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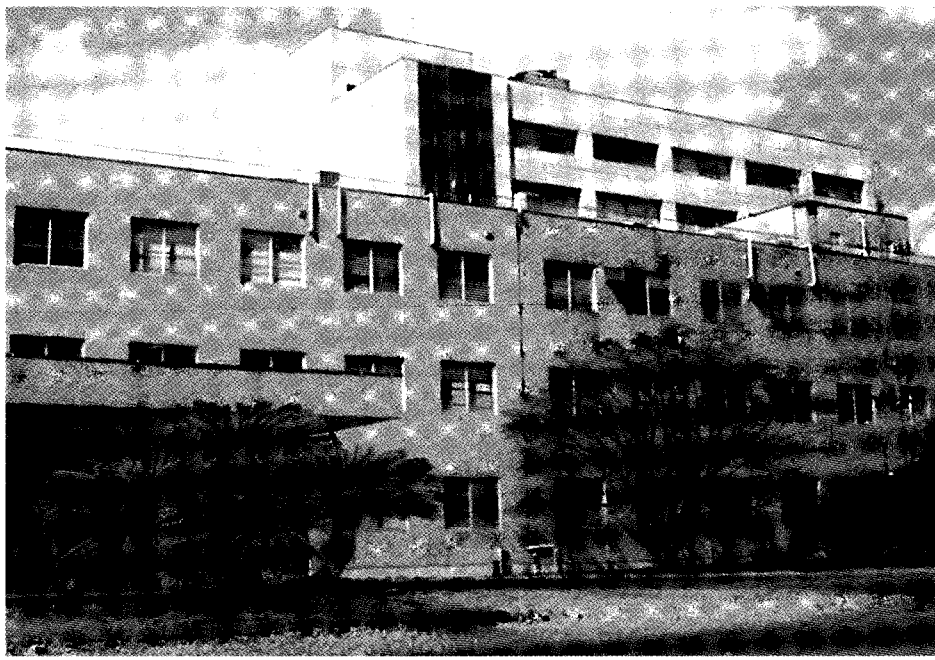

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GENERAL STATEMENT

National Institute of Genetics (NIG) was established 51 years ago as a center for genetics research. Major contributions of NIG in the past, especially in plant genetics, population and evolution genetics, 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 in the Graduate School of Life Science. This year, we have 37 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 9 foreign and 15 Japanese postdoctoral fellows actively participated in 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 related to DNA sequences. In recent years, it receives data input not only from Japanese institutions but also from institutes in other Asian countries. We have also 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.

In the past year we saw a number of changes in the NIG staff members. Prof. Akio Murakami retired at the end of March. We would like to express our

gratitude for his contribution to our institute. Newly appointed as professors are Drs. Yumiko Saga (Mammalian Genetics Lab.), Tetsuji Kakutani (Div. Of Agricultural Genetics), Naoko Imamoto (Gene Network Lab.), Hironori Niki (Radioisotope Center) and Hitoshi Ueda (Div. Of Gene Expression). Drs. Atsushi Kawakami, Katsumi Maenaka, Kumiko Sogawa, Nobuyuki Siina, Shingo Kose are appointed as research associates. On the other hand, the following members left NIG to take new positions. Among them are Drs. Tetsuichiro Saito (Kyoto Univ.), Takasi Tada (Kyoto Univ.) , Tsutomu Ohta (National Cancer Center) and Masayuki Hatta (Ochanomizu Univ.).

Japanese Government is planning to reorganize national universities and national institutes. We are willing to be engaged in the process actively to make the reform appropriate for better academic achievement.

Yoshiki Hotta, Director-General

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SHIROISHI, Toshihiko; Professor, National Institute of Genetics

RESEARCH ACTIVITIES IN 2000

A. Department of Molecular Genetics

A-a. Division of Molecular Genetics

(1) Monitoring of Growth-Coupled Changes in Promoter Activities in *Escherichia coli* Using Fluorescent Proteins as Reporters

Hideki MAKINOSHIMA, Akiko NISHIMURA[1] and Akira ISHIHAMA
([1]Laboratory of Microbial Genetics, Genetic Strains Research Center)

The pattern of gene expression in *Escherichia coli* changes markedly during the growth transition from exponential to stationary phase. Of about 4,000 genes on the *E. coli* chromosome, a total of about 1,000 genes are expressed in the exponential growth phase as measured by two-dimensional gel electrophoresis of whole cell extracts. The expression level of these growth-related genes decreases markedly in the stationary phase, whereas a new set of more than 100 genes which are not expressed in the exponential phase begins to be expressed. The growth-related genes are transcribed by the RNA polymerase holoenzyme containing σ^D, while the stationary phase genes are transcribed by Eσ^S holoenzyme. For detailed analysis of the growth phase-coupled regulation of global gene transcription, we set up a monitoring system of the *in vivo* promoter activity by using fluorescent proteins as reporters. Various kinds of the promoter were fused to the genes coding fluorescent proteins with different wave lengths. To measure the activity of test promoter at various growth phases, the reference and the test promoters were fused to the coding sequences for different fluorescent proteins with the emission maximum of different wavelength and both fusion genes were inserted into the same plasmid vector of various copy numbers. For instance, the reference promoter, *lac*UV5, was fused to the Red fluorescent protein and the test promoter was fused to the Green fluorescent protein. The ratio of Red and Green fluorescence was measured at

various growth phases . After analysis of various promoters from the known *E. coli* genes, we could show the time dependent change in promoter activity for a number of *E. coli* promoters, including *bolA*, *cbpA*, *dps*, *fic*, *hupA*, *mdh*, *osmY* and *rmf*. Results indicate that the pattern of time-dependent change in promoter activity is different among these promoters. For some details see Refs. 6, 8, 13, 14 and 17.

(2) Anti-sigma Factors for the σ^{70} and σ^{S} Subunits of *Escherichia coli* RNA Polymerase

Akira ISHIHAMA, Dipank DASGUPTA[1], Miki JISHAGE and Kaneyoshi YAMAMOTO
([1]On leave of absence from Saha Institute of Nuclear Physics, Calcutta)

The *Escherichia coli* genes expressed during entry of cell growth into the stationary phase is transcribed by the RNA polymerase holoenzyme containing the *rpoS*-coded σ^{S}, a close relative of σ^{D}, with a mutual overlap of promoter recognition properties. A set of more than 100 stationary phase-specific genes is expressed under the control of σ^{S}. The intracellular concentration of σ^{S} subunit increases during the transition from exponential growth to stationary phase. The level of σ^{D} subunit for growth-related gene transcription, however, stays constant, without being degraded in the stationary phase, suggesting that in addition to the level control, the activities of σ subunits must be controlled. So far various mechanisms have been identified, including association of the antisigma factors, changes in the cytoplasmic compositions, and modulation of the nucleoid configuration. Recently we discovered a novel *E. coli* protein, referred to Rsd (regulator of sigma D), which forms a complex with σ^{70} and represses its function. Purified Rsd protein formed complexes *in vitro* with σ^{70} but not with other σ subunits, and inhibited σ^{70}-dependent transcription *in vitro* to various extents depending on the promoters used. Both depletion and over-production of Rsd enhances or suppresses, respectively, σ^{70}-dependent transcription *in vivo*, with concomitant decrease or increase in σ^{S}-dependent transcription. After analysis of the σ^{79} cleavage sites by FeBABE tethered on Rsd and of the binary complex formation of Ala-substituted σ^{70} subunits with Rsd, the contact site of Rsd on the σ^{79} subunit was identified

to be within region 4.2, including amino acid residues L595 and L598.

As an extension of this line of studies, we have searched for anti-sigma factor for the σ^S subunit, and found that in late stationary-phase cells, a certain fraction of σ^S is associated with Dps (DNA-binding protein in starved cells). Purified Dps forms *in vitro* binary complexes with σ^S but not with other σ subunits, threby interfering with the formation of σ^S holoenzyme. Accordingly transcription *in vitro* of σ^S-dependent promoters was inhibited by the addition of Dps. In agreement with these *in vitro* observations, the expression *in vivo* of *lacZ* under the control of σ^S-dependent *fic* and *katE* promoters increased in mutants lacking Dps. In late stationary phase of cell growth, unbound cytoplasmic form of Dps was found to exist, which may form complexes with σ^S. These findings altogether raise a possibility that Dps is an anti-sigma factor for the stationary phase-specific σ^S subunit. For details see Refs. 1, 6, 7, 9 and 14.

(3) Search for Class-III (β -contact) and -IV (β '-contact) Transcription Factors in *Escherichia coli*

Akira KATAYAMA, Tasuku NOMURA, Nobuyuki FUJITA and Akira ISHIHAMA

The RNA polymerase holoenzyme of *Escherichia coli* is composed of the core enzyme with the subunit composition of $\alpha_2 \beta \beta'$ and one of seven different species of the σ subunit. For regulated transcription initiation of most inducible genes, additional protein or nucleotide factors are required. Most protein factors with the regulatory activities of transcription interact directly with the RNA polymerase. Transcription factors with enhancing or inhibiting activities of transcription were classified as activators or repressors. Several lines of evidence indicate that these transcription factors have dual functions, transcription activation and repression, depending on the sites of DNA binding. In *E. coli*, at least 100-150 transcription factors are considered to interact directly with the RNA polymerase. Since the mode of transcription regulation by transcription factors is related to the mode of protein-protein interaction, we proposed to classify these transcription factors on the basis of contact subunit of RNA polymerase. So far a number of class-I (α -contact) and -II (σ -con-

tact) factors have been identified and the mode of action were analyzed in details for some of these factors. This year we searched for class-III and -IV transcription factors which interact with the β and/or β' subunits, respectively. For this purpose, we employed an affinity isolation of candidate proteins from whole cell extracts of *E. coli* expressiong a low level of β - or β'-subunits fused with glutathione S-transferase (GST). After N-terminal amino acid micro-sequencing or mass-spectrometry, we identified 7 proteins as candidates for the class-III (β-contact) and 9 proteins as candidates for the class-IV (β'-contact) factors. Proteins identified by both screenings are considered to be associated with the assembled RNA polymerase. For instance, the 110 kDa RapA/HepA protein, the putative helicase, is one such example. Proteins that were identified with only either GST-β or GST-β' are the candidates for class-III or class-IV factors, which are analyzed in details. For some details see Refs. 6, 10, 14 and 23.

(4) Protein-protein Contact Site Mapping of *E. coli* Transcription Factors on the RNA Polymerase

Sujatha SITRAMAN, Katsunori YATA[1] and Akira ISHIHAMA
([1]Radioisotope Center)

Transcription regulation is a major cellular process by which a large number of genes in *Escherichia coli* are controlled. The RNA polymerase holoenzyme consists of 2 α, β, β' and one of seven molecular species of the σ subunit. The function of RNA polymerase holoenzyme is further modulated through interplay with one or two of about 100-150 transcription factors. Transcription factors have been classified into four groups, class I, II, III and IV, based on the subunit, α, σ, β and β', of RNA polymerase they contact in order to execute their functions. By a combination of genetic and biochemical approaches, a number of class-I and -II factors have been identified and their contact sites have been mapped on the α and σ subunit, respectively. Systematic search for class-III and -IV factors is in progress in this laboratory (see above). For systematic mapping of the transcription factors on the RNA polymerase, we have devised a novel experimental system, in which the transcription factor

contact sites can be detected after analysis of contact-dependent cleavage sites on each subunit by a chemical protease, FeBABE, conjugated to the test factors. For detection of the cleaved subunits and mapping of the cleavage sites, different epitope tags were added to all four RNA polymerase subunits, thereby allowing the detection of cleavage products after Western blotting. To test the new experimental system for mapping of the factor-contact sites, we analyzed the binding sites of all seven σ factors on the core enzyme, and the contact sites of DnaK, GroEL, IF2 and EF-TU, which all associates stably with the RNA polymerase in the absence of DNA, on the holoenzyme E σ^{70}. For details see Refs. 2, 6, 7, 10, 18, 22, 23 and 28.

