mouse primordial germ cells (PGCs) during migratory stages by using a culture

system. They show a temporary proliferation in the culture, but their growth is arrested when they differentiate into gonia after arriving at fetal gonads. We are developing a culture system of germ cells to investigate the regulation of the entry into meiosis by germ cells. Also, we have been trying to identify novel genes involved in sex-differentiation of germ cells and gonads at these stages.

After PGCs arrive at genital ridges at around 10.5 dpc they continue to divide mitotically until about 13.5 dpc. Then, they take different paths of development according to their surrounding gonadal sex. Germ cells in male gonads are considered to arrest at the G0 stage of the cell cycle as prospermatogonia. In females, germ cells enter into meiosis and arrest at the prophase of the first meiotic division. Surface markers of PGCs which are common to the early totipotent stem cells start to diminish gradually after these transitions into mitotic arrest or meiosis. Germ cells resume mitosis as spermatogenic stem cells or meiotic progress and oocyte maturation after birth. Previous studies have suggested that sex-differentiation of the fetal germ cells is determined by the surrounding gonadal somatic environments, rather than their own chromosomal constitution. However, studies on the differentiation of PGCs has been limited to experiments using organ or re-aggregate cultures. Thus, examination of meiotic transition by PGCs and identification of its regulative factors have been impeded, due to the lack of the dissociated culture system of PGCs in which meiotic transition could be detected and which enables precise analysis on meiotic transition by PGCs. We have developed a primary culture system in which PGCs isolated and dissociated from mitotically dividing stages entered into early phase of the first meiotic division under the two-dimensional dissociated culture condition. We have demonstrated, with this system, that expression of meiotic proteins by PGCs occurs in the absence of embryonic gonadal somatic cells implying that an autonomous program for transition into meiosis is already present before reaching embryonic gonads. It is also demonstrated that LIF-gp130 signal, previously known as an inhibitor of differentiation of embryonic stem cells and promoter of the PGC survival, represses meiotic transition by PGCs in vitro. Such in vitro systems developed in our study shall provide a base for analysis on mechanisms and factors involved in meiotic transition by

PGCs of mammalian embryos.

Sex-differentiation of mouse fetal gonads starts by an appearance of testis cords in testis at 12.5 dpc, followed by mitotic arrest of germ cells in testis and initiation of meiosis in the ovary starting at 13.5 dpc. We have been trying to identify such genes in mice by using the subtraction and differential hybridization method to obtain genes whose expression is specific for the 13.5 dpc testis after subtraction by the female gonad cDNA. So far, we have isolated several clones representing novel sequences. We chose a novel gene containing typical basic helix-loop-helix domains for further investigation. We named it *nephgonadin* because its strong expression was observed in the kidney and gonad. At 13.5 dpc, expression of *nephgonadin* was stronger in the testis than the ovary. In adults, however, the expression level was decreased in the testis, while it was increased in the ovary. We are now examining functions of *nephgonadin* in gonad development.

(2) Molecular mechanism controlling neuronal differentiation in mammals

Tetsuichiro SAITO, Taro HAMA, Shuichi SAKAMOTO, Rie SABA and Norio NAKATSUJI

The mammalian nervous system comprises an enormous number of cell types. We have been trying several approaches to understand how the cell types are generated at the molecular level. Members of the family of basic-helix-loop-helix (bHLH) transcription factors, such as MASH1 and Neurogenin, play important roles in mammalian neurogenesis. We have focused on the regulatory cascades of the neural bHLH genes.

MBH1 (mammalian *BarH* homologue) is expressed in an exactly complementary pattern to *Mash1* and overlapping with *neurogenin2* in the developing nervous system. Forced expression of *MBH1* down-regulates *Mash1* expression and up-regulates *neurogenin2* in differentiating P19 cells, suggesting that *MBH1* is a regulator of the neural bHLH genes. *Neurogenin2*, however, is also expressed in some areas which do not express *MBH1*. Besides, *Drosophila* has two *BarH* genes. These suggest that there may be more *BarH* genes in mammals. To identify another mammalian *BarH* gene, we have performed

PCR using degenerated primers and obtained a DNA fragment encoding a homeodomain similar to the MBH1 protein. Using the DNA fragment as a probe, several cDNA clones were obtained which encodes the same protein, hereafter designated as MBH2. The MBH2 protein is more closely related to the MBH1 protein than any other BarH proteins, suggesting that the *MBH1* and *MBH2* genes may have been duplicated after the divergence of insects and vertebrates. The FIL peptide motif is also detected in the N-terminal portion of the MBH2 protein. *MBH2* expression is transient during neurogenesis as well as *MBH1*. *MBH2* mRNA was detected in the developing diencephalon and dorsal spinal cord. More detailed analysis of *MBH2* expression is currently being processed.

MBH1 expression is restricted to specific regions in the developing nervous system, such as the dorsal diencephalon and retinal ganglion cell layer. In order to learn the mechanisms that regulate *MBH1* expression, *MBH1* genomic DNA clones were obtained and characterized. The *MBH1* gene contains at least two introns, one of which splits the homeodomain-coding region, like the *Drosophila BarH1* and *BarH2* genes. Reporter plasmids containing a portion of the *MBH1* gene have been constructed to find out regulatory sequences for *MBH1* expression using transgenic mice.

PHD1, a member of the Paired-like homeodomain (PHD) family, is expressed downstream of *Mash1* during the differentiation of both dorsal spinal cord and olfactory sensory neurons. Other PHD genes, such as *Phox2* and *DRG11*, are also expressed downstream of the bHLH genes, in different lineages of neurons, suggesting that the cascade from bHLH to PHD transcription factors may be important for neuronal identity determination. In order to clarify the relation between *Mash1* and *PHD1*, we are trying to identify downstream target genes of *Mash1*.

(3) Reprogramming of parental-origin-specific epigenotype in mouse germ-line development

Takashi TADA, Masako TADA[1] and Norio NAKATSUJI ([1]PRESTO; Precursory Research for Embryonic Science and Technology, JST)

In mammals, maternal and paternal genomes are not functionally equiva-

lent and both genomes are essential to complete normal development. This phenomenon known as genomic imprinting is caused by accumulated effects of imprinted genes, which are expressed exclusively from the maternal or paternal allele. Allele-specific transcription of imprinted genes is regulated by epigenetic modification (imprint) such as DNA methylation, histone acetylation and chromatin structure, but not by modification of the DNA sequence in itself. One of the important aspects is that parental imprints never endure for more than one generation, indicating that both paternal and maternal imprints must be reprogrammed to paternal imprint during spermatogenesis and to maternal imprint during oogenesis. To address the mechanism of reprogramming the parental imprints in the germ line, imprinting status of embryonic germ (EG) cells established from primordial germ cells (PGCs) soon after entering to gonads of E11.5 and 12.5 embryos was analyzed. Obvious overgrowth and morphological abnormalities of chimeric embryos with these XY and XX EG cells suggest that reprogramming of the parental imprints is initiated before the onset of meiosis. DNA methylation analysis reinforced this idea. Although the maternal or paternal allele is differentially methylated in somatic cells, both alleles of the majority of imprinted genes examined lost DNA methylation and this hypomethylation situation was maintained before and after cell differentiation. This biallelic hypomethylation resulted in biallelic activation in some imprinted genes and biallelic silencing in others as revealed by mRNA *in situ* hybridization. These findings clearly demonstrated that the initial key step of reprogramming of the parental imprints is the erasure of the existing parental imprints in the germ line (ref. 6).

Erasure of the parental imprints in EG nuclei led us to consider that the *trans*-acting factor(s) involved in the initial step may be maintained in EG cells. To address this question, somatic cells in which the parental imprints have been established were cell-hybridized with EG cells. Differential DNA methylation of imprinted genes in somatic nuclei was drastically erased and both alleles showed hypomethylation in the hybrid cells. Maternally methylated and repressed *Peg1/Mest* in somatic cells was demethylated and reactivated in chimeras with the hybrid cells. It is likely that EG cells retain dominant *trans*-acting factor(s) to induce erasure of parental imprints as similar to germ cells. Recent our unique approach to elucidate mechanisms of the im-

printing erasure is that a single maternal human chromosome 7, carrying an imprinted gene *PEG1/MEST* and tagged with the transgene *neo,* was introduced into mouse embryonic stem (ES) cells (ES/P) and EG cells (EG/P) by the microcell-mediated chromosome transfer technique. Transcription from maternal human *PEG1/MEST*, which is methylated and repressed in somatic cells was detected in E7.5 and 9.5 chimeras only with EG/P but not in chimeras with ES/P. Reactivation of human *PEG1/MEST* in mouse EG/P derivatives indicates that the mouse erasure factor(s) is capable of reprogramming the human maternal imprint. This trait may be unique to germ cells as seen in EG/P cells but not in ES/P cells.

Imprinting erasure is tightly linked to the genome-wide demethylation in premeiotic germ cells. Erasure of epigenetic modification including DNA methylation and imprints may play an important role to prevent inheritance of a predefined parental epigenetic modification to offspring. If so, this process is essential for the preparation to impose appropriate epigenetic modification on gametic genomes. We are now focusing our attention on the identification of the *trans*-acting factor(s) involved in the erasure event.

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F-c. Plant Genetics Laboratory

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

(1)-a. Functional Analysis of Homeobox Genes Expressed in Embryogenesis and Regeneration Process in Rice

Yukihiro ITO, Atsushi CHUJO, Kazuhiko TAKAYA, Yasuo NIWA[1], Nori KURATA
([1]Shizuoka Pref. Univ.)

To elucidate genetic programmes that control embryogenesis and regeneration of rice, we conducted cloning and structural and functional analyses of genes which encode transcription factors and protein kinases. We previously identified eight KN1-like homeobox genes and analysed their expression patterns during early embryogenesis and regeneration by RT-PCR and *in situ* hybridization (see ref.7). To gain insight into functions of these genes overexpression experiments in rice were carried out. When one of the cloned gene *OSH1/HOS24* under the control of a cauliflower mosaic virus 35S RNA promoter (P35S) were introduced into rice calli by *Agrobacterium*-mediated transformation, most of the transformed calli did not regenerate even on regeneration medium and continued to grow as mostly undifferentiated calli. As *OSH1/HOS24* is known to be expressed in a shoot meristem, we postulated that *OSH1/HOS24* functions to maintain cells in the shoot meristem in an undifferentiated state and its overexpression causes inhibition of the normal regeneration of calli. In addition we found that overexpression of *OSH1/HOS24* induced expression of other homeobox genes *HOS3* and *HOS16*. In

early embryogenesis these three homeobox genes are expressed in a same region where the shoot meristem will appear. This suggests that such a expression network may also occur in embryogenesis.

To understand relationship between regeneration and *OSH1/HOS24*, a promoter region of the gene was fused to a coding region of green fluorescent protein (GFP) and resultant chimeric gene was introduced into rice calli. Several days after the transfer of the calli to the regeneration medium, the fluorescent signal was detected from some of them, and generation of green spots were later observed only from the fluorescent-positive calli except one. This correlation of the expression of the gene and the regeneration suggests that OSH1/HOS24 may play an important roll during the regeneration processes and this gene can be used as a suitable molecular marker to study the regeneration process.

We also carried out structural and expression analyses of *HAZ1*, a homeobox gene classified into a PHD-finger type. Nucleotide sequence analysis of a genomic clone and a cDNA clone of *HAZ1* indicates that the predicted protein contains characteristic motives such as nuclear localization signals, Zn finger, acidic regions and homeodomain from N-terminus to C-terminus. This domain organization was same as a maize homeodomain protein Zmhox1. Expression of *HAZ1* was detected in all organs examined including leaf blade, leaf sheath, root and flowers containing an embryo at some stages. In situ hybridization analysis indicated that *HAZ1* was expressed in an entire embryo at a globular stage, specially at outer layers with a higher level. This indicates that in a globular embryo outer-inner differentiation was occurred, though no phenotypic organ differentiation was observed.

We started to isolate and characterize a protein kinase gene as a candidate to transduce a positional information which is postulated to be important for plant development. We focused on a receptor-like protein kinase gene such as *SOMATIC EMBRYOGENESIS RECEPTOR KINASE* of carrot and isolated its homologue from rice. Sequencing and expression analyses are in progress.

(1)-b. Cloning and Structural/Functional Analysis of Four LEC1 Related Genes in Rice

Kazumaru MIYOSHI, Nori KURATA

Arabidopsis LEC1 gene cloned recently revealed to encode a transcriptional factor HAP3. Most striking feature of this gene is that the over expression of LEC1 protein causes ectopic embryogenesis on leaves of transgenic plants. This is the first case of ectopic induction of plant embryogenesis by gene manipulation. In monocot spices, a HAP3 gene and other LEC1 homologous gene(s) are also isolated in maize and others. However, their roles and functions during embryogenesis in monocotyledonous plants are not known yet. It must be very significant to analyse the signaling pathways before and after the LEC1 homolog gene expression when embryogenesis occurs. We have isolated several cDNA clones showing high similarity to the LEC1 gene from a 3DAP embryo cDNA library using maize HAP3 cDNA probe, which is cloned by RT-PCR from maize developing embryo. Sequencing analysis and database search revealed that several, at least three, different LEC1 homologs are expressed during embryogenesis in rice. The isolation of full length cDNAs is now in progress. The investigation of expression pattern and the functional analyses using loss- and/or gain-of-function mutant plants of these genes is next subject.

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

(2)-a. Isolation and Structural Analyses for The Centromeric Region of Rice Chromosomes Toward Construction of Rice Artificial Chromosome (RAC)

Kenichi NONOMURA, Nori KURATA

The cereal centromeric sequence (CCS1) conserved in some *Gramineae* species contains a 17-bp motif similar to the CENP-B box, which is the binding site for centromere specific protein CENP-B in human. To isolate centromeric units of rice (*Oryza sativa* L.), we performed the polymerase chain reaction (PCR) using the CENP-B box-like sequence (CBLS) as primers. A 264-bp clone amplified in this method, called RCS1516, appeared to be a novel member of the CCS1 family, sharing about 60% identity with the CCS1 se-

quences of other cereals. Then, a 14-kb long genomic clone lRCB11 carrying RCS1516 sequence was screened and sequenced. It was found to contain 3 copies of a 1.9-kb direct repeat RCE1, with 5.1- and 1.7-kb intervening sequences. The 300 bp of 3' end of RCE1 were highly conserved among three copies (>90%) and almost identical with RCS1516 sequence including a CBLS motif. The copy number of RCE1 was estimated ranging from 10^2 to 10^3 in the haploid genome of rice. Cloned RCE1 units were provided for fluorescent *in situ* hybridization (FISH) analysis, and signals were observed on almost every primary constriction of rice chromosomes. Thus it was concluded that RCE1 is one of the significant components of rice centromere. The RCB11 clone contained at least four A/T-rich regions, which are candidate matrix attachment regions (MARs), in the intervening sequences between the RCE1 repeats. Other elements homologous with the short centromeric repetitive sequences, pSau3A9 and pRG5 detected in both sorghum and rice, were also found in the clone. These results were published in Ref. 5.

(2)-b. Production of Mini-Chromosome Rice Lines for Studying Centromere Structure

Kenichi NONOMURA, Nori KURATA

In the study of plant centromere, there is no way other than transformation to know whether the DNA fragments derived from centromeric sequences bear the centromeric function or not. We plan to induce mini-chromosome plant aiming to obtain minimal functional unit of rice chromosome. Ditelocentric alien addition lines (DtAALs), which contain a pair of telocentric chromosome from wild species (*Oryza punctata*, BB genome) in addition to the normal complement of *O. sativa* chromosome set (AA genome), were irradiated by gamma-ray. DtAALs were kindly provided by Dr. H. Yasui, Kyushu Univ. The M_2 population with over 3000 gamma-ray irradiated DtAALs will be used for selection of mini chromosome lines carrying diminished alien chromosomes.

(3) Analysis of Genetic Factors causing Segregation Distortion

(3)-a. Quantitative Analysis of genotype segregation for reproductive barriers

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 spite of the importance of reproductive barrier in speciation, a comprehensive survey of reproductive barriers lying inter- or intra- species have not been made because of lack of markers covering whole genome. The interaction between the different loci of the parents would be expected to play important role in reproductive isolation. However, identification of interactive loci has be seemed to be difficult in generally and has not been attempted.

In rice, a high density linkage map was constructed using an F_2 population derived from a cross between a *japonica* cultivar, Nipponbare, and an *indica* cultivar, Kasalath (NK) (ref. 1). In the last year, we developed a multi-response non-liner regression analysis program to explain segregation distortions of a linkage map by a few gametophytic reproductive barriers. We succeed to explain the segregation distortions on some chromosomes.

In this year, we have extended the regression analysis program considering zygotic reproductive barriers and succeeded to explain segregation distortions on all chromosomes in NK by 34 reproductive barriers. Moreover, we have estimated interactive loci for segregation by applying classical χ^2 independence tests for all marker combinations in the rice high density linkage map.

In order to ascertain the characteristics of the reproductive barriers between *japonica* and *indica* rice cultivars, we analyzed reproductive barriers in two other crosses; between Fl1087 and Dao Ren Qiao (FD) and between Fl1007 and Kinandang puti (FK). The frequencies of each allele in a whole genome were well explained by 31 and 38 reproductive barriers in FD and FK, respectively. To examine the possibility of common barriers lying between *japonica* and *indica* rice cultivar, the positions of the reproductive barriers

detected in FD and FK were estimated and located on the NK map using the markers common with those of the NK map, thus at least 92 out of 103 barriers detected in this study were at different loci. The regions of interactive pairs for segregation distortion were completely different among the three crosses. (in preparation for publication).

(3)-b. Positional Cloning of a Segregation Distortion Gene Detected in a Progeny of a Cross between *japonica* and *indica* rice

Yoshiaki HARUSHIMA, Nori KURATA

The molecular mechanisms of reproductive isolation have not analyzed without a few exceptions in *Drosophila*, etc. The aim of this study is isolation of the most prominent barrier, gametophyte gene 2 (*ga-2*), on chromosome 3 in NK by positional cloning, and elucidation of the molecular nature of the individual reproductive barriers. We specified ga-2 position between adjacent markers in the NK high density linkage map in last year. We have been developing the near isogenic lines that have Kasalath genome at *ga-2* locus in Nipponbare background by back-crossing preferable 6 progenies (BC_2F_6) that was selected from 276 plants with CAPS markers.

(4) 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. This *Ds* was flanked by a P35S and a coding region of a selectable chlorsulfuron resistance gene, so that excision of the *Ds* causes connection of the P35S and the coding region and can confer chlorsulfuron resistance. We also use a P35S-Ac transposes (AcTPase) gene together with a bialaphos re-

sistance 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*-flanking regions cloned only for single copy *Ds* carrying plants and sequenced indicated that some *Ds* (before transposition) integrated in the coding region of the genes and others in the repetitive sequences. We will conduct mapping of the integrated *Ds* sites on rice chromosomes to select chromosome specific *Ds* lines. The examination for transposition frequency of *Ds* elements when crossed by the P35S-AcTPase lines will be carried out in 1999.

(5) Generation and Screening of Retrotransposon Insertion Lines in Rice

Mitsugu EIGUCHI, Yasurou NAGATO[1], Kazumaru MIYOSHI, Yukihiro ITO, Nori KURATA ([1]Graduate School of Agriculture and Life Sciences, University of Tokyo)

To obtain a lot of mutants affecting the steps of embryogenesis, we used transposon-induced mutagenesis system of rice using retrotransposon *Tos17*. *Tos17* is activated under tissue culture condition and transposes several copies in each regenerant. Calli of the *japonica* variety Nipponbare, *Oryza sativa*, were induced from germinating rice seeds, transferred into N6 liquid medium for 3 months' suspension culture and then were transferred onto regeneration plates. We produced about 900 regenerated plants, each having several transposed copies of *Tos17*, and obtain M_2 seeds from all of the regenerated plants (NIG-TD lines). More than another insertion lines were also propagated as a colaborative work among several laboratories (NF lines).

To isolate valuable genes taking roles for early embryogenesis, we screened each 20 M_2 seeds of about 3000 *Tos17* lines. We obtained about 100 candidate lines defective in early embryogenesis. M_3 investigation will be continued in 1999. In addition, DNAs were extracted from pooled *Tos17* insertion lines and will also be used for screening mutant lines with disrupted gene sequences of unknown gene function (loss of function mutants).

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F-d. Microbial Genetics Laboratory

(1) Timing of cell division in *Escherichia coli*

Akiko NISHIMURA

We found previously that the *cfc* mutation uncouples DNA replication and cell division, and elevates the frequency of cell division. We further analyzed the structure and the role of the *cfc* genes. The cfc mutants divide before they reach the size at which cfc$^+$ cells divide, and produce many small cells-each with a single nucleoid. The mutations affect the timing of cell division, but not other processes of cell cycle, such as the length of a cell cycle and the initiation mass for chromosome replication. CfcA has a mutation in *glySa* which encodes the α-subunit of glycyl-tRNA synthetase, and cfcB1 has an IS2 insertion in *apaH* which encodes Ap4A hydrolase. The cfc properties of both *cfc* mutants were suppressed by a multicopy plasmid carrying *apaH*$^+$, and the intracellular level of Ap4A in cfcA was 15-fold higher, and cfcB was 100-fold higher than their parent. Experiments using a wild-type cell showed that a high level of Ap4A caused early cell division, and a low level of Ap4A caused delayed cell division. We have purified the GlyS-6xHis tagged proteins from cfc$^+$ and cfc$^-$ strains and analysed the catalytic activity *in vitro* for Ap4A synthesis and kinetic constants of tRNA aminoacylation catalyzed by GlyS. Mutant type GlyS synthesized more Ap4A than wild type GlyS but showed lower degradation activity of Ap4A to ADP than wild type GlyS. Catalytic activity for glycylation (Km/Kcat) of GlyS from cfcA is 20~100 times higher than that from wild type. Therefore, I conclude that Ap4A is a signal for induction of cell division. High level of Ap4A is responsible for the initiation of cell division. The *glyS* mutation allows efficient synthesis of Ap4A. (For details, see Ref. 2)

(2) Hsc66 Participates in The FtsZ Ring Formation in *Escherichia coli*

Takeshi UEHARA, Hiroshi MATSUZAWA[1], and Akiko NISHIMURA ([1]Department of Biotechnology, The University of Tokyo)

We found from the analysis of a temperature-sensitive mutant of cell division, *fts715*, that Hsc66, the DnaK/Hsp70 homolog, affected the septal ring formation. 1. The *fts715* mutant stopped cell division at 42℃ but did not chromosome replication or segregation. 2. Complementation, P1-phage mediated transduction, and sequencing analysis of mutant showed that the mutation existed in the *hscA* gene which encodes Hsc66, and 192 alanine was substituted to valine. 3. Immunofluorescence microscopy revealed that in wild type cells, both Hsc66 and FtsZ ring was localized at the potential division site. However, in the *fts715* filaments, FtsZ ring was rarely detected at the potential division sites except those of both ends, though Hsc66 was detected at the most potential division sites. These results indicate that Hsc66 affects the formation of FtsZ ring. The purified FtsZ is known to polymerize *in vitro* in the presence of GTP at 37℃. 4. We found that FtsZ could not polymerize under the condition at 42℃ and that the addition of wild type Hsc66 specifically alleviated the polymerization defect of FtsZ at 42℃. 5. Moreover, the polymerized FtsZ and Hsc66 were co-sedimented. 6. To purify the mutant type Hsc66, the maltose-binding protein-Hsc66 fusion (MBP-Hsc66) of wild type or mutant type Hsc66 were isolated. The mutant type MBP-Hsc66 could not suppress the polymerization defect of FtsZ at 42℃ but the wild type MBP-Hsc66 could. We propose that Hsc66 might promote or stabilize the polymerization of FtsZ at 42℃.

(3) Post genome project : Systematic analysis of cell division genes in *Escherichia coli*

Makiko HORIE[1], Kimiko SAKA, Maki TADENUMA, Kunio MATSUMOTO[1], Hiroaki ITO, Hirosada MORI[2], Hideaki SUGAWARA, Kousuke GOTO, Akiko NISHIMURA ([1]Kanagawa Inst. Tech., [2]Nara. Inst. Sci. Tech.)

The entire nucleotide sequence of *E. coli* was analyzed, and 4311 ORFs have been demonstrated, but the functions of more than half of these genes are still unknown. It is considered that the greater part of these genes are involved in coordinating cell proliferation. To analyze the hierarchy and network responses in expression of cell division genes as a model case, we have selected mutants defective in cell division (*fts*) from a temperature-sensitive mutant bank (*kts*) consisting of 5,000 strains established by Hirota *et al.*, and analyzed the physiological properties of the mutants and roughly mapped these genes. Furthermore, to assign a corresponding ORF to each *fts* mutation by genomic analysis, vectors were constructed and cloning has been performed for 360 ORFs.

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F-e. Invertebrate Genetics Laboratory

(1) Specification and proximo-distal pattern formation of the wing and leg in *Drosophila*

Satoshi GOTO, Kazumasa KUBOTA[1] and Shigeo HAYASHI([1]Tokyo University of Medicine and Dentistry)

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

ecules Decapentaplegic and Wingless. We are studying how various inductive signals are combined to specify diverse sets of cell types arranged in unique spacial patterns. Differential activation of Decapentaplegic and EGF receptor signaling appear to play key roles in this process.

The leg imaginal disc resumes cell proliferation in the second instar, maintaining the circular patterns of the proximal and distal marker expression, and elaborates its pattern by activating additional genes required for the differentiation of a subset of leg structures. We are investigating how the interaction between the proximal and distal cells maintain their specific gene expression, activate additional genes, and establish polarity along the proximodistal axis.

(2) Cell biological study of the tubular epithelial network formation in the tracheal system

Tomoatsu IKEYA, Takahiro CHIHARA, Miho TANAKA-MATAKATSU and Shigeo HAYASHI

Coordination of cell motility and adhesion is essential for concerted movement of tissues during animal morphogenesis. The *Drosophila* tracheal network is formed by branching, migration and fusion of tubular ectodermal epithelia. The tracheal tip cell is located at the end of each branch that are going to fuse. We are studying mechanisms of cell fate determination, patterned cell migration, and unique cell shape changes of tracheal cells by various genetic and histological techniques.

(3) Kinase-independent activity of Cdc2/Cyclin A prevents S phase in the *Drosophila* cell cycle

Shigeo HAYASHI and Masamitsu YAMAGUCHI[1]([1]Laboratory of Cell Biology, Aichi Cancer Center Research Institute, Chikusa-ku, Nagoya 464, Japan)

Cdc2 dependent inhibition of S phase is required in G2 for correct ordering of S phase and M phase in yeast and *Drosophila*. This function of Cdc2 has been ascribed to its activity to phosphorylate replication factors, preventing the assembly of preinitiation complex at the origin of replication. Whether

this is the sole mechanism of S phase inhibition by Cdc2 in higher metazoan is not known because pleiotropic functions of this essential cell cycle regulator make genetic analysis difficult. We show that Cdc2 coexpressed with Cyclin A inhibits S phase in *Drosophila* salivary glands and in diploid abdominal histoblasts. A kinase defective mutant of Cdc2 failed to promote mitosis, but still inhibited S phase with the same efficiency as the wild type protein. In addition, Cdc2 and Cyclin A cooperatively inhibit transcriptional activation by the essential S phase regulator E2F. Cdc2 binds to E2F in vitro, and post-transcriptionally promotes its accumulation in vivo. Furthermore, the inhibitory effect of Cdc2 on S phase is overridden by E2F. The results suggest that inhibition of S phase by Cdc2 is achieved in part by a kinase independent mechanism, which is likely to be mediated by inhibition of E2F. For detail, see ref. 3.

(4) *plexus*, a gene required for adult wing vein patterning

Hitoshi MATAKATSU[1], Sumiko GAMOU[1], Ryu-suke TADOKORO[2] and Shigeo HAYASHI ([1]Osaka Prefecural University, [2]Kitasato University)

Veins of the adult wing are a good landmark for the anteroposterior and dorsoventral positional informations. The gene *plexus* is required for suppression of vein formation in specific intervein positions. We have cloned the *plexus* gene. The structure and biochemical properties of Plexus protein, and its genetic interaction with various vein-patterning genes suggest that *plexus* is a component of a novel regulatory pathway of vein differentiation.

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

Satoshi GOTO, Hiroko TAKEUCHI, Misako TANIGUCHI 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 4000 lines were established in collaboration with groups in Japan and were examined for the activity of enhancers flanking the inserts in the embryo, larva and adult. The results are being assembled into a database.

(6) Interaction between *Drosophila* EGF receptor and *vnd* determines three dorsoventral domains of the neuroectoderm

Yoshimasa YAGI,[1] Toshiharu SUZUKI,[1] and Shigeo HAYASHI ([1] Laboratory of Neurobiophysics, School of Pharmaceutical Science, The University of Tokyo)

Neurogenesis in *Drosophila melanogaster* starts by an ordered appearance of neuroblasts arranged in three columns (medial, intermediate, and lateral) in each side of the neuroectoderm. Here we show that in the intermediate column, the receptor tyrosine kinase DER represses expression of proneural genes *achaete* and *scute* , and is required for the formation of neuroblasts. Most of the early function of DER is likely to be mediated by the Ras-MAP kinase signaling pathway which is activated in the intermediate row, since a loss of a component of this pathway leads to a phenotype identical to that in DER mutants. MAP-kinase activation was also observed in the medial column where *esg* and proneural gene expression is unaffected by DER. We found that the homeobox gene *vnd* is required for the expression of *esg* and *scute* in the medial column, and we have shown that *vnd* acts through the negative regulatory region of the *esg* enhancer that mediates the DER signal, suggesting the role of *vnd* is to counteract DER dependent repression. Thus nested expression of *vnd* and the DER activator *rhomboid* is cruicial to subdivide the neuroectoderm into the three dorsoventral domains. For detail, see ref. 1.

Publications

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3. HAYASHI, S. and YAMAGUCHI, M.: Kinase-independent activity of Cdc2/Cyclin A prevents S phase in the Drosophila cell cycle. Genes to Cells, 4, 111-122, 1999.