(5) Determination of Intracellular Concentrations of Transcription Factors in *Escherichia coli*

Akira ISHIHAMA, Katsunori YATA, Etsuko KOSHIO, Akira IWATA[1] and Susumu UEDA[1] ([1]Nippon Institute for Biological Science, Ohme, Tokyo)

Constitutively expressed genes in *Escherichia coli* are transcribed by the RNA polymerase holoenzyme alone without transcription factors, but transcription of inducible genes requires additional transcription factors. A total of about 100-150 DNA-binding proteins are considered to function as transcription factors by directly interacting with the RNA polymerase holoenzyme when both bind to the promoters so as to increase the local concentrations. On this view point, the expression level of those inducible genes is considered to be determined by the intracellular concentrations of individual transcription factors. At present, however, little is known about the intracellular levels and controls of transcription factors. We then initiated a systematic study of the level controls of transcription factors in *E. coli*. For this purpose, we purified more than 50 species of the transcription factor in His-tagged forms from overexpressing cells using the respective cloned genes, and raised polyclonal antibodies in rabbits against the purified transcription factors. The concentration of transcription factors in whole lysates of *E. coli* W3110 growing under various conditions were determined by using quantitative immuno-blotting. So far we determined the intracellular levels of ArcR, DeoR, DnaK, Fur, GreA,

GroE, IclR, InfB, LacI, LeuO, MerR, NusA, OxyR, PhoP, RpoZ, SdiA, SoxR, SoxS, ThiI and TufB. For some details see Refs. 6, 8, 23 and 24.

(6) Novel Mode of Transcription Regulation: Simultaneous Activation and Repression by a Single Transcription Factor

Kaneyoshi YAMAMOTO, Katsunori YATA[1] and Akira ISHIHAMA
([1]Radioisotope Center)

SdiA, the *Escherichia coli* homologue of quorum-sensing regulator, controls the expression of the *ftsQAZ* operon for cell division. Transcription of *ftsQAZ* is under the control of two promoters, σ^{70}-depedent upstream *ftsQ*P2 and σ^{S}-dependent downstream *ftsQ*P1, each being separated by 125 bp. SdiA activates transcription *in vivo* from *ftsQ*P2. SdiA was found to stimulate transcription *in vitro* from *ftsQ*P2, and in addition, to repress transcription from *ftsQ*P1. Molecular basis of the dual functions of SdiA on transcription regulation of *ftsQ*P1P2 was analyzed in details using reconstituted RNA polymerases lacking the contact surfaces for class-I or class-II transcription factors. Gel shift and DNase I footprinting assays indicated that only a single molecule of SdiA binds between -51 and -25 with respect to the upstream P2 promoter. In agreement with the influence on overall transcription, SdiA stimulated the RNA ploymerase binding to P2 but inhibited its binding to P1. Activation of *ftsQ*P2 transcription by SdiA was observed with a mutant RNA polymerase containing a C-terminal domain (CTD)-deleted α subunit (α 235) but not with RNA polymerase holoenzymes containing σ^{S} (E σ^{S}) and a CTD-deleted σ^{D} (E σ^{D}529). In good agreement with the transcription assay, DNA protection of P2 was also not observed with the RNA polymerase holoenzymes, E σ^{S} and E σ^{D}529. These observations altogether indicate that: (i) SdiA supports the RNA polymerase (E σ^{D}) binding to *ftsQ*P2; and (ii) unlike the well-known mechanism of RNA polymerase recruitment by protein-protein contact between class-I factors and αCTD, the recruitment of RNA polymerase to *ftsQ*P2 by SdiA depends on the presence of σ CTD. On the other hand, transcription inhibition of the *ftsQ*P1 promoter by SdiA was observed with all the RNA polymerases examined, including E σ^{D}, E σ^{D}529, E σ^{D} containing α235, and

E σ^S (and therefore in the absence of RNA polymerase-binding to the upstream P2). We predict that the P1 transcription inhibition is due to conformational change in P1 promoter DNA. For some details see Refs. 2, 6, 18, 19 and 28.

(7) Systematic Search for Zn^{2+}-binding Proteins in *Escherichia coli*

Akira KATAYAMA, Atsuko TSUJII[1], Akira WADA[2], Takeshi NISHINO[1] and Akira ISHIHAMA ([1]Nihon Medical School, Department of Biochemistry, Tokyo, [2]Osaka Medical School, Department of Physics, Suita)

As a part of the proteome analysis of *Escherichia coli*, attempts have been made to identify all Zn^{2+}-binding proteins. For this purpose, whole cell extracts of *E. coli* W3110 were fractionated by two methods of two dimensional gel electrophoresis, *i.e.*, the widely used O'Farrell method and the RFHR (radical-free and highly reducing) method. After gel electrophoresis, proteins were blotted onto PVDF membranes and after renaturation, exposed to binding assay with radioactive Zn^{2+}. A number of novel *E. coli* proteins so far unidentified as Zn^{2+}-binding proteins have been found to carry the Zn^{2+}-binding activity as detected by this conventional metal-binding assay. To examine the affinity and specificity of Zn^{2+} -binding, the genes for some newly identified Zn^{2+}-binding *E. coli* proteins were cloned into an expression vector and overexpressed in *E. coli* BL21(DE3) in His-tagged forms. The purified Zn^{2+}-binding proteins were analyzed, in parallel with the known Zn^{2+}-binding proteins such as carbonic anhydrase and Sp1, for the Zn^{2+}-binding affinity. The specificity of Zn^{2+}-binding was also tested by competition assay in the presence of other metal ions.

(8) Protein and mRNA Levels of All Twelve Subunits of RNA Polymerase II in *Schizosaccharomyces pombe*

Hitomi SAKURAI, Makoto KIMURA and Akira ISHIHAMA

The RNA polymerase II of eukaryotes is composed of twelve subunits (Rpb1 to Rpb12), of which five are shared among Pol I, Pol II and Pol III. At present,

however, little is known on the regulation of synthesis and assembly of the twelve subunits. To get insight into the regulation of synthesis of these twelve subunits in the fission yeast *Schizosaccharomyces pombe*, we analyzed protein and mRNA levels for all the *rpb* genes. Results of Western blot and competitive RT-PCR analyses indicate that: (i) the intracellular concentrations of twelve Rpb subunits in growing *S. pombe* cells are different about 15-fold between the least abundant Rpb3 and the most abundant Rpb12; (ii) at least certain fractions of the abundant RNA polymerase II subunits exist as unassembled states as analyzed by glycerol gradient centrifugation of whole cell extracts; and (iii) mRNAs for Rpb1, Rpb3, Rpb7 and Rpb9 are among the group of low abundance, while the levels of Rpb6 and Rpb10 mRNAs are about 5-fold and that of Rpb2 mRNA is about 40-fold higher than the Rpb3 mRNA level. The protein-to-mRNA ratio (or the translation efficiency) is low for the *rpb1*, *rpb2*, *rpb3* and *rpb11* genes, encoding the homologues of subunits β', β, α, and α, respectively of the prokaryotic RNA polymerase core enzyme.

As an extension, we also analyzed transcription organization by oligo-capping method. Results indicate that transcription of one group of genes including *rpb3*, *rpb4*, *rpb5*, *rpb6*, *rpb7* and *rpb10* is initiated mainly at a single site, while that of the other group of genes for *rpb1*, *rpb2*, *rpb8*, *rpb9*, *rpb11* and *rpb12* is initiated at multiple sites. The promoters of the first group genes contain the TATA box sequence between -26 to -62, while the second group genes carry TATA-less promoters. Several common sequence segments, tentatively designated "Rpb motifs", were identified in the promoter regions of the *rpb* genes. For details see Refs. 11, 12 and 20.

(9) Isolation and Characterisation of RNA Polymerase II-Associated Proteins in *Schizosaccharomyces pombe*

Makoto KIMURA, Hisako SUZUKI and Akira ISHIHAMA

Eukaryotic RNA polymerase II is a large complex with the molecular mass of about 500 kDa. In the light of comparison with many other smaller nucleic acid polymerases, we can assume that only a part within the RNA polymerase II molecule is involved in the catalytic reaction of RNA polymerization, and other

parts have regulatory functions. In transcription regulation, the RNA polymease II is supposed to be controlled by protein-protein interaction with a number of transcription factors. We tried to isolate such RNA polymerase II-interacting protein factors from polymerase complexes, which were isolated using an epitope-tagging method. For this purpose, an *S. pombe* strain carrying a FLAG-tagged *rpb3* gene on its chromosome was constructed by gene replacement and RNA polymerase II complexes were isolated from cell extracts using anti-FLAG antibody resin. By controlling the conditions for extraction, we succeeded to separate two types of the RNA polymerase II complex, one containing non-phosphorylated and the other containing phosphorylated form of Rpb1. We assume that the non-phosphorylated and the phosphorylated forms represent the RNA polymerase II molecules non-engaged and engaged, respectively, in transcription, because the C-terminal domain (CTD) of the largest subunit (Rpb1) is not phosphorylated before promoter entry but phosphorylated during promoter clearance. The proteins that have been identified as associated with the non-engaged RNA polymerase II complexes include the general transcription factor TFIIF and the CTD-specific phosphatase Fcp1. This complex fraction could be separated into RNA polymerase II complex containing both TFIIF and Fcp1 and that containing only Fcp1. The *S. pombe* TFIIF herewith identified consisted of three species of subunit as in the case of *Saccharomyces cerevisiae* TFIIF, but different from that in higher eukaryotes. TFIIF from these two yeast strains contains an additional small subunit, Tfg3, which is also a component of other several transcriptional complexes. See Refs. 11 and 12 for related subjects.

(10) Search and Analysis of Transcription Factors Interacting with Rpb7 Subunit of *Schizosaccharomyces pombe* RNA Polymerase II

Hiroshi MITSUZAWA, Emi KANDA and Akira ISHIHAMA

Rpb7 is an RNA polymerase II subunit that is not shared by RNA polymerase I or III. Rpb7 associates with another RNA polymerase II-specific subunit, Rpb4, to form a subassembly dissociable from the core assembly consisting of the remaining 10 subunits. Rpb7 is required for accurate transcrip-

tion initiation *in vitro* from the core promoter but is unnecessary for RNA synthesis. These observations suggest that Rpb7 has a role in transcription regulation. To elucidate the function of Rpb7 in transcription by RNA polymerase II, we have used the *S. pombe* RNA polymerase II as a model. Using the yeast two-hybrid system, we have isolated a number of Rpb7-interacting proteins. One of the isolated clones encodes a protein that has homology to the *S. cerevisiae* Nrd1 protein. Nrd1 has been implicated in pre-mRNA processing because it interacts with the C-terminal domain (CTD) of the largest subunit (Rpb1) of RNA polymerase II and contains RS and RNA-binding motifs, which are often found in proteins involved in splicing. We found that, like the *S. pombe* counterparts, the *S. cerevisiae* Rpb7 interacts with Nrd1 in the two-hybrid system, indicating that the interaction has been conserved between *S. pombe* and *S. cerevisiae*. The result suggests that the interaction of Rpb7 with Nrd1 is not an artefact in the two-hybrid system but has physiological relevance. Thus our results suggest a novel role of Rpb7 in pre-mRNA splicing in addition to transcription initiation. See Refs. 5 and 15 for related subjects.