G. CENTER FOR GENETIC RESOURCE INFORMATION

G-a. Genetic Informatics Laboratory

(1) Cross-species Database

Yukiko YAMAZAKI

The goal of this project is full cross-referencing of biological knowledge among different species. In 1996 an integrated database of wheat and rice genetic resource information was experimentally developed. The prototype of cross-species functional gene database was created in 1997. In order to integrate gene information from any different species, I've started developing the new system (SEEGEN) this year. The data was retrieved from i) the DNA sequence database, ii) the genetic resource databases and iii) the gene dictionaries of different species. Although the current program is written in PERL language, the SEEGEN system will be incorporated into object-oriented DB system.

(2) Genetic Resources Database

2-1. Wheat Genetic Resources Database -KOMUGI

Yukiko YAMAZAKI, Hisashi TSUJIMOTO[1], Taihachi KAWAHARA[2] and Yasunari OGIWARA[1] ([1]Kihara Institute for Biological Research, Yokohama City University, [2]Plant Germ-plasm Institute, Faculty of Agriculture, Kyoto University)

In addition to the update of the existing database, the KOMUGI 1.1 version offers a comprehensive interface for searching. Mutual mirroring between Graingenes provided by USDA, U.S.A. and KOMUGI has been in agreement. As a subgroup of KOMUGI, the Triticeae DNA repository database compiled over 900 clones and chromosome maps of common wheat have been added to the database. The next task of the working group will be setting up the gene dictionary tightly coupled to the KOMUGI database. The KOMUGI 1.1 version is available via WWW server at http://www.shigen.nig.ac.jp/wheat/wheat.html.

2-2. Rice Genetic Resources in Japan

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

The networking group of rice researchers in Japan has started to capture all available information about rice science, such as i) fundamental knowledge of rice science, ii) image data of mutant rice strain, iii) gene dictionary revised according to the new classification of gene function and iv) the integrated linkage map of phenotype and RFLP markers provided by Dr. Yoshimura. The existing rice genetic resource database will include the above information with cross-linking each other. We decided to use object-oriented database implementation to construct the next version of Rice DB because of complicated network of relationships between the contents.

2-3. Barley Germplasm Database

Kazuyoshi SATO[1], Mari SAITO and Yukiko YAMAZAKI ([1]Okayama University)

The first version of barley germplasm database has been released and is now available through the internet at http://www.shigen.nig.ac.jp/barley/Barley.html. The database lists 4250 germplasms and each strain consists of agro-morphological, physiological, and pathological information as well as genetic traits. Dr. Sato is working for setting up the original database on his own server machine at Okayama University and the data mirroring system, through which the data can be updated by connecting the remote computer, is under construction. Our site also plans to support a project to provide CORBA(Common Object Request Broker Architecture) access to other public databases.

2-4. Mouse Microsatellite Database

Toshihiko SHIROISHI, Rie TSUCHIYA and Yukiko YAMAZAKI

The mouse microsatellite database in Japan (MMDBJ ver.1.0) was publicly released with over 900 entries and is on-line available at http://www.shigen.nig.ac.jp/mouse/mouse.html. The database provides SSLP (simple sequence length polymorphism) information among different strains especially derived from Japanese wild mouse. Although the current database has an on-line direct data

submission system so that researchers can deposit their own data through the network, the revision in the structure of the database is necessary to either combine new data with different format or to provide new service requested by researchers. So we've started developing new database using object oriented database management system, ObjectStore.

2-5. Profiling of E.coli chromosome database

Junichi KATO[1], Tohru IKEGAMI[1], Takehiro YAMAKAWA, Kazu MITSUI, Yukiko YAMAZAKI ([1]University of Tokyo)

The PEC (Profiling of E.coli chromosome) database has been started construction in the collaboration with Dr. Kato's group. Among many different ways of genome analysis, the project focused on the following point of views, (1) the gene (or orf) is essential or not, (2) the codon analysis of genes, (3) the gene structural analysis such as motif, module and/or domain, and (4) the comparative analysis between different organisms. The goal of this project is to collect the relevant information as much as possible and provide reliable resources for further research. The PEC is object oriented database with java based applications. The information, which the current PEC database uses, is derived from E.coli strain MG1655 and the knowledge information was extracted from several different databases including DNA database and journal articles.

2-6. Arabidopsis genetic resource database

Shinji GOTO[1], Rie TSUCHIYA, Yukiko YAMAZAKI ([1]Miyagi Educational University)

Arabidopsis genetic resource database has been constructed in the collaboration with Dr. Goto this year. The first release contains about 1200 strains and the database is available at http:www.shigen.nig.ac.jp/arabidopsis. The collection includes AIS collection (Arabidopsis Information Service, Germany) and Sendai collection developed by Dr. Goto. The database is the first genetic resource DB of Arabidopsis in Japan and will incorporate other Brassica strains in the near future. Data submission system, through which the strain maintainer can update his/her own data by connecting to the remote computer

running the databases, has been developed.

Publications

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3. YAMAZAKI, Y.: Genetic Resource Database, Protein, Nucleic Acid and Enzyme. 43,2012-2015, 1998.

G-b. Genome Biology Laboratory

(1) Systematic analysis of mRNA distribution by whole mount *in situ* hybridization

Tomoko MOTOHASHI, Tokie OHBA, Ikuko SUGIURA, Masumi OBARA, Sayuri KITAYAMA, Takami SUZUKI, Tadasu SHIN-I and Yuji KOHARA

In this lab, *C.elegans* EST project has been performed, which has identified 10,955 cDNA species based on the comparison of tag sequences of some 65,000 clones. The distribution of the cDNA species among the six chromosomes is quite even; LG1: 1856, LG2:1855, LG3:1670, LG4:1645, LG5:1713, LGX:1424. To clarify their expression patterns, we have established an efficient protocol for *in situ* hybridization on whole mount embryos, larvae and adults (http://watson.genes.nig.ac.jp.8080/db/method), and have applied the procedure to the classified cDNA groups. Thus far, we have done the *in situ* analysis for 3500 cDNA groups mostly from an autosome LG3 and the sex chromosome LGX; LG1:6, LG2:522, LG3:1448, LG4:190, LG5:49 and LGX:1281. Analysis of the remaining 7000 cDNA groups is also in progress and will be finished within a year. Although the experimental part of the *in situ* hybridization is very efficient, analysis of the results including taking photos and giving annotations is a very time consuming process. Technical information on our *in situ* methods and statistics of the results of the expression patterns will be presented. All the data will become available at (http://watson.genes.nig.ac.jp:8080/db/).

(2) NEXTDB : The nematode expression pattern map database

Tadasu SHIN-I and Yuji KOHARA

We have developed a WWW-based database "NEXTDB" to integrate all the information of ESTs, gene expression patterns and gene functions of *C.elegans* which are being produced and analyzed in this laboratory.

Raw data of tag sequencing is transferred to the server machine from the ABI sequencers and then processed such that ambiguous regions which contain a larger rate of "N" (ambiguous base), cloning vectors, and poly-A tails are removed. The processed 3'-tags are classified to unique cDNA groups by applying a cumulative method by use of FASTA. Both 5'- and 3'-tag sequences are mapped to cosmid sequences by use of BLASTN. BLASTX searches are also done to confirm homologies of predicted CDSs to genes of other organisms. All the above processes are done automatically.

Images of whole mount *in situ* hybridization for detecting mRNA distribution were taken by CCD cameras equipped on Zeiss Axioplan microscopes (See Motohashi et al.), transferred to the server machine and then processed and arranged properly in the database. Annotations added to individual images were also stored in the database. Images of immunostaining taken on Zeiss LSM510 confocal microscope (See Onami et al.) and the data of RNAi phenotypes (See Hirono et al.) were also stored and arrange properly in the database. Finally, NEXTDB has the page for feedback information from the users of cDNA clones including full sequences, RNAi phenotypes, anomalies, gene functions, homologies and so on.

In order to integrate NEXTDB, we applied a hierarchical model to arrange all the clones and cDNA groups; 1) chromosome, 2) cosmid clone, 3) CDS, 4) cDNA group, and 5) cDNA clone. The cosmid map which connects 1) and 2) are obtained from AceDB. The relations about cosmids and their CDS are retrieved from the annotations of the Sanger Centre sequence data. All of the information is linked to each other in NEXTDB and displayed visually by use of JAVA applets. The latest version is available at the following URL. <http://watson.genes.nig.ac.jp:8080/db/index.html>

(3) Expressed Genes in *C. elegans*

Jean THIERRY-MIEG[1,2], Danielle THIERRY-MIEG[1,2], Tadasu SHIN-I[1] and Yuji KOHARA[1]([1]NIG, Mishima, Japan, [2]CNRS, Montpellier, France.)

A large normalized set of *C.elegans* cDNAs has been constructed and the two ends of each cDNA have been sequenced in Mishima. The resulting 118300 traces together with 6000 other cDNA sequences available from GenBank were aligned over 95.8 megabases genomic DNA available from the *C.elegans* consortium. The expressed genes were reconstructed, and the exact splicing established. As of March 99, there are hits to 9100 genes, among which 320 are in yet unsequenced genomic holes. That is about half of the 19000 genes predicted by the consortium, but not an exact subset, because the actual transcripts merge or split or differ in some finer way from the predictions in almost half the cases.

To fully exploit the data, we hand edit the basecall of the traces in view of the genomic sequence. About 4000 genes have been edited so far. Based on that set, a minimum of one third of the genes show alternative splicing and should generate more than one product. Alternative polyadenylation also frequently occurs. Transplicing to SL1 is apparent in only 35% of the probably complete genes. This may be an underestimate, but we do see SL1 in 12/14 of the complete genes directly upstream from the rare genes transpliced to SL2.

Because we have both the cDNA and the genomic sequence, we derive accurate data on the distribution of the genes along the genome, the exact structure of the introns, the transplicing and the 3' untranslated regions. Based on these independent sequence data, we confirm the high quality of the genomic sequence which presents less than one error per 40 kb and very few rearrangements. We expect that 3.2 Mb of the genome is still missing. The data on our servers can be accessed at http://alpha.crbm.cnrs-mop.fr or http://watson.genes.nig.ac.jp:8080/db/.

(4) Protein Expression Pattern Analysis of Maternal mRNAs

Shuichi ONAMI, Tamami NAGAOKA and Yuji KOHARA

Our cDNA/expression pattern project has identified many maternal mRNAs whose function is unknown. To study their function, we have started protein expression pattern analysis.

Our main interest is cell fate determination in early embryogenesis. Thus, we focus on genes whose mRNA is maternally supplied, and 1) disappears before 18cell-stage or 2) localizes to specific cells. We picked up 100 such genes out of 2000 genes based on our *in situ* hybridization results. We are raising rat antisera against all of these genes using bacterially expressed partial proteins (100-150 amino acids). Until now, we have obtained them for 54 genes and stained embryos and gonads. Fifty antisera (93%) showed detectable staining. The sub-cellular staining patterns are classified into perinuclear cytoplasm and plasma membrane (8), cytoplasmic granules (7), nucleus and plasma membrane (6), P-granules (6), cytoplasm (5), nucleus (5), nuclear membrane (5), plasma membrane (5), and egg shell (3). Thirty-one antisera (57%) showed tissue-specific staining before hatching and 9 of them showed cell-specific staining before 18cell-stage. Twelve antisera (22%) stained specific regions of the gonad. We also analyzed RNAi phenotype for those 100 genes (see Hirono et al.). We found 4 genes whose RNAi resulted in germ line defects out of 11 genes whose immunostaining pattern looked like P-cell specific. We believe that this approach provides fruitful information about maternal mRNAs. We are preparing to release these data on our WWW site (http://watson.genes.nig.ac.jp:8080/db/).

(5) Systematic RNAi experiments with maternal genes

Keiko HIRONO, Shuichi ONAMI and Yuji KOHARA

The accumulation of gene expression patterns that are being produced in this lab provides unique sets of limited numbers of genes that possibly participate in specific stages, lineages, tissues and so on. Such sets of genes could be subjected to systematic analysis to elucidate the molecular mechanisms governing the biological processes. Since we are particularly inter-

ested in early embryogenesis, we have focused on a set of genes whose mRNA are maternally supplied and disappear before 18cell-stage or localize to specific cells in early embryos. We selected 100 such genes out of 2000 genes based on our in situ hybridization analysis, and have analyzed them with respect to RNAi phenotype as well as protein distribution by immunostaining.

We micro-injected double-stranded RNA into the gonads of ten N2 worms per gene. Phenotypic analyses were performed on the injected worms themselves, F1 embryos, larvae and adults that survived (called "escapers") and F2 embryos, with respect to embryogenesis, larval growth, sterility, morphogenesis, the expression of several differentiation markers and so on. Out of the 100 genes, 45% showed F1 embryonic-lethality or the reduction of the number of F1, 34% showed various phenotype with only escapers, 18% showed sterility, and 2% did not show any phenotype.

The phenotypes of some genes were consistent with the results of protein distribution. For example, RNAi with the cDNA group CELK02786 caused no or a very fragile egg shell, and the antibody against the gene product stained the surface of entire embryo, probably underneath the egg shell, suggesting the gene is involved in the process of egg shell formation. RNAi of 4 out of 11 genes whose immunostaining patterns looked P-lineage specific caused germ line defect. We believe that this approach of the combination of the analysis of expression patterns and RNAi phenotypes provides highly useful information. We are preparing to release these data on our WWW site (http://watson.genes.nig.ac.jp:8080/db/).

(6) Toward a four-dimensional database of gene expression in *C. elegans*

Masahiro ITOH, Tomoko MOTOHASHI, Yohei MINAKUCHI and Yuji KOHARA

One of the most challenging targets in the post genomics era is computer simulation of development. We feel *C.elegans* is the most suitable system for this purpose, since the complete parts list of cells has been identified and the expression patterns and functions of all genes are being analyzed systematically. Aiming at the final goal and at integrating such information, we are constructing a computer graphics (CG)-based four-dimensional (3D +

time course) database of gene expression in *C.elegans*.

Thus far, we have made a CG for early embryogenesis up to 100 cell stage based on 4D Nomarski image data including the data kindly supplied by Kevin O'Connel at the University of Wisconsin at Madison. Since it was very hard for conventional image processing software to extract the contour of cells from the Nomarski images, we adopted a 'low-tech' procedure; firstly we traced the contour of individual cells and nuclei by hand-writing on thousands of the original images of the 4D microscopic data and then reconstructed 3D structures by piling up and making relationships among the contour data from different focal planes. The resulting 3D structures of various stage embryos were arranged along the time course of development and connected each other by an interpolating method to finally generate the CG of early embryogenesis. The graphics is faithful to the real embryogenesis, thus, it provides various information such as the volume of individual cells, the extent of cell-cell contact and so on.

To incorporate the information of gene expression patterns into the 4D graphics database, we have developed a procedure to superimpose a fluorescent-confocal data of the distribution of mRNA and proteins onto the CG. In the procedure, we use triple color staining, namely, DAPI and Cy-3 for nuclei and POS-1 (P-lineage specific) as internal markers for superimposing, respectively, and Cy-5 for a query gene product. By this fashion, we are incorporating the immunostaining patterns of maternal gene products. We are planning to make the 4D database accessible over the internet.

(7) *Pos-1* encodes a cytoplasmic zinc-finger protein essential for germline specification in *C. elegans*

Hiroaki TABARA, Russell J. HILL[1], Craig MELLO[2], James R. PRIESS[1] and Yuji KOHARA ([1]Fred Hutchinson Cancer Research Center, Howard Hughes Medical Institute, Seattle, WA 98109, [2]University of Massachusetts Cancer Center, Worcester, MA 01605)

In higher animals, sperm and oocytes differentiate from special cells called germ cells. Since the germ cells must reproduce the entire organism with each new generation, they are considered to be totipotent. Thus mechanisms

must exist that maintain totipotency in the embryonic cells destined to produce germ cells, although very little is understood about this process for any animal embryo.

In *C. elegans* embryogenesis, the germ cells arise from a stem cell-like lineage pattern. Blastomeres in the branch of the lineage that leads to germ cells are called germline blastomeres; the sisters of the germline blastomeres produce only somatic tissues and are called somatic blastomeres. The final germline blastomere is called P4 and it produces only germ cell descendants.

Several differences have been described between the germline blastomeres and somatic blastomeres of the *C. elegans* embryo. For example, many maternally-provided mRNAs persist longer in the germline blastomeres than in the somatic blastomeres. Cytoplasmic granules of unknown function called P granules are partitioned asymmetrically into the germline blastomeres during each of the early divisions, ultimately becoming localized into the P4 blastomere. The relationship between the persistence of maternal mRNAs, or P granule localization, and the totipotency of the germline blastomeres has not been determined.

Another difference between the germline and somatic blastomeres is in the initiation of embryonic gene transcription; after embryonic transcription initiates at the 4-cell stage of development, new mRNA transcripts are detected only in the somatic blastomeres. This result suggests that transcription is repressed or blocked in the germline blastomeres. The apparent lack of transcription provides an explanation for why germline blastomeres do not respond to transcription factors such as the SKN-1 and PAL-1 proteins. SKN-1 and PAL-1 are translated from maternally-provided mRNAs in both germline and somatic blastomeres. Although these transcription factors promote specific patterns of somatic differentiation in the somatic blastomeres, they appear to have no effect on the development of the germline blastomeres. Thus, transcriptional repression appears to be at least part of the mechanism that maintains the totipotency of the early germline blastomeres.

Mutations in the genes *pie-1* and *mex-1* cause the germline blastomeres to adopt somatic fates, suggesting that they function in maintaining the totipotency of the germline blastomeres. In *pie-1* mutants, embryonic transcription appears to initiate simultaneously in both somatic and germline blas-

tomeres, such that the germline blastomeres become competent to respond to SKN-1 and PAL-1. These results suggest that *pie-1*(+) functions in repressing transcription in wild-type germline blastomeres. *mex-1* appears to play a distinct role from *pie-1*, because at least some transcription is properly repressed in the germline blastomeres of *mex-1* mutants. PIE-1 is a novel, predominantly nuclear, protein of unknown function that is localized exclusively to a germline blastomere at each of the early embryonic cell divisions. MEX-1 is a novel, cytoplasmic protein that is localized predominantly to a germline blastomere at each early division. PIE-1 and MEX-1 are both components of P granules, and both proteins contain two copies of a CCCH-type "finger" domain originally described in a vertebrate protein of unknown function called TIS11/Nup475/TTP.

We have taken two complementary approaches to identify additional genes that establish germline/soma differences in *C. elegans*. As part of the *C. elegans* cDNA project, we have used molecular screens to look for mRNAs that are localized asymmetrically in the early embryo. In a second approach, we have used genetic screens to identify maternal-effect lethal mutations that prevent the development of germ cells. Here we describe the *pos-1* gene, which was identified independently in both screens. We show that the *pos-1* mRNA is distributed asymmetrically after the first cleavage of the embryo, and is present predominantly in germline blastomeres. Like MEX-1, POS-1 is a cytoplasmic protein localized predominantly to germline blastomeres. Like both MEX-1 and PIE-1, the POS-1 protein is a component of P granules and has two copies of the CCCH finger motif. We show that *pos-1* mutants have developmental defects that are distinct from both *mex-1* and *pie-1* mutants, suggesting that the POS-1, MEX-1 and PIE-1 proteins have at least some distinct roles in the specification of germline blastomeres.

(8) Studies on POS-1 interacting proteins

Ken-ich OGURA and Yuji KOHARA

The *pos-1* gene encodes a TIS11 type zinc finger protein similar to PIE-1 and MEX-1 (Tabara et al., Development, 126, 1-11, 1999). *Pos-1* mutants show maternal effect embryonic lethality with either reduced or excess pharynx, almost no intestine, no germ cell and extra hypodermis. POS-1 proteins localize to P-lineage, being a temporary component of P granules. Unlike PIE-1, POS-1 presents in cytoplasm but not in nuclei. POS-1 is suggested to function in translation of maternal mRNAs such as *apx-1*, however, the molecular process in which POS-1 functions is largely unknown.

Aiming at understanding the molecular process, we have identified the proteins that interact with POS-1 by using the yeast two hybrid system. These included POS-1 itself, MEX-3 (a KH type RNA binding protein), a novel RNA binding protein (named PIP-1 after POS-1 Interacting Protein), a serine/threonine protein phosphatase 2A catalytic subunit (PP2AC). We have confirmed that POS-1 directly bound to these proteins in vitro. The results indicated that POS-1 could form homodimer or homocomplex. MEX-3 appears to regulate the translation of maternal *pal-1* mRNA, POS-1 may function in the translation of maternal mRNA in cooperation with MEX-3.

RNAi experiments with *pip-1* yielded phenotypes similar to the *pos-1* mutants. The *pip-1* mRNA was detected abundantly in early embryos, and showed its localization to posterior blastomeres after 4 cell stage. Immunostaining with anti-PIP-1 antibodies showed that PIP-1 was present equally in the cytoplasm of both AB and P1 blastomeres at 2 cell stage, and began to localize to the posterior blastomeres at 4 cell stage; the staining was strong in P2 and EMS but weak in ABa and ABp. Interestingly, this staining pattern is in contrast with MEX-3. PIP-1 was not detected in late stage embryos. PIP-1 was suggested to be a temporary component of P granules. We confirmed that the staining disappeared in *pip-1*(RNAi) embryos. Furthermore, using the two hybrid system, we found that PIP-1 interacted with MEX-3 and also MEX-1.

Finally, we revealed that the PP2AC homolog is encoded by *let-92*, a gene required for early larval development (Clark et al., Genetics, 119, 345-353,

1988). Our analysis showed that LET-92 plays essential roles in the coordination of nuclear division and cytokinesis and P granule localization. It is interesting that POS-1 has putative phosphorylation sites of casein kinase 2 and PKC.

(9) *tbx-9* encodes a transcription activator

Yoshiki ANDACHI

tbx-9 is one of the T-box family genes that share a DNA binding motif, T-box. Genes of the family are found widely in metazoans, and many of them are involved in important events for development. Three T-box genes, Brachyury (T), VegT and *tbx-2*, have been shown to encode transcription factors. In *C. elegans*, the whole genome sequence predicts that this organism has about 20 T-box genes, and cDNA clones corresponding to the predicted genes are being isolated by the cDNA project. As I am interested in the regulation of gene expression, I have been studying one of the T-box genes, CELK02736 = *tbx-9*, which is the first one among T-box genes whose cDNA clones have been isolated. I previously showed that *tbx-9* is expressed in only a few cells of early-stage embryos and *tbx-9* deletion mutants produced by gene disruption show aberration of morphogenesis in the posterior body region in embryogenesis, including disturbance of the row of body wall muscles.

To test whether the *tbx-9* protein has a sequence-specific DNA binding activity, the *tbx-9* DNA binding sequence was determined as consensus among sequences of DNA fragments that were selected from a mixture of DNA fragments of random sequence by forming a complex with the *tbx-9* protein. The determined *tbx-9* binding sequence was 18 bases in length and had a palindromic structure. The T product binding sequence determined by a similar procedure has been reported to be palindromic, and the binding sequences of T and *tbx-9* are almost identical. To know whether the *tbx-9* protein is a transcription factor, transactivation of a reporter gene depending on the *tbx-9* binding sequence was examined. To this end, the following plasmids were constructed. For forced expression of *tbx-9*, the *tbx-9* gene was ligated downstream of the *hsp16-41* heat shock promoter. For assay of transactivation, the *tbx-9* binding sequence was inserted upstream of the *pes-10* minimal

promoter and the reporter *lacZ* gene. Transgenic lines containing both plasmids were produced by co-injection. Ectopic expression of *tbx-9* by subjecting transgenic embryos to heat shock caused activation of the *lacZ* gene, indicating that the *tbx-9* protein functions as a transcription activator. As the next goal, I am aiming at identifying target genes of *tbx-9*.

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H. STRUCTURAL BIOLOGY CENTER

H-a. Biological Macromolecules Laboratory

(1) Single Molecule Imaging of Biological Functions

Makio TOKUNAGA

In the post-genome era, novel methods are needed to elucidate functions of molecules found in genome. Recently, single molecule techniques have been developed out of *in vitro* researches of motor proteins. We are trying to spread out this new technique from *in vitro* to *in vivo* and from motor proteins to other biological molecules. 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*. It is essential to minimize the background light. 2) Imaging *in vivo* of interacting molecules and activated molecules.

(2) Single molecule capture, manipulation and force measurement of protein molecules

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We have developed a new instrument to capture and directly manipulate individual molecules using a scanning probe. A single myosin head molecule (myosin subfragment-1, S1) was captured onto the tip of a scanning micro-probe using a flexible glass microprobe, and manipulated with subnanometer resolution. Single molecule was confirmed by examining in the fluorescence microscopy at the same time using objective-type TIRFM. Movements and forces resulting from the interaction of a captured single S1 molecule with

actin filaments were measured. Our data are consistent with the unitary mechanical step during sliding along an actin filament of ~5.3 nm, but groups of 2 to 5 rapid steps in succession often produce displacements of ~11 to ~30 nm. The instances of multiple stepping are produced by single heads during one biochemical cycle of ATP hydrolysis. The results proves that the loose coupling mechanism is right. For details, see Ref. 2.

(3) Sliding movements of actin filaments undergoing fluctuations propelled by myosin subfragment-1 flexibly bound to the surface

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We have recently demonstrated that when skeletal muscle S1 is specifically attached to a glass surface in its tail end, it can move actin as fast as intact myosin. The specific binding was achieved by replacing a endogenous RLC by a recombinant fusion protein of biotin-dependent transcarboxylase (BDTC, a biotinylated peptide) and chicken gizzard RLC (cgmRLC). S1 was bound to an avidin-coated glass surface through BDTC-cgmRLC. In this work, a flexible peptide, 5G, which is expected to have a random coil structure and ~12 nm in expanded length, was inserted between BDTC and cgmRLC (BDTC-5G-cgmRLC). Thus, S1 was attached to a glass surface via the flexible chain. Insertion of 5G did not affect the actin-activated ATPase activity. Velocity of actin filaments caused by BDTC-5G-cgmRLC-S1 on a glass surface was similar to those by intact myosin and by BDTC-cgmRLC-S1, respectively. It was confirmed that the inserted chain was indeed flexible, using both electron microscopy and video image analysis. The present results don't support the widely accepted lever arm model.

(4) Molecular interactions measured by nanometer-sized probes using subpiconewton intermolecular force microscopy

Michio HIROSHIMA[1] and Makio TOKUNAGA ([1]Department of Biophysical Engineering, Osaka University)

Interaction between biological macromolecules involves several kinds of forces such as electrostatic forces, hydrophobic attractive forces, and so on. For a detailed understanding of the interactions between biological macromolecules, it is required to obtain information about the properties of these forces at the molecular level. We have refined 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. In this study, we have measured the forces exerted on a nanometer-sized probe using this novel microscopy. Forces between hydrophobic surfaces and between weakly charged surfaces were measured in aqueous solutions.

A long-range attractive force was observed between hydrophobic surfaces out to 150 nm with a decay length of 30 nm in water. Both the range and the decay length of the force were decreased with the increase of KCl concentrations or with the decrease of hydrophobicity of the surfaces. A linear relation was found between the decay length and the contact angle between the hydrophobic surfaces and drops of electrolyte solutions. This result suggests that the long-range attractive force results from the interaction between the hydrophobic surfaces and water.

(5) A New Model of Molecular Motors

Makio TOKUNAGA

Our single-molecule researches provided a direct evidence of the loose coupling mechanism on protein motors. I have shown that a new simple model named "asymmetric interaction model" can explain the results of the single-molecule experiments. Because this model is based only on a simple assumption that the interaction potential between motor proteins has a asymmetric shape, the theoretical formulation is very simple. Structural fluctuations of

protein molecules can play an important role as a fluctuation sensor, which realize the Maxwell's demon using energies supplied by ATP hydrolysis.

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2. KITAMURA, K., TOKUNAGA, M., IWANE, A. H., and YANAGIDA, T.: A single myosin head moves along an actin filament with regular steps of 5.3 nanometres. Nature, 397, 129-134, 1999.

H-b. Molecular Biomechanism Laboratory

(1) Branched pathway mechanism of transcription initiation by *E. coli* RNA polymerase, and promoter-arrested initiation complexes

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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 promoter binding is converted into a binary complex incapable of synthesizing full-length RNAs and synthesizing only abortive transcripts. These non-productive complexes designated "moribund" complexes (Nuc. Acids Res. 24, 1380-1381 (1996): J. Mol. Biol. 256, 449-457 (1996)).

We kinetically and biochemically isolated the nonproductive complexes at λP_R, lacUV5, T5N25antiDSR promoters, and promoters with the "best" -10 or -35 box selected by SELEX. But at T7A1, rrnBP1, and the one with both of the "best" -10 and the -35 boxes, moribund complexes, if any, decays synchronously to the productive complexes, and are not kinetically isolated. Thus

promoters can be classified into two classes, but this classification depends on reaction conditions; λP_R behaves like T7A1 in the presence of either a mutant σ^{70} or Gre factors, and T7A1 becomes similar to λP_R at low salt. Binary complexes with λP_R -type promoters are resistant to heparin while those with T7A1 type are not (SEN et al., J. Biol. Chem. (1998)).

Moribund complexes formed at λP_R readily convert into inactive dead-end complexes making no RNA. DNA and protein footprinting analyses showed that this dead-end complex is backtracked from the promoter, lacks the strand opening and has more exposed conserved region 3 of σ^{70}. This inactivation was mitigated in the simultaneous presence of a high concentration of initiating nucleotide and Gre factors prior to RNA synthesis. At T5N25antiDSR promoter moribund complex is predominant up to 2 hr, and retains the strand opening, suggesting moribund complex is a member of open complexes.

A minimalist model consistent with all these observations is that two subspecies of open binary complex exist either in productive or moribund states, forming a two-state switch. At promoters like λP_R these different forms cannot directly convert into each other. Gre factors introduce reversibility to moribund binary complex formation allowing rapid switching into productive binary complex which is consumed up by long RNA synthesis (SEN et al., J. Biol. Chem. (1998)).

(2) Role of the spacer region of σ^{70} and Inactivation by oligomerization

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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. The roles of the characteristic spacer region in the strong adhesive nature of σ^{70} were examined.