(11) Analysis of General Transcription Factor TFIID from *Schizosaccharomyces pombe*

Hiroshi MITSUZAWA, Hiroaki SEINO[1], Fumiaki YAMAO[1] and Akira ISHIHAMA
([1]Division of Muragenesis, Department of Molecular Genetics)

The general transcription factor TFIID plays a critical role in transcription initiation by RNA polymerase II. TFIID is a multiprotein complex comprising the TATA-binding protein (TBP) and multiple TBP-associated factors (TAFs). We have characterized two related TAFs in *S. pombe*, TAF72 and TAF73, which have homology to WD repeat-containing TAFs such as human hTAF100, *Drosophila* dTAF80, and *S. cerevisiae* yTAF90. We showed that TAF72 and TAF73 are present in the same complex with TBP and other TAFs, demonstrating that TAF72 and TAF73 are indeed TAFs. yTAF90 is also present in SAGA, a histone acetylase complex distinct from TFIID. This led us to test the possibility that TAF72 and TAF73 are shared by TFIID and other histone acetylase complexes. SPAC1952.05, a gene predicted by the *S. pombe* genome

sequencing project, encodes a protein that is 53% identical to *S.cerevisiae* Gcn5, the histone acetylase subunit of SAGA. We refer to this gene as *gcn5*$^+$ and examined whether its product Gcn5 is associated with TAF72 and TAF73. Co-immunoprecipitation experiments demonstrated that TAF72, but not TAF73, is associated with Gcn5. It thus seems that TAF72 is present in both TFIID and SAGA-like complexes while TAF73 is present only in TFIID. Interestingly, overexpression of TAF72 or TAF73 can suppress the cell cycle arrest in mitosis caused by mutations in the *ubcP4*$^+$ and *cut9*$^+$ genes. *ubcP4*$^+$ encodes a ubiquitin-conjugating enzyme whereas *cut9*$^+$ encodes a subunit of a ubiquitin-protein ligase complex known as anaphase-promoting complex. These results suggest that TAF72 and TAF73 may regulate the expression of genes involved in ubiquitin-dependent proteolysis during mitosis. Our study thus provides evidence for a possible role of WD repeat-containing TAFs in the expression of genes involved in progression through the M phase of the cell cycle. For details see Ref. 15.

(12) Differential Roles of vRNA and cRNA in Functional Modulation of Influenza Virus RNA Polymerase

Ayae HONDA, Atsushi ENDO[1] and Akira ISHIHAMA
([1]Daiichi Pharmaceutical Co., Exploratory Res. Lab., Tokyo)

The influenza virus RNA polymerase is involved in both transcription (vRNA-directed mRNA synthesis) and replication (vRNA-directed synthesis of complementary RNA (cRNA) and cRNA-directed vRNA synthesis). In transcription, the RNA polymerase catalyzes not only RNA synthesis but also the cleavage of capped host RNA to generate capped RNA primers and polyadenylation at the 3'-termini of mRNA. The RNA polymerase is composed of one molecule each of PB1, PB2 and PA. PB1 plays a central role in RNA polymerase assembly by providing the contact surfaces for both PB2 and PA. In the virus particles, this RNA polymerase is bound to a double-stranded region of viral RNA (vRNA) formed by base-pairing between its 5'- and 3'-termini. Since the RNA polymerase is tightly associated with vRNA, no purification method yielding large amounts of functional RNA polymerase has been established. For de-

tailed analysis of the structure-function relationship of each P protein, large amounts of functional RNA polymerase are required in a template-free form. We established the expression system of RNA polymerase by simultaneous infection of three kinds of the recombinant baculovirus insect cells, each expressinng PB1, PB2 or PA protein. For purification purpose of the 3P complex, the PA protein was expressed as a fusion with His_6 tag added at its N-terminus. The affinity-purified 3P complex showed the activities of capped RNA binding, capped RNA cleavage, v- and c-sense model RNA binding, model vRNA-directed and ApG-primed RNA synthesis, and polyadenylation of newly synthesized RNA. We conclude that a functional form of influenza virus RNA polymerase with the catalytic specificity of transcriptase is formed in baculovirus-infected insect cells. The capped RNA cleavage was observed in the presence of vRNA but not of cRNA, indicating that vRNA functions as a regulatory factor for the specificity control of viral RNA polymerase as well as a template for transcription.

Using the same set of recombinant baculoviruses, two kinds of binary 2P complex, PB1-PA and PB1-PB2, were isolated. Both 2P complexes showed partial activities of transcription and/or replication. See Refs. 3 and 4 for related subjects.

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

Ayae HONDA, Takuto OKAMOTO, Masako KAIDO and Akira ISHIHAMA

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

three viral P proteins can catalyze RNA synthesis in the absence of primers. We then proposed that an as yet unidentified host factor(s) is involved in the functional conversion of the RNA polymerase from transcriptase to replicase.

Attempts have been made to identify host proteins, which interact with each of the P proteins, using yeast two hybrid screening system. Several positive clones have been isolated for each P protein, and complate cDNAs for some of these putative PB1-, PB2- and PA-interacting host factors have been isolated. One of the PB1-interacting protein with the molecular mass of 54kDa (PB1c54) was overproduced in *E. coli*, and examined for possible influence on the functions of influenza virus RNA polymerase. Using anti-PB1c54 antibodies raised in rabbits, the intracellular localization of PB1c54 in both virus-infected and uninfected cells was analyzed by indirect immunofluorescent microscopy. After virus infection, the PB1c54 was found to colocalize with the viral RNA polymerase, indicating that PB1c54 interacts *in vivo* with the viral RNA polymerase.

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A-b. Division of Mutagenesis

(1) Comprehensive survey and functional analysis of ubiquitin-conjugating enzymes in *S. pombe*

Joon-Hyun PARK, Hiroaki SEINO and Fumiaki YAMAO

Ubiquitin-dependent proteolysis plays important roles in many biological processes such as cell cycle control, signal transduction, cell differentiation, and so on. The diversity and specificity of ubiquitination are based on the molecular heterogeneity of ubiquitin-conjugating enzymes (Ubc or E2) and ubiquitin-ligases (E3). Thirteen Ubc's has been identified and their functions has been characterized in budding yeast. In fission yeast, however, very little information about Ubc's has been accumulated. We, therefore, surveyed Ubc's to elucidate their functional diversity in fission yeast. In addition to four Ubc's

(UbcP1-P4) previously identified by us through screening by their enzymatic activities, other eight Ubc's (UbcP5-P12) and a non-canonical Ubc, mms2, have been found through homology search in the fission yeast genome data base. The functional analyses of Ubc's were carried out through disruption of their genes, showing that some genes are found essential for the cell growth while its ortholog in budding yeast are not. Some Ubc's in fission yeast have no structural homologue in budding yeast, and vice versa. Interestingly, phenotypic differences between the fission yeast Ubc's and their structural homologues in the budding yeast have been found in many cases, suggesting the importance of functional survey of fission yeast Ubc's.

We now focus on UbcP7 which functions together with Mms2 in repair of damaged DNA with the same epistasis to Rad6. Recently, by two hybrid screening, we identified new protein that interacts specifically with Mms2, dysfunction of which resulted in higher sensitivity to DNA damage. Functional analysis of this new repair protein with respect to UbcP7 and Mms2 are now undergoing.

(2) Ubiquitin-conjugating enzymes involved in mitotic cyclin degradation

Hiroaki SEINO and Fumiaki YAMAO

Biochemical analysis of mitotic cyclin ubiquitination using frog or clam egg extract showed that two ubiquitin conjugating enzymes, Ubc4 and Ubc-x/E2-C, work with ubiquitin ligase, APC (anaphase promoting complex), to ubiquitinate mitotic cyclin. However, functional differences between the two Ubc's has not yet been elucidated. We identified their homologues, ubcP4 and ubcP1/ubc4, respectively, in fission yeast. We found both of them were essential for cell viability. Cells depleted with either of these Ubc's exhibited mitotic defects with accumulation of mitotic cyclin. Even in G1 cells, mitotic cyclin was stabilized under depletion of one of them. Over-production of the each ubiquitin-conjugating enzyme was not able to rescue the defect of temperature-sensitive mutants of other one. These results indicate that the two Ubc's are involved in degradation of mitotic cyclin in non-redundant fashion.

Analysis of the functional relationship and differences between these two Ubc's in the mitotic cyclin destruction are now undergoing.

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

Tsutomu KISHI and Fumiaki YAMAO

SCF complexes, composed of Skp1, Cdc53 and one of the F-box proteins, have been implicated in the Cdc34-dependent ubiquitination in *Saccaharomyces cerevisiae*. We have found that 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 interacting with Skp1 through the F-box motif. Furthermore, we have found that Grr1 also interacts in vitro with phosphorylated Cln1 and Cln2. From these data, we have proposed that Grr1 is required for degradation of Cln2 through linking phosphorylated Cln2 to Skp1 in SCF^{Grr1} complex.

To isolate additional genes that are required for the degradation of Cln2, I have employed a genetic screen to select for stable Cln2-LacZ fusion protein. One of the mutants dog75 (Destabilization of G1 cyclin) was characterized. In dog75 mutants, Cln2 was stabilized at high temperatures. Ubiquitination of Cln2 was decreased in the mutants. However, Gic2 protein, another target ubiquitinated by SCF^{Grr1} complex, was not stabilized in the mutant. Sequence analysis revealed that the gene codes for a protein that has a homology with protein kinases.

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A-c. Division of Nucleic Acid Chemistry

(1) Host proteins involved in transcription of Sendai virus (SeV) genome

Kiyohisa MIZUMOTO (School of Pharmaceutical Sciences)

Cellular proteins (host factors) may play key roles in transcription of Sendai virus (SeV) genome. We have previously shown that the host factor activity, which stimulates *in vitro* mRNA synthesis of SeV, are separated into at least tree complementary fractions. Two of them were identified as a cytoskeletal protein, tubulin (Mizumoto *et al., J. Biochem.* **117**, 527, 1995) and a glycolytic enzyme, phosphoglycerate kinase (PGK) (Ogino *et al., J. Biol. Chem.* **274**, 35999, 1999). Here, the third factor activity was further resolved into two complementary fractions, and one of them was purified to an almost single polypeptide chain with an apparent molecular weight of 52,000 (p52) (Ogino *et al. Biochem. Biophys. Res. Commun.,* **285**, 447, 2001). From biochemical and immunological analyses, p52 was identified as a glycolytic enzyme, enolase. Recombinant human α-enolase, as did p52, acted synergistically to stimulate SeV mRNA synthesis with other three host factors. West-western blot analysis demonstrated that tubulin specifically binds enolase as well as PGK, suggesting that these two glycolytic enzymes stimulate SeV transcription through their interaction with tubulin which has been integrated into a transcription initiation complex (Takagi *et al., J. Biochem.* **118**, 390 1995).

(2) Transcriptional Regulation during the stationary growth phase in escherichia coli

Kan TANAKA

(2)-a. Structure and function of a sigma factor RpoS (σ^{38}, σ^{S})

Escherichia coli can grow in the presence of several nutrients, including organic carbon, nitrogen, sulfur and phosphate. However in most natural envi-

ronments, they always face shortage of some nutrients, and are found as resting cells. Therefore, survival strategy in the starved conditions must have been extremely important during the evolution of E. coli. We are trying to understand the characteristics of transcriptional apparatus in starved bacteria.

RNA polymerase of E. coli is composed of a core enzyme and one of seven sigma factors, and substitution of sigma subunits modulates promoter recognition specificity of RNA polymerase. A major portion of E. coli promoters contains 'TATAAT' type-10 consensus sequence, and this type promoters are recognized by two forms of RNA polymerase containing either σ^{70} or σ^{38}. σ^{70} is the principal sigma subunit, and essential for the cell viability. The expression of σ^{38} is induced at the stationary growth phase, and required for the induction of genes specific for this growth phase. Although the difference of the promoter specificity is obvious in vivo, both RNA polymerases could recognize many promoters in common in vitro. Thus, the molecular basis for the recognition specificity has not been well established.

During the stationary phase, cellular physiology changes in many aspects compared with the exponential growth phase. As for the internal condition, solutes such as potassium and glutamate are considered to accumulate. In a previous study, Kusano et al. as well as us studied the effects of ionic solvents on the activities of E σ^{70} and E σ^{38} in vitro, and found that E σ^{38} is more resistant to (or rather activated by) potassium glutamate at 150 mM or above. Thus, E σ^{38} appeared to be an RNA polymerase suitable for high ionic concentrations. We found a conserved 16-amino acid sequence specific for σ^{38} proteins at the C-terminus, and purified a σ^{38} variant lacking this region. An RNA polymerase holoenzyme containing this mutant sigma was found to be highly sensitive to the high salt concentration in vivo, and therefore, we concluded that this element was esseintial for the transcription at these physiological conditions (Ohnuma et al., *J. Bacteriol.* **182**, 4628-4631, 2000).

(2)-b. Gene silencing at the stationary growth phase

Many promoters that are actively transcribed during the exponential phase are silenced at the onset of the stationary growth phase. These gene repres-

sion mechanisms have been extensively analyzed for rrnBP1 promoter, where ppGpp, H-NS and intracellular NTP concentration are deeply involved. We analyzed the activity of the lacUV5 promoter, a consensus promoter, during the growth phase transition, and found that this promoter is shut off at the onset of the stationary phase. This shut off was dependent on the nucleoid protein H-NS, and numerous consensus promoters were considered to be silenced by the similar mechanism after the growth.

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B. Department of Cell Genetics

B-a. Division of Cytogenetics

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

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

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

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

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

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

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

strates. The activity was dependent on Manganese, but not ATP.

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

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

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

(2) Recognition of G-DNA Structure at the Yeast Telomere Ends and its Dissolution by Mre11 of *S. cerevisiae*

Tsutomu OHTA, Shigeo TANAKA, Daisuke TATSUDA, Hiroyuki OSHIUMI and Tomoko OGAWA.

Telomere locates at the ends of eukaryotic chromosomes and plays important roles in cell growth. It contains G-rich sequence repeats whose synthesis is mediated by a teromerease. In *Saccaromyces cerevisiae*, many genes are known to be involved in the maintenance of the length and mutations in these genes result in the shortening of the length and cell senescence. *MRE11*, which is involved in homologous recombination and repair of DNA damage, is responsible for the maintenance. The protein has two each of binding sites to DNA and to Rad50. Using various *mre11* mutants that are deleted either one or both DNA binding sites, site-A and site-B, we found that Mre11 binds at site-A to the telomere ends. The G-rich sequence of single-strand tails of telomeres is suggested to form the structure called G-DNA made by pairing of non-canonical guanine bases. We found that the site-A of Mre11 recognizes the DNA structure, but not its primary sequence. The binding of Rad50 to Mre11 provably controls the way of Mre11 binding at site-B to the G-structure at the telomere ends. We also show that Mre11 dissolved the G-structure and either one of DNA binding sites can serve for the reaction.

It has been reported that G-rich tails of more than 30 nucleotides at the 3' ends of telomeres appear in the wild-type cells only at the late S-phase of the cell-cycle. Similar tails were found in *mre11-7B* from the late S to G2 phases, whereas in *mre11*Δ or *mre11-6A*, the tails were almost undetectable throughout the cell cycle. We then examined whether Mre11 binds to the telomere in vivo using chromatin immuno-precipitation assay. In the wild-type and *mre11-7B* cells, the telomere DNA was found only in the S-phase, whereas in *mre11-6A* cells, very small amount of telomere DNA was detected throughout the cell-cycle. The ability of mutant Mre11 proteins to bind the telomere roughly correlates to the presence of the single-strand tails in corresponding mutant strains. Taken together with similar results of the telomere length obatined from the *hdf1*Δ (*yku70*Δ) strain, the results imply that the binding of Mre11

protects the tails.

In conclusion, the G-rich sequence at the end of telomere forms the G-DNA structure. Mre11 recognizes the structure and its binding protects the tails. Subsequently, Mre11 dissolves the structure for further elongation of telomere. The modes of binding of Mre11 to DNA show a selective role in the process. Thus, Mre11 plays critical roles in maintenance and elongation of telomere length. Ref. [3]

(3) Domain Structure and Dynamics in the Helical Filaments Formed by RecA and Rad51 on DNA

Xiong YU, Steven A. JACOBS, Stephen C. WEST, Tomoko OGAWA and Edward H. EGELMAN.

Both the bacterial RecA protein and the eukaryotic Rad51 protein form helical nucleoprotein filaments on DNA that catalyze strand transfer between two homologous DNA molecules. However, only the ATP-binding cores of these proteins have been conserved, and this same core is also found within helicases and the F1-ATPase. The C-terminal domain of the RecA protein forms lobes within the helical RecA filament. However, the Rad51 proteins do not have the C-terminal domain found in RecA, but have an N-terminal extension that is absent in the RecA protein. Both the RecA C-terminal domain and the Rad51 N-terminal domain bind DNA. We have used electron microscopy to show that the lobes of the yeast and human Rad51 filaments appear to be formed by N-terminal domains. While the lobes of the RecA subunit define a unique polarity for the filament formed on single stranded DNA, human Rad51 filaments appear to polymerize on single stranded DNA with a random polarity. In addition, these lobes are conformationally flexible in both RecA and Rad51. Within RecA filaments, the change between the "active" and "inactive" states appears to mainly involve a large movement of the C-terminal lobe. The N-terminal domain of Rad51 and the C-terminal domain of RecA may have arisen from convergent evolution to play similar roles in the filaments. Ref. [4]

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B-b. Division of Microbial Genetics

(1) Sld3, which Interacts with Cdc45 (Sld4), functions for chromosomal DNA Replication in *Saccharomyces cerevisiae*

Yoichiro KAMIMURA 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 forms a complex with essential DNA polymerase ε (Pol ε), is required for association of DNA polymerases with origins in DNA replication and for the control of the S-phase checkpoint (ref.1). To identify factors interacting with Dpb11, we have isolated 10 *sld* (synthetic lethality with *dpb11-1*) mutations which fall into 6 complementation groups. We have cloned *SLD1~6* and found that *SLD1* is identical to *DPB3* encoding the third subunit of Pol ε, *SLD4* is identical to *CDC45* required for the initiation and elongation step of chromosomal DNA replication and *SLD6* is identical to *RAD53* which has crucial role for the cell cycle checkpoint. The *SLD2, 3, 5* genes were new genes essential for the cell growth (Kamimura et al., Mol. Cell. Biol., 18, 6102-6109, 1998). We characterized *SLD3* encoding a 77kDa protein.

In order to characterize the *SLD3* gene, we isolated thermosensitive alleles of the *SLD3* gene. We isolated the *CDC45* gene as a multicopy suppressor of *SLD3* thermosensitive mutations. Conversely, high copy *SLD3* suppressed the thermosensitive growth defect of *cdc45* mutation. Using cross-linker reagent, the interaction between Sld3 and Cdc45 was detected throughout the cell cycle. Two-hybrid assay suggests that the Sld3 protein encoded by a thermosensitive allele reduced the efficiency of complex formation with Cdc45. Consistent with complex formation between Sld3 and Cdc45, Sld3 and Cdc45 associated simultaneously with replication origins in the chromatin immunoprecipitation (CHIP) assay: both proteins associated with early-firing origins in G1 phase and with late-firing origins in late S phase. Moreover, the origin

association of Sld3 and Cdc45 were mutually dependent. Since the thermosensitive *sld3* mutation conferred defect of DNA replication at the restrictive temperature, these results suggest that the Sld3-Cdc45 complex associates with replication origins and its association is required for DNA replication. Furthermore, at the restrictive temperature in *sld3-5* cells, RF-A, a single-strand DNA binding protein, did not associate with origins. Therefore, the origin association of Sld3-Cdc45 complex is prerequisite for origin unwinding in the initiation of DNA replication.

(2) Phosphorylation of Sld2 by cyclin-dependent protein kinase

Hiroshi MASUMOTO and Hiroyuki ARAKI

Cyclin-dependent protein kinases (Cdk) are key enzymes which control the cell cycle. For initiation of DNA replication, S-phase specific Cdk (S-Cdk) is required while real target of S-Cdk is obscure.

Since Sld2, which form a complex with Dpb11 (Kamimura et al., Mol. Cell. Biol., **18**, 6102-6109, 1998), has six conserved amino acid sequences for Cdk-phosphorylation, we examined whether Sld2 is phosphorylated by S-Cdk. As expected, in SDS-polyacrylamide gel Sld2 migrated slower in S phase than in G1 phase. Phosphatase treatment converted slow mobility species of Sld2 obtained from S phase to fast mobility specie as obtained from G1 phase, suggesting that Sld2 is phosphorylated in S phase. When we expressed Sic1, an inhibitor of S-Cdk, phosphorylated form of Sld2 did not appear. Thus, phosphorylation of Sld2 in S phase depends on S-Cdk activity. Next, we substituted the serine or threonine residues in six S-Cdk phosphorylation sites by alanine. The mutant protein containing six alanine substitutions did not support cell growth but also onset of S phase while three combinations of five alanine substitutions supported cell growth. Furthermore, slow mobility species of the mutant protein containing six alanine substitutions did not appear. It suggests that S-Cdk dependent phosphorylation of Sld2 is essential for DNA replication.

Since Sld2 and Dpb11 form a complex, we investigated their complex formation by tagging them and using co-immunoprecipitation assay. Co-immuno-

precipitation of Sld2 and Dpb11 was detected in S phase but not in G1 phase and Dpb11 immunoprecipitated only with phosphorylated form of Sld2. When we expressed wild-type and six-alanine substitution mutant proteins of Sld2 in the *sld2* mutant cells defective in complex formation between Sld2 and Dpb11, complex formation between the mutant protein and Dpb11 did not be detected while the wild type protein coprecipitated with Dpb11. These results suggest that phosphorylation of Sld2 is required for complex formation between Dpb11 and Sld2.

We previously showed that complex formation between Dpb11 and Sld2 is required for early step of DNA replication (Kamimura et al., Mol. Cell. Biol., **18**, 6102-6109, 1998), and Dpb11 associates with replication origins in S phase (ref,1). Using CHIP assay, we found that Dpb11 and Sld2 associate simultaneously with replication origins and their origin associations are mutually dependent. Because Dpb11 is required for loading of DNA polymerases to origins, this finding suggests that the Dpb11-Sld2 complex associates with origins and this association is required for DNA polymerase loading.

Taken all together, it seems likely that S-Cdk regulates complex formation between Sld2 and Dpb11 and consequently loading of DNA polymerases to replication origins.