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 (Δ SR) complemented the disruption at 30 and 25°C, proving that the region is not essential for growth allow temperature. At all tested temperature this protein predominantly exists as oligomers which are in equilibrium with a small fraction of monomer. Both forms bind to core enzyme but the holoenzyme formed with oligomers had 15% or less activity of the wild-type. Therefore, the role of the spacer region is the maintenance of active monomeric form.

The protein footprinting analysis of the ΔSR with a kinase-tag fused at the C-terminus, which also complements the disruption, indicated that at least region 4 and C-terminus are involved in oligomerization. According to an atomic force microscopic observation, the oligomer forms fibers as previously reported for wild-type σ^{70} at 60°C and RpoD800 at 46°C.

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

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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 nonspecific 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 nonspecific 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*

Masaya TAKE-UCHI, Akane OISHI, Takeshi ISHIHARA and Isao KATSURA

Fluoride-resistant (*flr*) mutations of *C. elegans* are recessive and grouped into two categories: class 1 mutations (strongly resistant mutations in *flr-1*, *flr-3* and *flr-4*) and class 2 mutations (weakly resistant mutations in *flr-2* and *flr-5*) (Katsura I. *et al*. (1994) Genetics 136, 145-154). From the results presented below, we think class 1 *flr* genes constitute an intestinal regulatory system that responds to fluoride ion and that controls various functions, including neural functions.

Besides resistance to fluoride ion, class 1 *flr* mutations show many phenotypes: slow growth, short defecation cycle periods, frequent skips of the expulsion step of defecation, and synthetic abnormality in dauer formation (See (2) below). *flr-1* encodes an ion channel belonging to the DEG/ENaC (*C. elegans* degenerins and mammalian amiloride-sensitive epithelial sodium ion channels) superfamily, while *flr-4* codes for a novel Ser/Thr protein kinase having a hydrophobic domain on the carboxyl-terminal side. A genomic DNA fragment that rescues *flr-3* mutations produces a polycistronic mRNA encoding a kinase-like protein and a novel protein. A *flr-1*::GFP fusion gene is expressed only in the intestinal cells from the comma stage of embryos to the adult stage. A *flr-4*::GFP fusion gene 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. In these fusion genes, GFP cDNA is connected in frame to the 3' end of the coding region of the *flr* genes. Since they can rescue the corresponding mutant phenotypes, the expression at the site and time mentioned above should be sufficient for the animal to show wild-type phenotypes.

Class 2 *flr* mutations interact with class 1 mutations. Of class 1 phenotypes, class 2 mutations suppress slow growth and synthetic abnormality in dauer formation, but do not suppress strong resistance to fluoride ion or abnormalities in defecation cycles.

In our studies last year, the following results were obtained.

(i) We introduced a nonsense mutation into each ORF in the genomic DNA fragment that rescues the *flr-3* mutant phenotypes. By checking the rescue activity of the mutant DNA fragments, we concluded that *flr-3* encodes a kinase-like protein.

(ii) We killed AUA neurons in wild-type and *flr-4* animals. These animals had the same defecation cycle periods and growth rate as those which suffered no cell-killing. Hence, we concluded that AUA neurons are not required for these phenotypes.

(iii) Fluoride ion increased the defecation cycle periods of wild-type animals but did not change those of class 1 *flr* mutants. The results suggest that fluoride ion probably enhances the activity of class 1 *flr* gene products in wild-type animals and thereby increases their defecation cycle periods, eventually causing their death.

(iv) Mutations in *flr-4* gene consisted of a mutation at a splice-acceptor site, two missense mutations in the kinase domain, and a missense mutation in the C-terminal hydrophobic domain. All of them had the phenotypes of strong fluoride-resistance and defecation cycle abnormalities. The two mutations in the kinase domain, which were temperature-sensitive, had almost normal growth rates and showed low penetrance in synthetic dauer abnormality. The mutation in the hydrophobic domain was special in that its abnormality in the expulsion step of defecation was suppressed by fluoride ion.

(v) The phenotypes of *flr-3* mutants were suppressed by multiple copies of the wild-type *flr-4* gene. The results can be explained, if FLR-3 is an activator for FLR-4. FLR-3 has a weak but overall homology to FLR-4. This may be relevant, if FLR-3 interacts with FLR-4 directly.

(vi) We cloned *flr-2* gene by the rescue method and found that it encodes a homolog of a TGF-β antagonist belonging to the gremlin/DAN/cerberus family. Two mutations in *flr-2* were missense mutations at conserved amino acids. Since there are four TGF-β homologs in the *C. elegans* genome, we are determining which is the one that interacts with FLR-2.

(2) Analysis of Synthetic Dauer-constitutive Mutations

Kouji MIYAHARA, 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 mutations, we investigated those in *sdf-1* (1 allele) and *sdf-13* (2 alleles) in detail. It was known that the *sdf-1* mutant avoids benzaldehyde, which is an attractant of wild-type animals. This year we found that it also avoids isoamylalcohol, butanone, NaCl, and lysine, all of which are attractants of wild-type animals. It behaved as thermophilic in thermotaxis. However, it showed normal behavior to diacetyl, pyrazine, thiazole, and high osmotic pressure, and avoided octanol more strongly than wild-type animals. In con-

trast, the *sdf-13* mutants showed normal chemotaxis to benzaldehyde, isoamyl alcohol and diacetyl. However, they showed almost no adaptation to the former two attractants despite showing normal adaptation to the latter. Thus, we could obtain various behavioral mutants by isolating Sdf-c mutants. We cloned *sdf-13* gene by the rescue method and found that it encodes a homolog of mammalian Tbx2 and *Drosophila* Omb, a transcription factor containing the T-box domain.

(3) Analysis of *che-2*, a gene required for the sensory cilia in *C. elegans*

Manabi FUJIWARA, Takeshi ISHIHARA and Isao KATSURA

Mutants in the *C. elegans che-2* gene have extremely short cilia with an abnormal posterior projection in many sensory neurons, and show defects in behaviors that are mediated by ciliated sensory neurons, such as chemotaxis and osmotic avoidance. We cloned *che-2* gene and found that it encodes a new member of the WD40 protein family. Analysis of mutation sites showed that both the N-terminal WD40 repeats and the C-terminal non-WD40 domain are necessary for the CHE-2 function. CHE-2-tagged green fluorescent protein, which can rescue the mutant phenotypes, is localized at the cilia of almost all the ciliated sensory neurons (except AFD and BAG). Expression of *che-2* in a subset of sensory neurons in a *che-2* mutant by using a heterologous promoter resulted in restoration of the functions and cilium morphology of only the *che-2*-expressing neurons. Thus, *che-2* acts cell-autonomously. This technique can be used in the future for determining the function of each type of *che-2*-expressing sensory neurons. Using a green fluorescent protein as a marker, we found that the extension of cilia in wild type animals took place at the late embryonic stage, whereas the cilia of *che-2* mutant animals remained always short during development. Hence, the abnormal posterior projection is made due to the inability of cilium extension, rather than the degeneration of cilia once formed correctly. Expression of *che-2* in a *che-2* mutant under a heat shock promoter showed that the extension of cilia, surprisingly, can occur even at the adult stage and that such cilia can function in behavior. However, some cilia made at the adult stage had an abnormal curved shape, which

suggests that these cilia may not pass through the socket cell, unlike those made in normal development.

(4) Functional Analysis of Interneurons as Studied by Selection between Two Behaviors

Takeshi ISHIHARA and Isao KATSURA

The nematode *C. elegans* shows avoidance from 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 elucidate 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 present knowledge on the neural circuitry of *C. elegans* and the identity of the sensory neurons that act in these behaviors.

This assay method was used for the analysis of mutants in an AMPA-type glutamate receptor gene (*glr-1*), which is expressed only in interneurons. They showed a preference for odorants, as compared with wild-type animals. However, if each behavior was assayed separately, the dose response curves of *glr-1* mutants were the same as those of wild-type animals. The results suggest that GLR-1 plays a role in the interaction between the two responses.

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 than well-fed animals. 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 reasonable, because starved worms can look for food over a wider area.

To elucidate the mechanism of selection between two behaviors and that of the effect of starvation, we are isolating and analyzing mutants that show abnormality in this selection. The mutant *ut236* had a tendency to choose avoidance from copper ion rather than chemotaxis to odorants, although it showed no abnormality in each behavior. On the other hand, the mutant *ut235* lacked the effect of starvation in the above assay, but was essentially

normal in the change of locomotion speed by starvation and in the response 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 from copper ion, regardless of starvation. To identify the gene responsible for the *ut236* mutation, we are now attempting the rescue of the phenotype by microinjection of cosmid clones from the 1 Mb region where the mutation was mapped.

We also isolated new mutants from EMS (ethylmethane sulfonate)-treated worms and worms containing movable transposons (*mut-7* worms). Some of them had a preference for chemotaxis to odorants over avoidance to copper ion, while others had little starvation effect. We are cloning the genes of the *mut-7* mutants by the transposon-tagging method.

(5) Reverse Genetics of Genes Containing PDZ domains

Ryuichi HISHIDA, Takeshi ISHIHARA and Isao KATSURA

PDZ domains are thought to act in protein-protein interaction, often forming clusters of membrane-bound receptors, ion channels, etc., and localizing these molecules to a specific site on the membrane. We searched genes that encode proteins containing PDZ domains, in the genomic sequence of *C. elegans* and investigated the site and time of their expression by using GFP fusion genes. Two of the genes, which we are interested in, were subjected to gene disruption. One of them encoded a protein having a MDM1(one of intermediate filaments)-like sequence on the C-terminal side of PDZ and was expressed in many neurons and the excretory cells as well as at the edge of the vulva, from larvae to adults. The protein exists as granules in cytoplasm, often flanking nuclei on the anteroposterior axis. We isolated a mutant in which the MDM1-like sequence was deleted. However, it showed no clear phenotype. The other gene encoded a MAGI-1 homolog, which contained a guanylate kinase-like sequence, two WW domains and five PDZ domains. Its GFP fusion gene was expressed in the intestine from embryos to larvae, in lateral hypodermis from embryos to adults, in some head neurons, the anus, ventral hypodermis, the uterus, the spermatheca and the distal tip cells of the gonad from larvae to adults. Although a mutant lacking the guanylate kinase-like sequence had no conspicuous phe-

notype, overexpression of the gene by multicopy transformation resulted in low-penetrance morphological abnormality in the lateral seam cells.

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H-d. Biomolecular Structure Laboratory

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

Yasuo SHIRAKIHARA

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 transmembrane 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 are extending the structural study to a nucleotide-bound form of the $\alpha_3\beta_3$ sub-assembly and to an $\alpha_3\beta_3\gamma$ sub-assembly.

We have examined structure of the nucleotide-soaked crystal of the $\alpha_3\beta_3$ sub-assembly, hopefully assuming the structure in it to represent an nucleotide-bound form. Diffraction data from the soaked crystals were collected before they cracked eventually, as the $\alpha_3\beta_3$ sub-assembly is destabilized by presence of nucleotides. 12 different soaking conditions were tried. In the ATP and MgADP soaked crystals, the $\alpha_3\beta_3$ sub-assembly were placed with orientations different from that of the nucleotide-free sub-assembly. However the structures themselves in these soaks were very similar to the structure of the nucleotide-free form. Close inspection of electron density map revealed

that the density for the sulfate bound to the P-loop (phosphate binding loop) of the β-subunit got significantly weaker in comparison with the equivalent density in the nucleotide-free crystal. In contrast, the density for the sulfate bound to the α-subunit's P-loop looked very similar between nucleotide-soaked and nucleotide-free forms. These features were commonly observed for all 12 maps except for Pi soaked one. It is likely that some transient state between nucleotide-free and nucleotide-bound form has been captured in this experiment.

As the $\alpha_3\beta_3\gamma$ sub-assembly exhibits kinetic properties very similar to those of F1, and a number of interesting mutant sub-assemblies have been engineered, the sub-assembly is thought to be a good target for structural study. We have searched crystallization conditions for the wild type sub-assembly, but have experienced extreme difficulty. Both preparation method and crystallization conditions have been examined extensively. Among various trials to improve preparations, heat treatment and ammonium sulfate fractionation were very effective, but for example, additional columns (dye-ligand columns and gel filtration column) and control in bound nucleotides were only marginally effective. The following points have been noticed during crystallization experiments. As a binding ligand, ATP γ S was very useful, and so were CDTA,DTT,methanol as general additives. Crystals were easily formed at 15 degree, but they were unstable when transferred to 25 degree environment. It was possible to grow crystals at 25 degree, but only in different crystallization conditions. Crystals at the current growing conditions seem now ready for a diffraction study, judged from their sizes.

These structure studies were done in collaboration with Masasuke Yoshida, Toyoki Amano, Eiro Muneyuki, Yasuyuki Kato and Satoshi Tsunoda at Research Laboratory of Resource Utilization, Tokyo Institute of technology.

(2) Crystallographic Study of the Transcription Activator, PhoB

Toshihiko AKIBA and Yasuo SHIRAKIHARA

PhoB Protein is a positive transcriptional activator for the genes in the phosphate (*pho*) regulon of *E. coli*, such as *phoA* and *pstS*, that are induced by phosphate starvation. PhoB binds to the *pho* box in the promoter region,

which is the consensus sequence shared by the regulatory regions of *phoA*, *phoB*, *phoE* and *PstS*. PhoB is activated by phosphorylation by PhoR. The N terminal domain of PhoB is responsible for this regulatory role, whereas the C terminal domain has a DNA binding ability.

From particular interest to interactive regulatory mechanisms between the N- and the C- domains, crystallization of an intact form of the protein has been attempted. After establishing overproducing conditions and purification procedure as described previously, crystallization conditions for unphosphorylated form were screened employing Hampton crystal screen, but so far without success. In an attempt to crystallize a phosphorylated from, conditions to obtain the phosphorylated form were examined. A problem was that in the presence of acetylphosphate, which is an phosphate donor, part of PhoB population forms a dimer. Efforts has been made to overcome this problem.

These works have been done in collaboration with Kozo Makino, Research Institute for Microbial Diseases, Osaka University.

(3) Crystallographic Study of the Transcription Repressor, CamR

Koji FUKUSHI and Yasuo SHIRAKIHARA

CamR protein is a repressor that regulates transcription of the cytochrome P-450cam hydroxylase operon of *Pseudomonas putida*. Expression of the *camDCAB* operon and the *camR* gene is regulated through interaction of the CamR protein with the single operator located in the overlapping promoter region between the *camDCAB* operon and the *camR* gene. D-camphor is an inducer, and binds cooperatively to CamR. CamR is a homodimer with a molecular mass of 40 kDa.

Two crystal forms have been obtained, one from polyethyleneglycol solution in the presence or absence of D-camphor and the other from Na-K-phosphate solution in the presence of D-camphor. By switching of the *E.coli* over-expression system from a P_L promoter system to a more efficient T7 (pET system) promoter system, and by minor modifications in purification procedure, we eliminated a problem of poor reproducibility in crystallization. Although crystal forms had similar unit cell parameters and diffracted to better than 2.5 Å

resolution, they were very sensitive to temperature change, thus prohibiting full data collection. Further efforts have been made to get rid of the problem.

This work has been done in collaboration with Hironori Aramaki, Daiichi College of Pharmaceutical Sciences.