(3) Functional analysis of sld5 and Psf1

Yuko TAKAYAMA, Yoichiro KAMIMURA and Hiroyuki ARAKI

The *SLD5* gene isolated as a synthetic lethality with *dpb11-1* (see section (1)) encodes an essential 34kDa protein. To elucidate the function of Sld5, we isolated the *PSF1* (Partner of *SLD5*) gene as a multicopy suppressor of the thermosensitive *sld5-12* mutation. *PSF1* encodes a 24kDa protein which is essential for cell growth. The protein-level of Psf1 was roughly constant during the cell. Co-immunoprecipitation assay revealed that the Sld5 and Psf1 proteins coexist in the same complex throughout the cell cycle. We also isolated a thermosensitive *psf1-1* mutation by the plasmid shuffling method. High-copy *SLD5* suppresses thermosensitive growth of *psf1-1*, suggesting that the *psf1-1* mutation is defective in the interaction between Sld5 and Psf1. Actually, in

psf1-1 cells, co-existence of Sld5 and Psf1 in the complex is hardly detected. Moreover, when we prepared the extract from *psf1-1* cells harboring high copy *SLD5*, a complex containing Psf1 and Sld5 was detected.

Psf1-1 mutant cells arrested with a dumbbell shape at the restrictive temperature as did *sld5-12* cells. FACS analysis showed that *psf1-1* cells are defective in early step of DNA replication. Indirect immunofluorescent assay revealed that the Psf1 protein is localized in nucleus throughout cell cycle, suggesting that Psf1 plays a role in the place close to chromatin DNA. As expected, in CHIP assay, the Psf1 and Sld5 proteins associated simultaneously with replication origin in S phase. It strongly suggests that both proteins function directly for DNA replication. Moreover, the Psf1 association with a late-firing origin was significantly delayed behind the association with early-firing origins, suggesting that Psf1 associates with replication origins immediately before firing. Furthermore, in *psf1-1* cells, Pol ε did not associate with replication origin. Taken all together, the complex containing Psf1 and Sld5 functions directly in DNA replication, before loading of DNA polymerase.

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B-c. Division of Cytoplasmic Genetics

(1) Derivation of the relationship between neutral mutation and fixation solely from the definition of selective neutrality

Jun-ichi TOMIZAWA

A mutation whose fixation is independent of natural selection is termed a neutral mutation. Therefore selective neutrality of a mutation can be defined

by independence of its fixation from natural selection. By the population genetic approach, Kimura [Kimura, M. (1962) *Genetics* **47**, 713-719] predicted that the probability of fixation of a neutral mutation (u) is equal to the frequency of the new allele at the start (p). The approach traced the temporal sequence of the fixation process, and the prediction was obtained by assuming the selective equality of neutral mutant and wild-type alleles during the fixation process. However, because the definition concerns only mutation and fixation, an ideal approach should deal only with these and not with the intervening process of fixation. The approach begins by analysis of the state of fixation of a neutral mutation, and its relation with the initial state is deduced logically from the definition. The approach shows that the equality of the alleles during fixation process is equivalent to the equality of probability of their ultimate fixation in a steady state. Both are manifestations of the definition of selective neutrality. Then, solely from this dual nature of the definition, the equality between u and p is derived directly. Therefore, the definition of selective neutrality can be represented by the equation $u = p$.

The use of the assumption based on the future probability of fixation of alleles in characterizing their behavior in the process of fixation by Kimura caused tautology.

(2) Studies on behavioral disorders of knockout mice

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

In this year, we found that the analgesic effects of mu-opioid-receptor agonist (morphine) and kappa-opioid-receptor agonist (U50488) were reduced in *weaver* mutant mice having mutant G-protein activated inwardly rectifying potassium channels (GIRK channels) in the brain, suggesting that the intracellular signal pathway via GIRK channels is important for opioid-induced analgesia.

We also investigated the molecular mechanisms underlying reduced opioid-induced analgesia in CXBK mice that were considered to lack mu-opioid receptor. We found that an abnormal non-coding region of mu-opioid-receptor mRNA causes the reduced opioid-induced analgesia in CXBK mice.

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C. Department of Developmental Genetics

C-a. Division of Developmental Genetics

(1) A common basis for formation of Drosophila sensory organs

Nao NIWA, Masataka OKABE and Yasushi HIROMI

Insect sensory organs such as eye and antenna are specialized for particular functions, and form at specific positions within each segment. Since structurally diverse segments are considered to have evolved from repeated units in a worm-like ancestor, organogenesis of segment-specific sensory organs may share a molecular basis that is common to all segments. In Drosophila compound eye formation, the *eyeless* gene has been identified as a master control gene, whose mis-expression can induce ectopic eyes in several regions of the body. Using the ectopic eye formation by *eyeless* mis-expression as an experimental paradigm, we visualized spatial and temporal conditions for organ formation in segments that normally do not produce an eye.

Competence for ectopic eye formation by *eyeless* was clearly restricted, and required Dpp and ecdysone signals. Similar constraints were found for the formation of the endogenous eye and also for other sensory organs, such as auditory receptors (Johnston's organ) and tension receptors (chordotonal organ). These signals are integrated and result in the expression of a common proneural gene *atonal* in each sensory organ precursor. Our results define a molecular pathway for a common neurogenic activity, and suggest that segment-specific sensory organs originated from an ancient prototype organ.

(2) The relationship between sensory organ identity and positional information in Drosophila

Masataka OKABE and Yasushi HIROMI

The embryonic peripheral nervous system has three major types of sensory

organs; mechanosensory organ, chemosensory organ and chordotonal organ, each generated from respective sensory organ precursors that form at specific positions within each segment. The identity of sensory organs is determined at the precursor stage, by instructive transcription factors such as a homeodomain protein Cut and a Paired-type transcription factor Pox-neuro (Pox-n). We are analyzing the regulation of *cut* and *pox-n* expression to identify molecules that link positional information and the organ identity.

We have analyzed *cis*-regulatory elements of *cut* and *pox-n* using transgenic flies carrying reporter gene constructs. We identified a 120bp genomic fragment of the *cut* gene at 30kb upstream from its transcription initiation site as a *cis*-element sufficient for expression in mechano- and chemosensory precursors. We also found that a *cis*-regulatory element of *pox-n* gene for expression in chemosensory precursors is located within a 4.2kb genomic fragment upstream of its transcription initiation site. In future we plan to identify *trans*-regulatory factors that act through these *cis*-regulatory elements. A combination of *trans*-acting factors is likely to comprise a network of positional information that direct specific gene expression at particular positions.

(3) Translational repression determines a neuronal potential in Drosophila asymmetric cell division

Masataka OKABE and Yasushi HIROMI

Asymmetric cell division is a fundamental strategy for generation of cellular diversity during animal development. Daughter cells manifest their asymmetry in their differential gene expression. The importance of transcriptional regulation in this process has been the focus of many studies, but cell-type specific "translational" regulation has held a more minor role. In Drosophila sensory organ development, Notch-signaling directs the asymmetry between neuronal and non-neuronal lineage, and a zinc finger transcriptional repressor Tramtrack69 (TTK69) acts downstream of Notch as a determinant of non-neuronal identity. We found that repression of TTK69 protein expression in the neuronal lineage occurs translationally rather than transcriptionally. This translational repression was achieved by a direct interaction between *cis*-

acting sequences in the 3' untranslated region of *ttk69* mRNA and its trans-acting repressor, the RNA binding protein Musashi (MSI). Although *msi* can act downstream of Notch, Notch-signaling does not affect MSI expression. Thus, Notch-signaling is likely to regulate MSI activity rather than its expression. Our results define cell-type specific translational control of *ttk69* by MSI as a novel downstream event of Notch-signaling in asymmetric cell division.

(4) Transcriptional regulatory mechanism of a nuclear receptor, Seven-up

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

Neuronal subtype selection within the Drosophila eye depends on the nuclear receptor Seven-up. Seven-up is expressed in only four of the eight photoreceptor neurons in each ommatidium, and its loss of function results in the transformation of these cells into another photoreceptor subtype. Furthermore, ectopic expression of the Seven-up ligand binding domain causes several cell fate changes in the developing eye, depending on the cell types and their developmental stages. However, little is known about the molecular mechanisms of Seven-up action, such as the transcriptional regulatory mechanism or its downstream target genes, that underlie regulatory effects of Seven-up on cell fates. To learn more about the role of Seven-up in gene regulation, we have initiated genetic analyses of two proteins, the p52 subunit of TFIIH and a novel SAM-domain protein Samuel, that interact with the ligand binding domain of Seven-up in yeast cells. We have identified loss of function alleles of *p52* and *samuel*, and have shown that they are both essential for viability. These proteins may play a role in transcriptional regulation by Seven-up. We are also searching for the direct target genes of Seven-up by using differential display techniques. Since Seven-up ligand binding domain has been shown to have a transcriptional repression activity in the developing eye, we are examining genes whose expression levels are upregulated when Seven-up function is removed.

(5) Control of glial differentiation by the homeodomain protein REPO

Yoshihiro YUASA, Masataka OKABE and Yasushi HIROMI

In Drosophila, glial determination of all neuroectodermally derived glia depends on the transcription factor Glial cells missing (GCM), which serves as a binary switch between neuronal and glial cell fates. Since the expression of GCM is transient, other factors must be responsible for the terminal differentiation of glia. Three transcription factors, Reversed Polarity (REPO), Tramtrack69 (TTK69), and PointedP1 (PNTP1), are induced by GCM in glial cells. REPO is a paired-like homeodomain protein, expressed exclusively in glial cells. Mutant phenotypes of *repo* suggest that REPO is required for the migration and differentiation of embryonic glial cells. In order to understand how REPO functions in glial terminal differentiation, we have analyzed the mechanism of gene regulation by REPO. We showed that REPO can act as a transcriptional activator through the CAATTA motif in glial cells, and defined three reporter genes whose expression depends on REPO function. In different types of glial cells, REPO can act alone, or cooperate with either TTK69 or PNTP1 to regulate different target genes. Since TTK69 and PNTP1 are expressed only in a subset of cells that express REPO, their cooperation with REPO may contribute to the diversity of glial cell types. In addition, we found that REPO is also necessary to suppress neuronal development, cooperating with TTK69. We propose that REPO plays a key role in glial development by linking glial determination and their diversification.

(6) Sprouty regulates photoreceptor neuronal number through positive and negative effects on induction

Masaki IWANAMI and Yasushi HIROMI

Neuronal differentiation of photoreceptor cells in the Drosophila eye is triggered by a secreted inducer, Spitz EGF, that results in activation of the ras/MAPK pathway in the induced cells. The result of induction is remarkably constant; every ommatidia has precisely eight photoreceptor neurons located at stereotyped positions within the ommatidium. To achieve such constancy,

the induction process must be tightly regulated in the inducing cells and the induced cells. Sprouty is an intracellular molecule that negatively regulates ras signaling. Sprouty is required cell-autonomously in lens-secreting cone cells to prevent these cells from assuming the neuronal fate. However, *sprouty* is also expressed in presumptive photoreceptor neurons, in particular R2/R5/R7 cells. This cell-type specificity of *sprouty* expression is achieved by the repression of *sprouty* transcription by the nuclear receptor Seven-up, in R3/R4/R1/R6. Loss of *sprouty* function in R2/R5 results in the up-regulation of Argos expression, whose transcription is induced by the ras signal that Sprouty inhibits. Since Argos is a non-autonomous antagonist of the receptor for the inducing signal (EGFR), Sprouty also positively regulates neuronal induction through controlling *argos* expression. The dual function of Sprouty is likely to have an important role in ensuring that the induction by a secreted signal generates a constant outcome.

(7) Systematic identification of peptide signaling molecules in *Hydra*

Toshitaka FUJISAWA, Masayuki HATTA, Hiroshi SHIMIZU, Seungshic YUM, Naoe HARAFUJI[1], Osamu KOIZUMI[2], Yoshitaka KOBAYAKAWA[3], Fumihiro MORISHITA[4] and Osamu MATSUSHIMA[4] ([1]The Grad. Univ. for Advance Studies, [2]Fukuoka Women's Univ, [3]Kyushu Univ, [4]Hiroshima Univ)

We have continued our efforts to systematically screen and characterize peptide signallimg molecules from *Hydra magnipapillata*. This year we have isolated over 100 peptides and determined amino acid sequences of about 50 of them. Five genes encoding peptides were cloned and characterized. The detailed characterization was carried out on the following two peptides described below.

(8) Morphogenetic peptides, Hym-323 and Hym-346 that are involved in foot formation

Naoe HARAFUJI, Toshio TAKAHASHI, Hiroshi SHIMIZU, Masayuki HATTA and Toshitaka FUJISAWA

It is generally thought that *Hydra* patterning is regulated primarily by small diffusible substances called morphogens, We have found two morphogen candidates, Hym-323 and Hym-346 that are involved in foot formation. Both peptides are derived from epithelial cells and thus are termed epitheliopeptides. They are different in amino acid sequences and coded by two independent genes. When each of them is exogenously added to hydra, it alters the positional value and widens the foot area thus favoring foot formation. In situ hybridization and/or immunostaining analyses in intact hydra showed that *Hym-323* was expressed both in the ectedermal and endodermal epithelial cells throughout the body except for the basal disk, whereas *Hym-346* was expressed in the foot endoderm. However, *Hym-346* was also expressed in the endoderm of the tentacle bases, suggesting its involvement in tentacle formation. During foot regeneration *Hym-323* continued to be expressed until the complete foot formation and then ceased. This suggests that *Hym-323* is required only for foot formation but not for its maintenance. In contrast, *Hym-346* started its expression early and continued thereafter, suggesting that *Hym-346* is required for both foot formation and its maintenance.

(9) Universal occurrence of the vasa-related genes among metazoans and their germline expression in *Hydra*

Kazufumi MOCHIZUKI, Chiemi FUJISAWA and Toshitaka FUJISAWA

The *vasa* (*vas*)-related genes are members of the DEAD box protein family and are involved in germ cell formation in higher metazoans. In the present study, we cloned the *vas*-related genes as well as the *PL10*-related genes, other members of the DEAD box protein family, from lower metazoans: sponge, *Hydra* and planaria. The phylogenetic analysis suggested that the *vas*-related genes arose by duplication of a *PL10*-related gene before the appearance of sponges but after the diversion of fungi and plants. The *vas*-related genes in *Hydra*, *Cnvas1* and *Cnvas2* were strongly expressed in germline cells and less strongly expressed in multipotent interstitial stem cells and ectodermal epithelial cells. These results suggest that the *vas*-related genes occur universally among metazoans and that their expression in germline cells was estab-

lished at least before cnidarian evolution.

(10) Digestive movements of hydra similar to peristalsis and mass peristalsis in vertebrates

Hiroshi SHIMIZU and Toshitaka FUJISAWA

Digestive movements in vertebrates' intestine such as peristalsis occur as the concerted contraction of longitudinally and circumferentially running muscle processes under the control by myenteric plexus lying between them. Although very primitive, gastrovascular cavity of hydra has structural similarities to the intestine such as pattern of muscle processes running and diffuse nerve net between. Despite these, hydra's digestive process is thought to be a static event. In this study we obtained evidence that behaviors similar to peristalsis and mass peristalsis occur in digestive process of hydra. The peristalsis pushed the contents forward in the cavity whereas mass peristalsis pushed them toward the mouth resulting in defecation. Tissue excised from an animal also underwent the two movements demonstrating that body column tissue has endogeneous activity of these movements. Interestingly, animals having no nerve cells showed a weak peristaltic movement suggesting a formerly unknown non-neuronal mechanism of peristalsis. These observations suggest that hydra gastrovascular cavity is, as to dynamic movements, functionally equivalent to vertebrates' intestine and that the diffuse nerve net having no ganglia distributed in the body column controls the movements as enteric nervous system.

(11) Metamorphosis regulation of reef-building corals by peptide hormones

Masayuki HATTA and Toshitaka FUJISAWA

Among a number of peptides isolated from hydra, a family of peptides with the GLWa motif in the C-ternimus have the metamorphosis-inducing activity for marine hydrozoans. We tested biological activities of those peptides on reef-building corals, and found that one of the GLWa family, Hym-248, can

induce metamorphosis of coral larvae in the genus *Acropora*. It is suggested that the GLWa neropeptides act as internal mediators to convert external cues and to trigger metamorphosis hormonally, and this mechanism is conserved in cnidarians. In addition, this finding provides a possible technique to turn coral seedlings production into reality, for the first time in the long history of the reef restoration research.

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C-b. Division of Gene Expression

(1) MBF2 is a tissue- and stage-specific coactivator that is regulated at the step of nuclear transport in the silkworm *Bombyx mori*

Qing-Xin LIU, Hitoshi UEDA and Susumu HIROSE

Coativators MBF1 and MBF2 mediate BmFTZ-F1-dependent transcriptional activation *in vitro* by interconnecting BmFTZ-F1, TATA binding protein TBP, and TFIIA. Here, we analyzed temporal and spatial expression patterns of MBF2 during embryonic and larval development of the silkworm *Bombyx mori*. MBF2 was detected in unfertilized eggs and embryos until stage 26. In stage 22 embryos, MBF1, MBF2 and BmFTZ-F1 colocalize in neural cells. During the larval stage, MBF2 was not expressed in the fat body and trachea. In the silk gland, MBF2 mRNA was constitutively expressed, but MBF2 protein appeared in the period between the second day and the molting D3 stage in both the third and fourth instars, and then disappeared. MBF2 was also detected on the second and third days of the fifth instar. Immunostaining during the fourth molt showed that MBF1, MBF2 and BmFTZ-F1 localize in the nucleus only at the D3 stage, while the two cofactors are present in the cytoplasm at other stages. Immunoprecipitation experiments suggested that MBF1, MBF2 and BmFTZ-F1 form a complex at the D3 stage. Transient expression of these factors in Schneider cell line 2 revaled that MBF1 and MBF2 localize to the nucleus and enhance BmFTZ-F1-dependent transcription only when all three factors are present. These data illustrate the functional regulation of MBF1 and MBF2 at the step of nuclear transport and implicate MBF2 in tissue- and stage-specific transcription. For details, see Ref. 1.

(2) The conserved nuclear receptor Ftz-F1 is required for embryogenesis, molting and reproduction in *Caenorhabditis elegans*

Masako ASAHINA[1,2], Takeshi ISHIHARA[3], Marek JINDRA[1,4], Yuji KOHARA[5], Isao KATSURA[3] and Susumu HIROSE[1] ([1]Depertment of Developmental Genetics, [2]Institute of Parasitology, [3]Laboratory of Multicellular Organization, [4]Institute

of Entomology, Czech Academy of Science, Ceske Budejovice, 37005 Czech Republic, [5]Laboratory of Genome Biology, National Institute of Genetics, Mishima, Shizuoka-ken 411-8540, Japan)

Nuclear receptors are essential players in the development of all metazoans. The nematode *Caenorhabditis elegans* possesses more than 200 putative nuclear receptor genes, several-fold the number known in any other organism. Very few of these transcription factors are conserved with components of the steroid response pathways in vertebrates and arthropods. Ftz-F1, one of the evolutionarily oldest nuclear receptor types, is required for steroidogenesis and sexual differentiation in mice and for segmentation and metamorphosis in *Drosophila*.

We employ two complementary approaches, direct mutagenesis and RNA interference, to explore the role of *nhr-25*, a *C. elegans* ortholog of Ftz-F1. Deletion mutants show that *nhr-25* is essential for embryogenesis. RNA interference reveals additional requirements throughout the postembryonic life, namely in molting and differentiation of the gonad and vulva. All these defects are consistent with the *nhr-25* expression pattern, determined by *in situ* hybridization and GFP reporter activity.

Our data link the *C. elegans* Ftz-F1 ortholog with a number of developmental processes. Significantly, its role in the periodical replacement of cuticle (molting) appears to be evolutionarily shared with insects and thus supports the monophyletic origin of molting. For details, see Ref. 2.

(3) Temporally restricted expression of transcription factor β FTZF1: significance for embryogenesis, molting and metamorphosis in *Drosophila melanogaster*

Masa-aki YAMADA, Takehide MURATA, Susumu HIROSE, Giovanni LAVORGNA[1], Emiko SUZUKI[2,3] and Hitoshi UEDA ([1]Laboratory of Morecular Cell Biology, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892-4255, USA, [2]Department of Fine Morphology, Institute of Medical Science, University of Tokyo, 4-6-1. Shirokanedai, Minato-ku, Tokyo 108-8639, Japan, [3]CREST, Japan Science and Technology Corporation)

FTZ-F1, a member of the nuclear receptor superfamily, has been implicated in the activation of the segmentation gene *fushi tarazu* during early embruogenesis of *Drosophila melanogaster*. We found that an isoform of FTZ-F1, β FTZ-F1, is expressed in the nuclei of almost all tissues slightly before the first and second larval ecdysis and before pupation. Severely affected *ftz-f1* mutants display an embryonic lethal phenotype, but can be rescued by ectopic expression of β FTZ-F1 during the period of endogenous β FTZ-F1 expression in the wild type. The resulting larvae are not able to molt, but this activity is rescued again by forced expression of β FTZ-F1, allowing progression to the next larval instar stage. On the other hand, premature expression of β FTZ-F1 in wild-type larvae at mid-first instar or mid-second instar stages causes defects in the molting process. Sensitive periods were found to be around the time of peak ecdysteroid levels and slightly before the start of endogenous β FTZ-F1 expression. A hypomorphic *ftz-f1* mutant that arrests in the prepupal stage can also be rescued by ectopic, time-specific expression of β FTZ-F1. Failure of salibary gland histolysis, one of the phenotypes of the *ftz-f1* mutant, is rescued by forced expression of the *ftz-f1* downstream gene *BR-C* during the late prepupal period. These results suggest that β FTZ-Z1 regulates genes associated with ecdysis and metamorphosis, and that the exact timing of its action in the ecdysone-induced gene cascade is important for proper development. For details, see Ref. 3.

(4) *Cryptosporiium parvum*: Functional complementation of a parasite transcriptional coactivator CpMBF1 in yeast

Guan ZHU[1], Michael J. LAGIER[1], Susumu HIROSE and Janet S. KEITHLY[1]
([1]Wadsworth Center, New York State Department of Health, P. O. Box 22002, Albany, New York 12201-2002)

We report here the identification of a novel multiprotein bridging factor type 1 from the apicomplexan *Cryptosporidium parvum* (CpMBF1), one of the opportunistic pathogens in AIDS patients. In slime molds, insects, and humans, MBF1-regulated systems have been associated with cell differentiation, which indicates that CpMBF1 could be responsible for the activation of similar sys-

tems in *C. parvum* during its complex life cycle. Because of the difficulties and high cost in obtaining sufficient and purified *C. parvum* material for molecular and biochemical analyses, well-characterized yeast genetic systems may be useful for investigating the functions of *C. parvum* genes.