(4) Crystallization of RNA Polymerase and its subunits

Katsuhiko MURAKAMI, Makoto KIMURA, Akira ISHIHAMA and Yasuo SHIRAKIHARA

The *Escherichia coli* RNA polymerase (RNAP) is a large assembly (a molecular weight for the core enzyme ($\alpha 2\beta\beta'$) is 380 kD and that for the holo enzyme ($\alpha 2\beta\beta'\sigma^{70}$)is 450kD). α subunit is included as a dimer into RNAP. The amino (N)-terminal domain from residue 20 to 235 is responsible for the enzyme assembly through the interactions with β and β' subunits and the carboxyl (C)-terminal domain of 94 residues in length is important for transcription regulation. The solution structure of the C-terminal domain is already resolved by NMR study.

For the purpose of analysis the RNAP assembly mechanism, we have tried to crystallize α subunit. As reported previously, PEG400/CaCl2 and PEG8000/ NaAcetate gave thin-plate crystals. However, the crystals were too thin to give diffraction and further efforts have been made this year to get thicker crystals, but so far without success.

For crystallization of RNAP, an overproducing system of the core enzyme has been constructed(BL21(DE3)/pGEMABC), allowing 5-fold yield increase and therefore concomitant impurity decrease. Crystallization conditions for the core and the holo enzymes were screened employing Hampton Crystal Screen Kit, but with no sign of crystalline materials formed. A subsequent Dynamic light scattering study showed that both forms of the enzyme exhibited a complicated pattern of aggregation depending on pH, Mg concentration, NaCl concentration, and species of counter ion in the Tris buffer. This led us to explore crystallization conditions around least aggregating conditions. The experiment is under way.

Pol II from yeast is a larger and more complicated assembly, comprising 12 subunits. We tried to crystallize Rpb11, which is a homologue of the E.coli α

subunit, and Rpb6. These were selected on the basis of availability of overproduction system. From dynamic light scattering and gel filtration experiments, Rpb11 was found to be polydisperse with respect to aggregation state and thus not suitable for crystallization experiment. Rpb6, with no such apparent difficulty for crystallization, gave no crystals so far.

(5) Crystallization of Mre11 fragment from yeast

Katsuhiko MURAKAMI, Tsutomu OHTA, Tomoko OGAWA and Yasuo SHIRAKIHARA

Mre11 is involved in the recombination process in yeast. We tried to crystallize a 70 amino acid fragment of Mre11, which is capable of binding DNA, however, was overproduced as a GST fusion protein in *E.coli*. It was anticipated that the fusion protein may be easy to crystallize due to crystallizing capability of the GST part, in view of the relative size of the fragment to the remaining GST part. However actual crystallization experiments failed, and we think that it is better to deal with the fragment itself.

(6) 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. Preparation methods are being improved to get better crystals.

This work has been done in collaboration with Kakunori Tanaka and Ichiro Yamato, Science University of Tokyo.

Publication

None

I. CENTER FOR INFORMATION BIOLOGY

I-a. Laboratory for DNA Data Analysis

(1) Different evolutionary histories in two subgenomic regions of the Major Histocompatibility Complex

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

The alpha and beta blocks, contain members of the multicopy gene families HLA Class I, human endogenous retroviral sequence-16 (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, indels 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 -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 organisation in the two blocks. We then found that the beta block contains four distinct duplications from two separate events, whereas the alpha block is characterised by multi-segment duplications. We will discuss these results in relation to the genetic content of the two blocks. For details, see ref. 1.

(2) Coevolution of PERB11 (MIC) and HLA Class I Genes with HERV-16 and Retroelements by Extended Genomic Duplication

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

The recent availability of genomic sequence information for the class I region of the MHC has provided an opportunity to examine the genomic organisation of HLA class I (HLAcI) and PERB11/MIC genes with a view to explaining their evolution from the perspective of extended genomic duplications rather than by simple gene duplications and/or gene conversion events. Analysis of genomic sequence from two different regions of the MHC (the alpha- and beta-block) revealed that at least six PERB11 and 14 HLAcI genes, pseudogenes and gene fragments are contained within extended duplicated segments. Each segment was searched for the presence of shared (paralogous) retroelements by Repeat Masker in order to use them as markers of evolution, genetic rearrangements and evidence of segmental duplications. Shared Alu elements and other retroelements allowed the duplicated segments to be classified into 5 distinct groups (A to E) that could be further distilled down to an ancient preduplication segment containing a HLA and PERB11 gene, an endogenous retrovirus (HERV-16) and distinctive retroelements. The breakpoints within and between the different HLAcI segments were found mainly within the PERB11 and HLA genes, HERV-16 and other retroelements suggesting that the latter have played a major role in duplication and indel events leading to the present organisation of PERB11 and HLAcI genes. On the basis of the features contained within the segments, a coevolutionary model premised on tandem duplication of single and multipartite genomic segments is proposed. The model is used to explain the origins and genomic organisation of retroelements, HERV-16, DNA transposons, PERB11 and HLAcI genes as distinct segmental combinations within the alpha- and beta-blocks of the human MHC. For details, see refs. 2 and 3.

(3) Extensive nucleotide variability within a 370 kb sequence from the central region of the Major Histocompatibility Complex

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

The recent availability of the genomic sequence spanning the central and telomeric end of the major histocompatibility complex (MHC) has allowed a detailed study of its organisation, gene content and level of nucleotide variability. Previous analyses of nucleotide variability in the MHC has focused on the coding regions of the human leukocyte antigen (HLA) Class I and II genes. Non-coding nucleotide variability has been considered a by-product of exonic diversity. However, with the advent of genomic sequencing, the extent of non-coding nucleotide variability within the MHC has just begun to be appreciated. In this study, we compared different human haplotypes in 370 kb of sequence in the central region of the MHC to show the following: 1) unusually high levels of non-coding nucleotide variability, up to 80 times greater than elsewhere in the genome; 2) non-coding nucleotide variability greater than 1% at nucleotide sites distant to the Class I genes; 3) nucleotide variability greater than 1% maintained over regions containing highly linked loci; and 4) distinct troughs and peaks in the level of nucleotide variability. We will discuss these observations in relation to a possible role of nucleotide variability in the organisation of the MHC. For details, see refs. 4 and 5.

(4) Elevated levels of non-coding polymorphism within the Major Histocompatibility Complex

Silvana GAUDIERI, Roger DAWKINS[1], Kaori HABARA and Takashi GOJOBORI ([1]Centre for Molecular Immunology and Instrumentation, University of Western Australia)

The Human Major Histocompatibility Complex (MHC) is characterised by polymorphic multicopy gene families including the Class I and II Human Leucocyte Antigens (HLA), rearrangements, insertions and deletions (indels), and uneven rates of recombination. Within the HLA Class I and II genes, positive

selection has been shown to operate on the peptide binding region, with polymorphism at linked loci attributed to balancing selection. Recent evidence suggests that non-coding polymorphism is unusually high within the MHC, up to fifty times greater than elsewhere in the genome. To examine the relationship between HLA and non-coding polymorphism, we analysed continuous sequence from different human haplotypes up to 200kb distant from the HLA genes. We report here the most extensive analysis of polymorphism within the genome. We show that the MHC exhibits large regions of high polymorphism (>1%) that contain linked loci. Polymorphism peaks do not necessarily coincide with the HLA genes and in some cases occur within human endogenous retroviral sequences (HERVs). Polymorphism >1% appears to be negatively correlated to recombination rate within the MHC. These results support the hypothesis that the MHC consists of polymorphic frozen blocks, where recombination rarely, if at all, occurs within the block due to the inhibition of recombination by high levels of polymorphism. For details, see ref. 6.

(5) Characterisation of the PERB11 gene family in Primates

S. K. CATTLEY[1] , N. LONGMAN[1], Silnava GAUDIERI, Roger L. DAWKINS[1] and C. LEELAYUWAT[1] ([1]Centre for Molecular Immunology and Instrumentation, University of Western Australia)

PERB11 (MIC) is a member of the Class I-like superfamily in the MHC region. The PERB11 gene family has 5 members in the human genome, two are transcribed and polymorphic (PERB11.1 and PERB11.2). These genes have been implicated in autoimmune diseases (Behcet's disease, Psoriasis. Chimpanzees appear to have fewer copies of PERB11 based on southern hybridisation analysis. Given the low prevalence of autoimmune diseases in chimpanzees, an examination of the number and position of PERB11 members in primates (esp chimpanzee) will be used to evaluate PERB11 as a disease candidate gene in this region. PERB11.1 and PERB11.2 specific primers (designed from human copies) were used to amplify 7 chimpanzees, 1 orangutan and 6 macaques, to examine the level of inter- and intraspecies differences. The resultant PCR products from four different PCR reactions have been cloned. For details, see ref 7.

(6) Identification of Neural pathways in Planarian

Akira TAZAKI[1], Silvana GAUDIERI, Kazuho IKEO, Takashi GOJOBORI, Kenji. WATANABE[1] and Kiyokazu AGATA[1] ([1]Laboratory of Regeneration Biology, Department of Life Science, Faculty of Science, Himeji Institute of Technology)

The Planarian contains a primitive central nervous system and has been used as a model to examine the developmental stages within the brain. We are collaborating with Dr. Agata from the Department of Life Science, Himeji Institute of Technology, Hyogo. His group is developing the technology of isolating cDNA libraries from single cells. The experimental plan for the brain involves thousands of individual cell cDNA libraries. These libraries will be compared for expression levels in different developmental stages. This plan requires a great deal of computer automation from sequence output to BLAST searches and database construction. To facilitate the construction of the automated analysis, we have started a preliminary project using the eye cell of the planarian. The initial project will involve 1000 cDNA sequences. We have already sequenced the 1000 cDNA inserts and are currently forming databases of the completed sequences. Interesting sequences will be further analysed for important motifs, domains. The preliminary project will provide new data on sensory developmental genes, organ specific transcripts and other interesting new sequences. For details, see ref. 8.

(7) A low rate of nucleotide changes in *Escherichia coli* K-12 estimated from a comparison of the genome sequences between two different substrains

Takeshi ITOH and Takashi GOJOBORI

The molecular mechanisms of mutation in bacteria have been extensively studied. However, there is a paucity of information on the evolutionary rates of bacteria when compared with that of other organisms such as mammals and viruses. Recently, two genomes of *Escherichia coli* K-12 have been independently sequenced by Japanese and American teams. These two closely related genomes gave us a unique opportunity to investigate the evolutionary rates of *E. coli* by directly comparing the genome sequences.

In this study, we attempted to estimate the rate of nucleotide changes by a direct comparison of the DNA sequences of two mutant derivatives of *E. coli* K-12, W3110 and MG1655. According to laboratory history records, these substrains originated from W1485 approximately 40 years ago. Of course, W1485 is also the derivative of the K-12 wild type. Only a few differences were found between the two genomes in our comparative analysis. When using 40 years as the divergence time between W3110 and MG1655, we estimated the rate of nucleotide changes to be at most about 10^{-7} per site per year. The rate of nucleotide changes estimated herein is relatively low compared to those of viruses. We also compared partial genome sequences between *E. coli* and *Shigella flexneri*. The divergence time between these two enteric bacteria has been supposed to be 25 million years ago. We then estimated the rate of nucleotide changes to be about 10^{-9} per site per year, which is consistent with the rate of nucleotide changes that we have estimated between the two *E. coli* substrains.

Of all the indels (insertions or deletions), except insertion sequences (ISs) and one prophage, 98.3% were indels of eight- or fewer nucleotides and 93.0% were indels of a single nucleotide. We found 10 long indels of about one thousand or more nucleotides in length, which are mainly caused by ISs. Eight ISs were found only in the W3110 genome, while one IS and one prophage were found only in MG1655. Since the sequence examined herein is approximately 45% of the entire genome and nine large indels by ISs were found in that region, the total number of large indels by ISs between the two substrains is estimated to be 20. Given that the divergence time is 40 years, this indicates that an *E. coli* genome has experienced IS-derived indel events at the rate of 0.25 per year. It is of particular interest to know that *E. coli* has accepted such drastic alterations frequently, on average once in four years. As has been reported, it is likely that ISs play an important role in genome evolution, because they provide homologous regions large enough to mediate recombination events. In fact, recent sequencing of a 93-kb plasmid pO157, which was extracted from *E. coli* O157:H7, revealed that pO157 carried 18 IS elements. It also revealed that pO157 has been extensively subjected to rearrangements of the plasmid genome. Furthermore, in the *E. coli* K-12 chromosomes, we found that ISs have been actively transposed and have been interspersed in the

genome during short-term evolution. Therefore, the major changes of the genome structures may be frequently caused by IS-mediated recombination events. For details, see ref. 28.

(8) Slow Evolutionary Rate of GB Virus C/Hepatitis G Virus

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With the aim of elucidating evolutionary features of GB virus C/hepatitis G virus (GBV-C/HGV), molecular evolutionary analyses were conducted using the entire coding region of this virus. In particular, the rate of nucleotide substitution for this virus was estimated to be less than 9.0×10^{-6} per site per year, which was much slower than those for other RNA viruses. The phylogenetic tree reconstructed for GBV-C/HGV, by using GB virus A (GBV-A) as outgroup, indicated that there were three major clusters (the HG, GB, and Asian types) in GBV-C/HGV, and the divergence between the ancestor of GB and Asian type strains and that of HG type strains first took place more than 7,000~10,000 years ago. The slow evolutionary rate for GBV-C/HGV suggested that this virus cannot escape from the immune response of the host by means of producing escape mutants, implying that it may have evolved other systems for persistent infection.

(9) Intragenic Recombinations in Rotaviruses

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Groups A to F rotaviruses are members of the genus *Rotavirus* in the family *Reoviridae*. Among these viral groups, groups A, B, and C are known to infect humans, and, moreover, group A rotavirus is one of the most important aetiological agents of gastroenteritis in infants worldwide. Nucleotide substitution and

gene reassortment have been reported to be the major mechanisms of producing antigenic variants for rotavirus strains; in particular, recombination has been reported to occur in the "all or none" fashion. In the present study, evidence for intragenic recombination in the VP7 gene between rotavirus strains bearing different serotypes is demonstrated for the first time. To identify the border of the recombination, four sequences were used for analysis; one putative recombinant sequence, one representative sequence from each of the two parental lineages considered to be involved in the recombination, and one sequence from known outgroup. An alignment was made of the four sequences, and the informative sites were collected along the alignment. The informative site supported either of the three phylogenetic relationships; the clustering of the recombinant with one of the two parental sequences, with the other parental sequence, and with the outgroup sequence. The relative frequencies of the former two types of sites were compared between the regions before and after each breakpoint along the alignment, using the Fisher's exact test. If the value was statistically significant at some breakpoints, the optimum position of the recombination was determined as the point which minimize that value. Intragenic recombination may be one of the escaping mechanisms from the host immune system for rotavirus. This process involves exchanging antigenic regions, and thus questioning the use of multivalent vaccines for the prevention of rotavirus infection. For details, see ref. 24.