In this study, the function of CpMBF1 as an interconnecting element between a DNA-binding regulator and TATA-box-binding protein (TBP) was confirmed using a yeast complementation assay. Under conditions of histidine starvation, an MBF1-deficient strain of *Saccharomyces cerevisiae* was unableto activate the *HIS3* gene, which encodes imidazoleglycerol-phosphate dehydratase (IGPDH), and thus became sensitive to 3-amino triazole, an inhibitor of this enzyme. Upon introduction of parasite CpMBF1 into *S. cerevisiae*, 3-amino triazole resistance of the MBF1-deficient strain was restored to wild-type levels, and Northern blot analysis revealed that CpMBF1 was able to activate *HIS3* transcription in response to histidine starvation. For detail, see Ref. 4.

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C-c. Division of Early Embryogenesis

(1) *In situ* screen for novel genes expressed in the yolk syncytial layer

Takuya SAKAGUCHI, Atsushi KUROIWA[1] and Hiroyuki TAKEDA ([1]Nagoya University)

Mesoderm induction and its dorsoventral specification are important process of vertebrate early development. When transplanted onto the animal-pole region of host blastula embryos, the zebrafish yolk cells induce ectopic formation of the mesoderm and the organizer, indicating that the yolk cell, especially yolk syncytial layer (YSL), is responsible for mesoderm induction and its dorsoventral patterning. Recently, genetic studies with mice and zebrafish demonstrated that Nodal could be an endogenous mesoderm inducer. However, we still do not know how the expression of nordal-related genes are regulated in the YSL and/or it's neighboring blastomeres. To address these questions, we carried out a large scale screening of genes expressed in the zebrafish YSL. By combination of a subtracted cDNA library with our *in situ* screening method, we have successfully obtained more than 10 independent-positive clones out of 200 clones tested (>5%). Sequencing analyses suggest that they encode some enzymes, amino acid transporting proteins, plasma membrane binding protein, transcription factor, RNA binding protein, and many novel genes. Functional analyses of isolated genes are now underway.

(2) Role of FGF/MAPK signaling in the developing telencephalon of zebrafish embryos

Minori SHINYA, Sumito KOSHIDA[1], Atsushi KUROIWA[2] and Hiroyuki TAKEDA ([1]Kondoh Differentiation and Signaling Project ERATO, [2]Nagoya University)

The telencephalon is formed in the most anterior part of the central nervous system (CNS) and is organized into ventral subpallial and dorsal pallial domains. In mice, it has been demonstrated that Fgf signaling has an important role in induction and patterning of the telencephalon. However, the precise role

of Fgf signaling is still unclear, due to overlapping functions of Fgf family genes. To address this, we examine, in zebrafish embryos, the activation of Ras/MAPK, one of the major downstream targets of Fgf signaling. Immunohistochemical analysis reveals that an extracellular signal-regulated kinase (ERK), a vertebrate MAPK, is activated in the anterior neural boundary (ANB) of the developing CNS at early segmentation stages. Experiments with Fgf inhibitors reveal that ERK activation at this stage is totally dependent on Fgf signaling. Interestingly, a substantial amount of ERK activation is observed in *ace* mutants in which *fgf8* gene is mutated. We then examined the function of Fgf signaling in telencephalic development by use of several inhibitors to Fgf signaling cascade including dominant-negative form of Ras (Ras^{N17}) and Fgf receptor (Fgfr), and a chemical inhibitor of Fgfr, SU5402. In treated embryos, the induction of telencephalic territory normally proceeds but the development of the subpallial telencephalon is suppressed, indicating that Fgf signaling is required for the regionalisation within the telencephalon. Finally, antisense experiments with morpholino-modified oligonucleotides suggest that zebrafish *fgf3*, which is also expressed in the ANB, co-operates with *fgf8* in subpallial development.

(3) FGF/MAPK signaling and somite maturation in vertebrate segmentation

Atsushi SAWADA, Minori SHINYA, Yun-Jin JIANG[1], Atsushi KAWAKAMI, Atsushi KUROIWA[2] and Hiroyuki TAKEDA ([1]Imperial Cancer Research Fund, London, [2]Nagoya University)

In vertebrate, segmentation process of the somite is thought to be governed by a clock-and-wavefront mechanism. Through Notch signaling, the oscillation in each cell is coordinated and translated into the cyclic wave of gene expression, such as *hairy*-related genes, sweeping caudorostrally through the presomitic mesoderm (PSM)(Sawada et al., 2000). This cyclic wave then arrests and furrow formation initiates in very anterior PSM, the region in which PSM cells mature and become competent to segment (a maturation wavefront).

We have examined the role of Fgf/MAPK signaling, activated in the posterior

PSM, in zebrafish somitogenesis. Transient treatment with FGF-receptor inhibitor promotes maturation of the PSM. In this condition, the *her1* (*hairy*-related gene) wave prematurely terminates in the intermediate PSM, leading to formation of large somites. Complementary to this, transplantation of FGF8-soaked beads produces smaller somites around the bead. From these results, we conclude that Fgf/MAPK signaling activated in the posterior PSM plays a crucial role in positioning a maturation wavefront to the anterior PSM.

(4) Molecular analysis of zebrafish midline mutant *chameleon*

Atsushi KAWAKAMI, Rolf KARLSTROM[1], Hiroyuki TAKEDA, William S. TALBOT[2] and Alexander F. SCHIER[3] ([1]University of Massachusetts, [2]Stanford University, [3]New York University)

Midline tissues, including the floor plate and notochord, produce inducing signals and pattern the neural tube and somites. A group of zebrafish mutants (midline mutants) has common defects in the ventral neural tube, somites and dorsal aorta. It is thought that these similar phenotypes are caused by mutations in one signaling pathway. To date, several midline mutants, *sonic-you*, *you-too*, *detour* and *smooth-muscle-omitted* have been characterized, and their responsible genes are *sonic hedgehog* (*shh*), *gli2*, *gli1* and *smoothened*, respectively, all of which are molecules involved in the shh signaling pathway. As shown in these examples, the midline mutants are a useful resource to study a complex signaling cascade and its regulation in vivo. We are now focusing on another midline mutant, *chameleon* (*con*). The rescue experiments together with the decreased expression of target genes suggest that the mutant phenotypes of *con* are caused by reduced Shh signaling. To identify the *con* gene, we mapped the mutation on LG20. We also mapped and compared the positions of candidate genes that may play roles in the Shh pathway. To identify the con gene by positional cloning, we generated DNA markers linked to *con* by AFLP. By genomic walking from the markers, we identified two YAC clones that cover the mutated region.

(5) EST project in medaka fish

Takanori NARITA, Tetsuaki KIMURA and Hiroyuki TAKEDA

Medaka (*Oryzias latipes*) has several advantages to zebrafish. For examples, medaka has about half size of genome (800 Mb; zebrafish - 1700 Mb) and the number of genes are expected to be lower than that of zebrafish. Tolerance to cold temperature make it easy to obtain a temperature-sensitive mutant which would be a powerful tool for developmental genetics. To gain insight into genetic system of medaka, we perform a large scale isolation of genes expressed in developing medaka embryos.

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C-d. Division of Physiological Genetics

(1) Transcriptional Modulation by Periodic Bent DNA through Chromatin Structure

Yoshiaki OHNISHI, Megumi KATO, Chanane WANAPIRAK and Ryoiti KIYAMA
(National Institute of Advanced Industrial Science and Technology)

DNA bend sites appear nearly periodically at an average interval of 680 bp, corresponding to a length of four nucleosomes, in the human β-globin locus as well as other loci (Kiyama, 1998; Wada-Kiyama *et al.*, 1999). These sites have a high affinity for histone core particles and their deletion results in disappearance of not only the phase at the bend site but also those in the immediate vicinity (Onishi *et al.*, 1998). These lines of evidence suggest that periodic bent DNA plays a key role in chromatin folding or nucleosome formation (Kiyama, 2000). Here we show two examples where the presence of these bend sites is closely associated with *cis*-acting elements in transcription.

The first example is the enhancer of the locus, which is located 11 kb upstream of the ε-globin gene at HS2, a DNase I-hypersensitive site (HS), and contains the binding site for an erythroid specific transcription factor, NF-E2. The enhancer was located between two DNA bend sites whose distance was longer than the average and can accommodate five nucleosomes (Kiyama, 2000). The nucleosomes in this region were regularly phased except the one that is located in the middle which corresponded to the precise location of HS2 and included the binding site for NF-E2. Several phases were adopted in this region in the reconstituted chromatin and in erythroid K562 cells where the globin genes are expressed, whereas only one phase was adopted in non-erythroid HeLa cells. Meanwhile, almost unique phases were adopted at the flanking bend sites *in vitro* as well as *in vivo*. This suggests that HS2 is placed at a region of weak nucleosome phasing activity along with factor binding sites and could be influenced by the nucleosomal phases determined by those located at the bend sites. Interestingly, changing the distance between one of the bend sites and NF-E2 site affected transcription efficiency, further supporting the significance of this site.

The other example is the silencers which are present in the promoter region of the globin genes. The most characterized silencer in this locus is located 200 to 300 bp upstream of the ε-globin and overlaps the first DNA bend site from the cap site (Wanapirak et al., 2000). This co-localization of the silencer or the repressor activity is true for the β-globin gene, and also for the c-*myc*, erythropoietin receptor and estrogen receptor genes. Furthermore, co-localization was observed among all of the human β-like globin genes and the globin genes of the other species where the locations of the first bend site upstream from the cap site are conserved. Although these sites contain transcription factor binding sites and binding sites for less characterized proteins, the mechanism of silencing gene expression is not clear. Therefore, co-localization of DNA bend sites with silencers could become a clue for understanding the regulation.

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D. Department of Population Genetics

D-a. Division of Population Genetics

(1) Genetic screens for factors involved in the notum bristle loss of interspecific hybrids between *Drosophila melanogaster* and *D. simulans*

Toshiyuki TAKANO-SHIMIZU

Interspecific cross is a powerful means to uncover hidden within- and between-species variation in populations. one example is a bristle loss phenotype of hybrids between *Drosophila melanogaster* and *D. simulans*, though both the pure species have exactly the same pattern of bristle formation on the notum. There exists a large amount of genetic variavility in the *simulans* populations with respect to the number of missing bristles in hybrids, and the variation is largely attributable to *simulans x* chromosomes. Using nine molecular markers, I screened the *simulans x* chromosome for genetic factors that were responsible for the differences between a pair of *simulans* lines with high (H) and low (L) missing bristle numbers. Together with duplication-rescue experiments, a single major quantitative locus was mapped to a 14-15 region. Importantly, this region accounted for most of the differences between H and L lines in three other independent pairs, suggesting segregation of H and L alleles at the single locus in different populations. Moreover, a deficiency screening uncovered several regions with factors that potentially cause the hybrid bristle loss due to epistatic interactions with the other factors. For details, see Ref. 1.

(2) Region-dependent regulation of mutation and crossover frequencies along Drosophila chromosomes

Toshiyuki TAKANO-SHIMIZU

Eukaryotic chromosomes are organized into discrete high-order domains of several different levels and categories such as nucleosomes, transcription units, and replication units. On a larger scale, cromosomes of warm-blooded vertebrates, particularly those of humans, are divided into GC-rich and GC-poor segments of sizes varying from 200 kb to more than 1 Mb, the so-called isochore structure. By contrast, Drosophila genomes have been considered to be much more homogeneous with respect to G+C content along chromosomes. However, I found evidence of irregular silent-site substitution rates and a regional heterogeneity in GC⇔AT substitution pattern in Drosophila genomes. Substitutions in Drosophila are generally biased toward A and T; the estimated number of A/T → G/C substitutions in restricted small *x* telomeric regions completely outnumbered that of the reverse substitutions independently in two Drosophila lineages. The data in noncoding regions, further, suggested mutational biases as a cause of the substitution biases.