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I-b. Laboratory for Gene-Product Informatics

(1) Feasibility of the inverse protein folding protocol

Motonori OTA and Ken NISHIKAWA

There are two protocols available in the protein structure (3D) - sequence (1D) compatibility method. One of them to recognize the protein fold, called forward-folding protocol, has been widely used in the structure prediction of new proteins. However, the other one in which a structure recognizes its homologous sequences among a sequence database, called the inverse-folding protocol, is a more difficult application. We have investigated the feasibility of the latter approach. A structural library, composed of about four hundred well resolved structures with mutually dissimilar sequences, was prepared. We examined whether each one of proteins in the library could correctly seek their homologs by both forward- and inverse-folding protocols. The results showed that the inverse-folding protocol is more effective than the forward-folding protocol, once the reference states of the compatibility functions are appropriately adjusted. This adjustment only slightly affects the ability of the forward-folding search. We noticed that the scoring, in which a given sequence is remounted onto a structure according to the 3D-1D alignment determined by the dynamic programming method, is only effective in the forward- folding protocol, and not in the inverse-folding protocol. Namely, the inverse-folding search works significantly better with the score given by the 3D-1D alignment *per se*, rather than that obtained by the remounting. See Ref. 4 for the details.

(2)Genes from nine genomes are separated into their organisms in the dinucleotide composition space

Hiroshi NAKASHIMA[1], Motonori OTA and Ken NISHIKAWA ([1]The School of Health Sciences, Faculty of Medicine, Kanazawa University)

The dinucleotide composition composed of 16 components was used to analyze the protein-encoding DNA sequences in nine complete genomes: *Escheri-*

chia coli, Haemophilus influenzae, Helicobacter pylori, Mycoplasma genitalium, Mycoplasma pneumoniae, Synechocystis sp., Methanococcus Jannaschii, Archaeoglobus fulgidus and Saccharomyces cerevisiae. The dinucleotide composition was significantly different between the organisms. The distribution of genes from an organism was clustered around its center in the dinucleotide composition space. The genes from closely related organisms such as Gram-negative bacteria, mycoplasma species and eukaryotes showed some overlap in the space. The genes from nine complete genomes together with those from human were discriminated into respective clusters with 80% accuracy using the dinucleotide composition alone. The composition data estimated from a whole genome was close to that obtained from genes, indicating that the characteristic feature of dinucleotides holds not only for protein coding regions but also noncoding regions. When a dendrogram was constructed from the disposition of the clusters in the dinucleotide space, it resembled the real phylogenetic tree. Thus, the distinct feature observed in the dinucleotide composition may reflect the phylogenetic relationship of organisms. See Ref. 1 for the details.

(3) Constructing the Protein Mutant Database

Takeshi KAWABATA, Motonori OTA and Ken NISHIKAWA

Currently the protein mutant database (PMD) contains over 81,000 mutants, including artificial as well as natural mutants of various proteins extracted from about 10,000 articles. We recently developed a powerful viewing and retrieving system, integrated with the sequence and tertiary structure databases. The system has the following features: (i) mutated sequences are displayed after being automatically generated from the information described in the entry together with the sequence data of wild-type proteins integrated. This may be convenient for user because it allows one to see the position of altered amino acids (shown in a different color) in the entire sequence of a wild-type protein; (ii) for those proteins whose 3D structures have been experimentally determined, the 3D structure is displayed to show mutation sites; (iii) a sequence homology search against PMD can be carried out with any query sequence; (iv) a summary of mutations of homologous sequences

can be displayed, which shows all the mutations at a certain site of a protein, recorded throughout the PMD. See Ref. 2 for the details.

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I-c. Laboratory of Gene Function Research

(1) DNA Data Bank of Japan at Work on Genome Sequence Data

Yoshio TATENO, Kaoru FUKAMI-KOBAYASHI, Satoru MIYAZAKI, Hideaki SUGAWARA and Takashi GOJOBORI

We at the DNA Data Bank of Japan (http://www.ddbj.nig.ac.jp) have recently begun receiving, processing and releasing EST and genome sequence data submitted by various Japanese genome projects. The data include those for human, *Arabidopsis thaliana*, rice, nematode, *Synechocystis* sp., and *Escherichia coli*. Since the quantity of data is very large, we organized teams to conduct preliminary discussions with project teams about data submission and handling for release to the public. We also developed a mass submission tool to cope with a large quantity of data. In addition, to provide genome data on WWW, we developed a genome information system using Java. This system (http://mol.genes.nig.ac.jp/ecoli/) can in theory be used for any genome sequence data. These activities will facilitate processing of large quantities of EST and genome data. (See Pub.1 for details)

(2) Formal design and implementation of improved DDBJ DNA database with a new schema and object oriented library

Toshitsugu OKAYAMA, Takurou TAMURA, Takashi GOJOBORI, Yoshio TATENO, Kazuho IKEO, Satoru MIYIAZAKI, Kaoru FUKAMI-KOBAYASHI and Hideaki SUGAWARA

New schema and systems of the database management for DDBJ were constructed using an object-oriented design approach. The design was accomplished in accordance with ANSI/SPARC three-level schema architecture. First, the conceptual schema was designed using a functional model named AIS (associative information structure) and was visualised in extended diagram format. The model is a natural extension of an ER (entity relationship) model and describes real-world objects in binary associations between entities with the concept of order. Second, the schema was mapped on a relational database as a physical schema. All details are concentrated in this schema and the layer lying above enjoys physical independence. Finally, as another layer, external modelling was introduced for the database application interface. It provides set-at-a-time basis operations and was implemented as a C++ object-oriented library. On this common framework of a new schema, a new annotator's workbench named Yamato II and a World Wide Web (WWW) submission system named Sakura have been successfully developed to improve drastically daily transactions in the DDBJ.

Publications

1. TATENO, Y., FUKAMI-KOBAYASHI, K., MIYAZAKI, S., SUGAWARA, H. and GOJOBORI, T.: DNA Data Bank of Japan at work on genome sequence data. Nucleic Acids Res, 26, 16-20, 1998.
2. OKAYAMA, T., TAMURA, T., GOJOBORI, T., TATENO, Y., IKEO, K., MIYAZAKI, S., FUKAMI-KOBAYASHI, K. and SUGAWARA, H.: Formal design and implementation of improved DDBJ DNA database with a new schema and object oriented library. Bioinformatics, 14, 472-478, 1998.

I-d. Laboratory for Molecular Classification

The laboratory for molecular classification is composed of Professor Hideaki Sugawara and Professor Assistant Satoru Miyazaki and carries out research and development in the field of bioinformatics. They especially concentrate in the development of information system for the capture, accumulation, evaluation and analysis of data aiming at squeeze information and knowledge from data. By utilizing the accomplishment of the research and development, the laboratory also carries out such important international tasks as DNA Data Bank of Japan (DDBJ) and WFCC-MIRCEN World Data Centre for Microorganisms (WDCM). They carefully design modular systems which will be flexible, scalable and effective in daily use. Last but not least, contribution of Dr. Y. Ogawa, Ms R. Tanabe and Ms. Y. Fujisawa to the activities of the laboratory should be mentioned.

(1) Data processing system for DDBJ

Hideaki SUGAWARA, Satoru MIYAZAKI, Takashi GOJOBORI, Yoshio TATENO, Kazuho IKEO, Kaoru FUKAMI-KOBAYASHI, Taku TAMURA[1] and Toshitugu OKAYAMA[2] ([1]Idengaku-fukyukai, [2]Hitachi SK)

Sequence data have increased tremendously and also become complex more and more these years. It is not an easy task to develop a robust data management system to cope with this change in quantity and quality. We challenged this task by utilizing object-oriented technology (ref (1)-1). We also developed a dedicated system to microbial genomes data (ref (2)-3).

(2) Information system for the study of biodiversity

Hideaki SUGAWARA, Satoru MIYAZAKI and Hiroyuki OGAWA

The laboratory rans the World Data Center for Microorganisms (WDCM) of theWorld Federation for Culture Collections (WFCC) and Microbial Resources Centers Network (MIRCEN). The WDCM maintains the on-line World Directory of Collections of Cultures of Microorganisms, which include 498 orga-

nizations from 60 countries. WDCM also provides a distributed and parallel search engine of the Agent for Hunting Microbial Information on the Internet (AHMII).

In addition to the on-line directory and the agent, the laboratory has developed a polyphasic analysis system by integrating conventional taxonomy based on phenotypes and phylogenetic analysis based on molecular data. A database system for the integration was developed by use of object-oriented technology (ref (3)-1). The laboratory did also sequence of microbial rRNA in order to accumulate the know-how of experimentalists. For the improvement of phylogenetic analysis, information theory was applied to the analysis of HIV evolution (ref(1)-4).

The laboratory was invited to a study group that aimed at establishing alternative methods to animal testing and contributed to the group by the development of a database and statistical analysis tool (ref (1)-5:12).

Publications

1. SUGAWARA, H., MIYAZAKI, S., GOJOBORI, T. and TATENO, Y.: DNA Data Bank of Japan dealing with large-scale data submission, Nucleic Acids Research,Vol.27, No.1, 25-28,1999.
2. OKAYAMA, T., TAMURA, T., GOJOBORI, T., TATENO, Y., IKEO, K., MIYAZAKI, S., FUKAMI-KOBAYASHI, K. and SUGAWARA, H.: Formal design and implementation of an improved DDBJ DNA database with a new scheme and object-oriented library, Bioinformatics,14, 472-478, 1998.
3. SUGAWARA, H. and MIYAZAKI, S.: Towards the Asia-Pacific Bioinformatics Network, Pacific Symposium on Biocomputing '98,(eds.) Altman R. B., Dunker A. K., Hunter L. and Klein T. E., 759-764, Hawaii, 1998.
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12. OHNO, T., FUTAMURA, Y., HARIHARA, A. and et al.: Validation Study on Five Cytotoxicity Assays by JSAAE - VIII. Details of the Neutral Red Uptake Assay, Altern. Animal Test. Experiment, 5, 131-145, 1998.

Others

1. SUGAWARA, H.: International Nucelotide Sequence Database, Experimental Medicine, Vol.16, No.1, 30-37, 1998.
2. SUGAWARA, H.: Culture collections reflect the culture of countries, Bioscience and bioindustry, 56, 272-275, 1998.
3. MORI, H., SUGAWARA, H. and UCHIYAMA, I.: E. coli genome, Tanpakushitu-kakusan-koso, 43, 1381-1387, 1998.

Oral presentation

1. MIYAZAKI, S. and SUGAWARA, H.: Prototype of a workbench for sharing information by use if object technology, Japan Society for Culture Collections, Hiroshima, June.
2. SUGAWARA, H.: Evolution of sequence database, Denshi-joho-tuushin-gakkai, Yamanashi, September.
3. SUGAWARA, H.: Role of a public database in the study of microbial genomes, Frontier of the Genome Biology of Escherichia coli, Okazaki, Japan, November.
4. SUGAWARA, H.: Information highway and its application to biological informatics - WDCM experiences and global biodiversity information facility, 16th International CODATA Conference, New Delhi, India, November.
5. SUGAWARA, H.: Future of in silico biology The Ninth Parallel Computing Workshop, Kawasaki, November.

J. RADIOISOTOPE CENTER

(1) Functional analysis of the *phoB/cotA* region of the *Bacillus subtilis* chromosome

Yoshito SADAIE, Katsunori YATA and Hideyuki OHSHIMA

The *phoB-rrnE-groESL-gutR-cotA* region (70kb) of the *Bacillus subtilis* genome contains several operons of unknown or not well known function. We disrupted 57 orfs of unknown function and determined their transcriptional activity with *lacZ* and Northern analysis. Some features of the operons found are as follows.

A large operon involved in degradation and incorpolation of konjac glucomannan consists of eight orfs whose predicted products include PTS system proteins as well as mannan digesting extracellular enzyme. The expression of this operon was induced by konjac glucomannan, especially by dissaccharides and repressed by glucose or mannose.

Inherent restriction/modification system genes *hsdMR/hsdMM* consist of tondemly located modification genes *ydiO* and *ydiP*, and separately located restriction gene *ydiS* which make an operon with two other genes, *ydiR* and *ydjA*. *ydiO* and *ydiP* make an operon and they were co-transcribed with upstream *groESL* gene at T0 and T1 stage.

Five orfs, *ydiA* to *ydiE*, make an operon down stream of the *rrnE* operon. YdiA and YdiD are not essential for growth but YdiB, YdiC, and YdiE are essential. The last gene codes for a protein similar to *P.haemolytica* o-sialoglycoprotein endopeptidase. This operon is transcribed during growth.

The expression of *ydjF,ydjG,ydjH*, and *ydjI* showed a peak at T1 stage. LacZ activity and the signal of Northern analysis coincided. They constitute an operon down stream of the glucitol utilization operon. Some genes such as *ydgK*, *ydhJ*, and *ydhL* are transcribed during growth and other genes and operons such as *ydhD*, *ydhF*, *ydhU* and *ydjP/Q* are transcribed after T4 stage.

(2) Molecular Organization of Restriction and Modification System Genes of *Bacillus subtilis*

Hideyuki OHSHIMA, Katsunori YATA and Yoshito SADAIE

Bacillus subtilis has one set of inherent restriction/modification system genes consists of *hsdMM* and *hsdMR* on a prophage-like region at around 55 degree, a desert area of 12 kb long which has lower GC content and few orfs except for these genes. DNA target is a six base sequence Py/TCGAPu where C is methylated. *hsdMM* consists of an operon composed of tandemly located genes, *ydiO* and *ydiP*, for cytosin methylases, and *hsdMR* is another operon made of three genes involving *ydiS*, coding for a restriction endonuclease. A classical RM mutant strain lacks this region.

Disruption of *ydiS* resulted in high transformability and disruption of *ydiO* or *ydiP* was successful only in the background of disrupted *ydiS*.

Expression of *ydiO* or *ydiP*, and *ydiS* was examined using *bgaB* or *lacZ* as reporter genes or by Northern analysis. Transcription of hsdMM and hsdMR operons was observed during growth and early sporulation phase. In addition to these transcriptions, hsdMM operon was found to be co-transcribed with upstream groESL operon at T0 and T1 stage during sporulation.

The above prophage-like region carries pseudogenes reminiscent of an integrase or a terminase of bacteriophages and more clearly of phosphomannomutase (*yhxB*).

(3) Rapid isolation of RNA polymerase from sporulating cells of *Bacillus subtilis*

Masaya FUJITA and Yoshito SADAIE

A highly ordered program of temporal and spacial gene activation during sporulation in *Bacillus subtilis* is governed by the principal RNA polymerase, and RNA polymerases containing at least five developmental sigma factors that appear successively during sporulation. We have developed a rapid procedure for extracting RNA polymerase from sporulating *B. subtilis* cells, which involves the construction of hexahistidine tagged β'subunit of RNA polymerase and the isolation of RNA polymerase holoenzyme with $Ni2^{+}$-NTA

resin. In *in vitro* transcription of various promoters with the RNA polymerase thus purified, we observed the temporal change of each RNA polymerase activity during sporulation. This procedure enables an isolation of RNA polymerase within 4hours with cell pellets. Our results indicated that a principal sigma factor, σ^A, could be detected in a holoenzyme form during all the stages of growth and sporulation while the other sigma factors, σ^H, σ^E, σ^F σ^G, and σ^K involved in sporulation could be detected sequentially during sporulation. Moreover, Spo0A, the central transcription factor of commitment to sporulation, was also co-purified with RNA polymerase at the early stages of sporulation.

Publications

1. FUJITA, M. and SADAIE, Y.: Rapid isolation of RNA polymerase from sporulating cells of *Bacillus subtili*. Gene 221, 185-190, 1998.
2. ASAI, K., FUJITA, M., KAWAMURA, F., TAKAHASHI, H., KOBAYASHI, Y. and SADAIE, Y.: Restricted transcription from Sigma H or phosphorylated Spo0A dependent promoters in the temperature-sensitive *secA341* mutant of *Bacillus subtilis*. Biosci. Biotech. Biochem. 62, 1707-1713, 1998.
3. SADAIE, Y.: *secA341* mutation inhibition of expression of the *Bacillus subtilis* protease gene, *aprE*; Biosci. Biotechnol. Biochem. 62, 1784-1787, 1998.
4. HAN, W-D., KAWAMOTO, S., HOSOYA, Y., FUJITA, M., SADAIE, Y., SUZUKI, K., OHASHI, Y., KAWAMURA, F. and OCHI, K.: A novel sporulation-control gene (*spo0M*) of *Bacillus subtilis* with a σ^H-regulated promoter. Gene 217, 31-40, 1998.
5. FUJITA, M. and SADAIE, Y.: Promoter selectivity of the *Bacillus subtilis* RNA polymerase σ^A and σ^H holoenzymes. J. Biochem. (Tokyo) 124: 89-97, 1998.
6. FUJITA, M. and SADAIE, Y.: Feedback loops involving Spo0A and AbrB in *in vitro* transcription of the genes involved in the initiation of sporulation in *Bacillus subtilis*. J. Biochem. (Tokyo) 124, 98-104, 1998.
7. NANAMIYA, H., OHASHI, Y., ASAI, K., MORIYA, S., OGASAWARA, N., FUJITA, M., SADAIE, Y. and KAWAMURA, F.: ClpC regulates the fate of a sporulation initiation sigma factor, σ^H protein in *Bacillus subtilis*. Mol. Microbiol. 29,505-513,1998.

K. EXPERIMENTAL FARM

(1) Development and reevaluation of the genetic stocks of rice

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

We have conducted the reproduction and distribution of genetic stocks of wild and cultivated rice. From October 1, 1997, we try to include new system of rice genetic stock generation and application. Additional resources we are conducting to produce, utilize and distribute are enhancer trap and gene trap rice line. Another trial to analyze the composition of the centromeric heterochromatin in rice chromosomes is also progressed. These projects are cooperated with the plant genetics laboratory. For details, see the reports of plant genetics lab.

Publication

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.

ABSTRACTS OF DIARY FOR 1998

Biological Symposium

Jan. 14	A manifold of codes in genetic sequences (Edward N.Trifonov)
Feb. 2	The 2nd Decade of Human Genome Informatics Support:Reflections and Future Directions relevant to other Genome efforts. (Tom Slezak)
Feb. 17	CREB-mediated gene expression:a mechanism underlying hippocampal Synaptic activity-induced formation of long-term memory? (Haruhiko Bito)
Feb. 18	From growth cone to synapse (Motojiro Yoshihara)
Feb. 26	Roles of Islet-1 transcription factors in the regional specification and neuronal differentiation in the brain (Hitoshi Okamoto)
Mar. 5	Oligonucleotide arrays and the discovery of efficient antisense reagents (E.M.Southern)
Mar. 12	Development and evolution of the skull (Shigeru Kuratani)
Mar. 17	Genetically engineered foodcrops:problems and prospects (Peter Day)
Mar. 17	Genetic dissection of *Drosophia* sexual behavior (Daisuke Yamamoto)
Mar. 19	The St.Louis,Sanger,Mishima worm sequencing project (Jean Thierry-Mieg)
Apr. 13	V(D)J RECOMBINATION AND ITS BIOLOGICAL RELATIVES (Martin Gellert)
Apr. 15	The role of Deltex in Notch signaling (Kenji Matsuno)
Apr. 22	Generation and positioning of cortical neurons:a cellular and histological study (Masaharu Ogawa)
Apr. 22	Inactivation at promoters and control of transcription initiation(Ranjan sen)

Apr. 23	Molecular mechanisms of synaptic connection:analysis of the neuromuscular junction in *Drosophia* (Toshinao Nose)
Apr. 27	Molecular and neural mechanisms of thermotaxis in the nematode *C.elegans* (Ikue Mori)
May 8	Supramolecular Structure of the *Salmonella typhimurium* TypeⅢ Protein Secretion system (Tomoko Kubori)
May 13	Genetic information controlling animal behavior -the analysis of Fyn-deficient mouse (Takeshi Yagi)
May 13	Reductive evolution of endocellular parasites and the origin of mitochondria (Charles G.Kurland)
May 14	Molecular mechanism of pattern formation revealed by analysis of the Cadherin superfamily (Tadashi Uemura)
May 18	Detection and analysis of mutation in human and mouse (Youichi Gondoh)
May 18	Genomic imprinting:gene regulation at the level of chromosome domain based upon genome modification (Hiroyuki Sasaki)
June 3	Dorsalization mechanisms in vertebrates:Genetic analysis by using zebrafish mutants (Yasuyuki Kishimoto)
June 16	Maintaining and integrating morphogen gradients.Cross regulation of Wg and Dpp. (J. Lawrence Marsh)
June 19	Analysis of functional sequences in human centromere and construction of artificial chromosomes (Hiroshi Masumoto)
June 22	Mechanism of Mouse Central Olfactory Projection (Tatsumi Hirata)
July 6	Mechanisms of Zebrafish central nervous system development:Embryo logical and genetic approach (Hiroyuki Takeda)
July 8	Regulation of neural plasticity by Ras (Atsu Aiba)
July 15	The initiation of meiotic recombination in *Saccharomyces cerevisiae* (Bernard Massy)
July 23	The role of *Pax-6* gene in neuronal differentiation and guidance (Noriko Ohsumi)

July 23	Higher order regulation of expression of HoxD gene cluster (Takashi Kondoh)
July 24	Developmental genetics of quaking mutation affecting neurological function and embryonic development (Kuniya Abe)
July 27	Programmed cell death in the nematode *C.elegans* :a search for new genes (Asako Sugimoto)
July 29	Chromatin structure and transcriptional regulatory mechanisms in the yeast genome (Mitsuhiro Shimizu)
July 30	ProteinX-New player in prion propagation (Kiyotoshi Kaneko)
July 30	Constructing trees from subtrees(with applications to mammalian evolution) (Dan Graur)
July 31	Relative Contributions of Mutation and Selection to the Evolution of Bacterial DNA Base Composition (Noboru Sueoka)
Aug. 3	Roles of the paraxial protocadherin(PAPC)gene in morphogenetic movement during gastrulation of the zebrafish, Xenopus and mouse embryos. (Akihito Yamamoto)
Aug. 7	Histo-blood type ABO system:from glycosyl chain antigens to genes and glycosyltransferases (Fumi-ichiro Yamamoto)
Aug. 28	Hedgehog protein biogenesis and signaling (Philip A.Beachy)
Sep. 1	Genetic control of programmed cell death in the nematode *C.elegans*. (Michael Hengartner)
Sep. 14	Genome imprinting and germ cell development (Takashi Tada)
Sep. 17	Functional analysis of Dpb11 which is involved in chromosomal DNA replication and cell cycle checkpoint in *Saccharomyces cerevisiae* (Youichiro Uemura)
Sep. 30	A common regulatory mechanism controls expression of *Drosophia* master control genes and establishment of organ identities (Syoichiro Kurata)

Oct. 5	Analysis of nuclear proteins which control the plant photomorphogenesis (Minami Matsui)
Oct. 5	Structural and functional genomics in *Arabidopsis thaliana* (Hiroyuki Tabata)
Oct. 6	Interaction between enhancer and promoter in *Drosophia* embryos (Sumio Ohtsuki)
Oct. 8	The Telomeres of *Drosophila* and the Mosquito *Anpheles gambiae* (Harald Biessmann)
Oct. 13	Signaling by *wnt* Genes in Drosophila:Common and Pathways (Roel Nusse)
Oct. 13	FGF signaling in the vertebrate embryo (Gail Martin)
Oct. 13	The *C.elegans* Genome Project (Stephanie L.Chissoe)
Oct. 14	Genetic and molecular mechanisms that pattern and organize in *Drosophia* (Thomas B.Kornberg)
Oct. 15	Control of meiotic recombination in Saccharomyces cerevisiae (Michael J.Lichten)
Oct. 16	Establishing polarity in the *C.elegans* embryo (Kenneth J.Kemphues)
Oct. 19	Control of developmental pathways by Hox proteins and their cofactors (Richard S.Mann)
Oct. 19	Understanding plant zygotic embryogenesis through analysis of somatic embryogenesis (Hiroshi Kamata)
Oct. 19	Expression and function of homeobox genes in rice (Shin Matsuoka)
Oct. 20	Specification of the dorsal axis of *Xenopus* by the Wnt signaling pathway (Randall T.Moon)
Oct. 22	The role of signaling factors in the development of kidney (Tomoko Ishihara-Obara)
Oct. 28	*Drosophila* ELAV protein is a major posttranscriptional regular in neurons (Kalpana White)
Nov. 2	Analysis of structure,function and development of the *Drosophia* brain,using GAL4-UAS technique (Kei Itoh)
Nov. 2	The End of Mitosis in Budding Yeast(Leland H.Johnston)

Nov. 9	"Genome fusion" of two plant species beyond one hundred million year's evolution.-Does it waste money and time? Or,is it informative for genome biology?- (Daisuke Shibata)
Nov. 9	Genome DNA methylation,plant development,and transposons:an approach using *Arabidopsis* mutants (Tetsuhito Kakutani)
Nov. 10	Signaling at the synapse:identification of HIG and SIF through low-activity mutans (Chihiro Hama)
Nov. 11	Evolution of Rh genes and antigenes in nonhuman primates (Antonie Blancher)
Nov. 19	Computer simulation of development processes (Hiroaki Kitano)
Dec. 1	Investigation of a set of genes essential for cell growth in *Escherichia coli* :Identification and characterization of the novel hda gene encoding a Dna-related protein,which is essential for chromosome replication (Jun-ichi Katoh)
Dec. 1	Mechanism of signal transduction of the phosphate regulon in *Escherichia coli* (Kouzou Makino)
Dec. 1	Estimation of past demographic parameters when the mutation rates vary among sites (Laurent Excoffier)
Dec. 7	FAST-1 is an essential component of activin/TGF β-mediated mesoderm induction in the early Xenopus embryo (Minoru Watanabe)
Dec. 10	Evaluation of Threading Specificity and Accuracy (Stephen H.Bryant)
Dec. 15	Isochores,selection and rendom drift. (Girogio Bernardi)
Dec. 22	The roles of PGL-1,a component of the germ granules,in the germline development of the nematode *C.elegans* (Ichiro Kawasaki)
Dec. 24	The roles of a organaizer specific homeo-box gene,Xlim-1,in the nerve induction and the anterior-posterior pattern formation (Masanori Taira)

Dec. 28 Yeast as a model for functional interpretation of the genomes :Approach from gene-gene and protein-protein interactions (Takashi Itoh)

Dec. 28 Molecular mechanisms of homologous recombination in *Escherichia coli* :Structure and functions of Holliday junction processing enzymes,RvuA, RvuB,and RvuC proteins (Hiroshi Iwasaki)

Mishima Geneticists' Club

Jan. 16 Post-translational regulation of nitrate reductase(NR) in higher plant (Kengo Kanamaru)

Feb. 25 Transcriptional regulation of Breathless,the FGF receptor required for the tracheal development in *Drosophia* (Tomokazu Ohshiro)

Mar. 11 Wnt3a and Wnt7a regulate chicken limb patterning through two distinct signaling pathways (Mineko Kengaku)

Mar. 12 tmRNA,a small RNA functioning as both tRNA and mRNA (Akira Mutoh)

FOREIGN VISITORS IN 1998

Jan. 11-14	Yuan Yi-da, Institute of Genetics, Chinese Academy of Sciences, China
Jan. 20-21	Zhang Zhi-xin, Beijing Blood Center, China
Jan. 19-Mar. 20	Jean Thierry-Mieg, CNRS, Laboratoire de Physique Mathematique, Universite Montpellier Ⅱ, France
Feb. 2	Tom Slezak, Human Genome Center, Lawrence Livemore National Laboratory, U.S.A.
Mar. 5-6	E.M.Southern, University of Oxford, U.K.
Mar. 17-18	Peter Day, The State University of New Jersy, U.S.A.
Mar. 17-18	Helmut Knuepffer, IPK, Gatersleben, Germany
Apr. 12-14	Martin F.Gellert, Laboratory of Molecular Biology, NIH, U.S.A.
May 26-Jun. 13	Dipankar Chatterji, Centre for Cellular and Molecular Biology, India
Jun. 3-4	Yasuyuki Kishimoto, Max Plank Institute of Tuebingen, Germany
Jun. 15-16	Bernard de Massy, Institut Curie, France
Jun. 16	J.Lawrence Marsh, University of California at Irvine, U.S.A.
Jul. 17-Aug. 23	Shozo Yokoyama, Syracuse University, U.S.A.
Jul. 23	Takashi Kondoh, University of Geneva, Switzerland
Jul. 30-31	Kiyotoshi Kaneko, University of California San Francisco,U.S.A.
Aug. 3-4	Akihito Yamamoto, University of California Los Angeles,U.S.A.
Aug. 28-29	Philip A.Beachy, Johns Hopkins University, U.S.A.
Aug. 31-Sep. 1	Michael Hengartner, Cold Spring Harbor Laboratory, U.S.A.
Sep. 28-30	Jin Feng, Institute of Genetics, Chinese Academy of Sciences, China

Sep. 28-30	Cao Jing-Long, Institute of Genetics, Chinese Academy of Sciences, China
Oct. 6-7	Sumio Ohtsuki, University of California Berkeley, U.S.A.
Oct. 8-9	Harald Biessmann, University of California at Irvine, U.S.A.
Oct. 12-13	Roel Nusse, Stanford University Medical Center, U.S.A.
Oct. 13	Stephanie L.Chissoe, Washington University School of Medicine, U.S.A.
Oct. 13-14	Gail Martin, University of California San Francisco, U.S.A.
Oct. 14	Thomas B.Kornberg, University of California San Francisco, U.S.A.
Oct. 14-15	Michael Lichren, NIH, U.S.A.
Oct. 15-16	Kenneth J.Kemphues, Cornell University, U.S.A.
Oct. 19-20	Richard S.Mann, Columbia University, U.S.A.
Oct. 20-21	Randall Moon, University of Washington School of Medicine, U.S.A.
Oct. 28-29	Kalpana White, Brandeis University, U.S.A.
Oct. 31-Nov.2	Leland H.Johnston, National Institute for Medical Research, U.K.
Nov. 10-13	Antoine Blancher, Hospital Purpan, France
Nov. 30-Dec. 3	Laurent Excoffier, University of Geneva, Switzerland
Dec. 7-8	Minoru Watanabe, Harvard Medical School, U.S.A.
Dec. 10-11	Steven H.Bryant, NCBI, NIH, U.S.A.
Dec. 10	Vijaya Gopal, Centre for Celler and Molecular Biology, India
Dec. 21-22	Ichiro Kawasaki, Indiana University, U.S.A.

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