Local changes in recombination rates also contribute to the irregular synonymous substitution patterns. This is because linkage associations between selective loci reduce the efficacy of natural selection (the so-called Hill-Robertson effect) and most synonymous changes in Drosophila as well as in unicellular organisms are not strictly neutral but under weak selection. I found evidence that an about 20-fold reduction in the *x*-telomeric crossover frequencies have occurred in the lineage leading to *D. melanogaster*. This is a region-wide but only local effect because the total map length of the *x* chromosome did not differ so much among the compared Drosophila species.

In sum, the above regional variations along the Drosophila chromosomes suggest the presence of region-dependent regulation mechanisms of mutation and crossover frequencies, which, in turn, lead to a regional variation in evolutionary rate within the Drosophila genomes. For details, see Ref. 2.

I should add that Ms Yuriko Ishii has contributed significantly to the above two works.

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D-b. Division of Evolutionary Genetics

(1) Functional analyses of centromere of higher vertebrate cells

Tatsuo FUKAGAWA, Atsushi OKAMURA, Ai NISHIHASHI, Yoshikazu MIKAMI[1] and Toshimichi IKEMURA ([1]Grad. Univ. Advanced Studies)

The centromere plays a fundamental role in accurate chromosome segregation during mitosis and meiosis in eukaryotes. Its functions include sister chromatid adhesion and separation, microtubule attachment, chromosome movement and mitotic checkpoint control. Although chromosome segregation errors cause genetic diseases including some cancers, the mechanism by which centromeres interact with microtubules of the spindle apparatus during cell division is not fully understood.

To understand the function of the centromere, we were led to develop a genetic analysis method that utilizes the hyper-recombinogenic chicken B lymphocyte cell line DT40. The high level of homologous recombination in DT40 cells allowed targeted disruption of genes for several centromere proteins. We attempted to generate several conditional knockout cell lines of several centromere proteins, including CENP-C, CENP-H, ZW10 and Mis6. We also created several temperature-sensitive CENP-C mutants with DT40 cells. Phenotypic analysis of these mutants revealed several things.

I) CENP-C is necessary but not sufficient for the formation of a functional centromere and the structure of CENP-C may be regulated during the cell cycle.

II) CENP-C may serve further functions during G1 of the cell cycle in addition to its role in mitosis.

III) Centromere assembly in vertebrate cells proceeds in a hierarchical manner in which localization of the centromere-specific histone CENP-A is an early event that occurs independently of CENP-C and CENP-H.

IV) ZW10 is required for a spindle checkpoint function in vertebrate cells.
V) We identified a chicken counterpart of Mis6 that is centromere protein of fission yeast. We found that chicken Mis6 localizes to centromere during cell cycle and is essential for function and assembly of centromere.
For detail, Fukagawa et al. EMBO J., (2001) and Fukagawa et al., Nucl. Acids Res. (2001).

(2) Analysis of DNA replication timing of centromere region of mammalian artificial chromosomes

Tatsuo FUKAGAWA, Ai NISHIHASHI, Toshimichi IKEMURA, Megumi NAKANO[1] and Hiroshi MASUMOTO[1] ([1] Univ. Nagoya)

There are two alternative approaches to the construction of mammalian artificial chromosomes (MACs). In the first, "bottom-up" approach, homologous recombination in the yeast is used to assemble MACs from candidate sequences that have been cloned in YACs. In the second, "top-down" approach, assembly of a chromosome from cloned constituents is avoided and, instead, a natural mammalian chromosome is whittled down to a mini-chromosome by telomere-directed chromosome breakage. We are using both MACs to study centromere function.

H. Masumoto and his colleague had developed a MAC by "bottom-up" approach (Ikeno et al., Nature Biotech., 1998). In the process of experiments, they created two kinds of cell lines; one line contains a stable mini chromosome with alpha satellite sequence that functions as centreomere DNA. The other line contains a chromosome in which alpha satellite sequence is integrated on random site and does not function as centromere. We measured DNA replication timing of alpha satellite sequence in both cell line. We found that replication timing of alpha satellite sequence in the centromere is late in S phase, but it is faster when alpha satellite sequence is integrated on random site in the chromosome. We are now studying the significance of DNA replication timing for centromere assembly.

(3) Chromosome-wide assessment of replication timing for human chromosomes 11q and 21q reveals disease-gene-rich regions

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

Mammalian DNA replication proceeds in a precisely regulated manner whereby Mb-sized clusters of replicons are activated at distinct times in S phase. The replicons are heterogeneous in size, but most are 50 to 450 kb in length. Several to 10 (or more) contiguous replicons with origins that fire synchronously at a specific time comprise a Mb-sized domain that can be visualized cytogenetically as a band by the replication-banding method. We measured replication timing of the entire lengths of human chromosomes 11q and 21q. Megabase-sized zones that replicate early or late in S phase (thus early/late transition) were defined at the sequence level. Early zones were more GC-rich and gene-rich than were late zones, and early/late transitions occurred primarily at positions identical to or near GC% transitions. In the early/late transition regions, concentrated occurrence of cancer-related genes including CCND1, FGF4, TIAM1 and FLI1 was observed. The transition regions contained other disease-related genes including APP, SOD1 and PTS. We also found the single nucleotide polymorphism (SNP) frequency was higher in the transition regions. These findings are discussed with respect to the prediction that increased DNA damage occurs in replication-transition regions.

(4) Codon usage and tRNA genes in eukaryotes: correlation of codon usage diversity with translation efficiency and with CG-dinucleotide usage as assessed by multivariate analysis

Shigehiko KANAYA[1], Yuko YAMADA[2], Makoto KINOUCHI[1], Yoshihiro KUDO[1] and Toshimichi IKEMURA ([1]Department of Bio-System Engineering, Faculty of Engineering, Yamagata Univ., [2]Department of Biochemistry, Jichi Medical School)

Species-specific diversity of codon usage in five eukaryotes (*Schizosaccharomyces pombe*, *Caenorhabditis elegans*, *Drosophila melanogaster*, *Xenopus laevis*, and

Homo sapiens) was investigated with principal component analysis. Optimal codons for translation were predicted on the basis of tRNA-gene copy numbers. Highly expressed genes, such as those encoding ribosomal proteins and histones in *S. pombe*, *C. elegans*, and *D. melanogaster*, have biased patterns of codon usage, which have been observed in a wide range of unicellular organisms. In *S. pombe* and *C. elegans*, codons contributing positively to the principal component with the largest variance (Z1-parameter) corresponded to the optimal codons which were predicted on the basis of tRNA gene numbers. In *D. melanogaster*, this correlation was less evident, and the codons contributing positively to the Z1-parameter corresponded primarily to codons with a C or G in the codon third position. In *X. laevis* and *H. sapiens*, codon usage in the genes encoding ribosomal proteins and histones was not significantly biased, suggesting that the primary factor influencing codon usage diversity in these species is not translation efficiency. Codon usage diversity in these species is known to reflect primarily isochore structures. In the present study, the second additional factor was explained by the level of use of codons containing CG dinucleotides, and this is discussed with respect to transcription regulation via methylation of CG-dinucleotides, which is observed in mammalian genomes. For details, see Kanaya et al., J. Mol. Evol. (2001).

(5) Analysis of codon usage diversity of bacterial genes with a self-organizing map(SOM): characterization of horizontally transferred genes with emphasis on the *E. coli* O157 genome

Shigehiko KANAYA[1], Makoto KINOUCHI[1], Yuko YAMADA[2], Yoshihiro KUDO[1] and Toshimichi IKEMURA ([1]Department of Electrical and Information Engineering, Yamagata Univ., [2]Department of Biochemistry, Jichi Medical School)

With increases in the amounts of available DNA sequence data, it has become increasingly important to develop tools for comprehensive systematic analysis and comparison of species specific characteristics of protein-coding sequences for a wide range of genomes. In the present study, we used a novel neural-network algorithm, a self-organizing map (SOM), to efficiently and comprehensively analyze codon usage in approximately 60,000 genes from 29 bacterial species simultaneously. This SOM makes it possible to cluster and

visualize genes of individual species separately at a higher resolution than can be obtained with principal component analysis. The organization of the SOM can be explained by the tRNA compositions and genome G+C% of the individual species. Using SOM, we examined codon usage heterogeneity in the *E. coli* O157 genome, which contains "O157-unique segments (O-islands)", and showed that the SOM is a powerful new strategy for characterization of horizontally transferred genes. For details, see Kanaya et al., Gene (2001).

(6) Skew and principal component analysis of prokaryotic and eukaryotic genomes

Yuta ICHIBA, Yoshihisa WATANABE and Toshimichi IKEMURA

GC skew that has been connected with asymmetries in base composition between the leading and the lagging strands was reported previously in many prokaryotic genomes. We analyzed mono-nucleotide and di-nucleotide skew of yeast and human genomes, as well as of several prokaryotic genomes in the following strategy. To find the most skewed nucleotide, we conducted multivariate analysis (principal component analysis; PCA) on the 5 - 100 kb segments of individual genomes. By using the skew and PCA, we have found that a significant proportion of the skews and the factor scores of PCA can be attributed to replication orientation. The present skew and the factor score can predict several ARSs of *Saccharomyces cerevisiae* chromosome VI. In addition, the correlation of the skew and factor score pattern with replication-timing of human genomes was observed. There also seems to be correlation between factor scores and SNPs. In the cyanobacterium *Synechocystis sp*, the most skewed di-nucleotide and factor score can predict an origin of replication which could not be identified by GC and AT skew.

(7) Programs for constructing phylogenetic trees and networks of closely related sequences

Naruya SAITOU

Nowadays increasingly many closely related sequences are deposited to the

DDBJ/EMBL/GenBank nucleotide sequence database. To deal with those vast number of closely related nucleotide sequences, I recently developed a series of programs to process closely related mass sequence data. We start from BLAST homology search. Program p0 extracts sequences homologous to query sequence and produce FASTA format output file. After retrieving those homologous sequences, multiple alignment usually follows. However, when we restrict our search only to closely related sequences, multiple alignment is not necessary, for BLAST already extracted homologous regions and those rarely have gaps. Therefore, we can skip multiple alignment process which often takes a very long computer time compared to BLAST search. After format transformation via programs p1 and p2, program p3 eliminates invariant sites. When we deal with closely related sequences, many invariant site are expected to exist, and this procedure can reduce the data file extensively. Program p4 then examines sequence identity, and all the identical sequences are joined. Consequently, only different sequences remain. The next step is to eliminate 'single polymorphic' sites in which only one sequence have different character and all others have the same character. This is conducted by program p5. In this fashion, we can rapidly extract so-called phylogenetically informative sites for maximum parsimony methods. For details, see ref. 13.

(8) CAMUS DB: Development of structural database for homology search

M KIKUCHI[1], S MISU[1], Tadashi IMANISHI[1] and Naruya SAITOU ([1]DNA Data Bank of Japan, Center for Information Biology, National Institute of Genetics)

DDBJ/EMBL/GenBank International Nucleotide Sequence Database (INSD) is increasing with unexpected rate (doubling time is only slightly longer than one year), and computation time for homology search is becoming longer and longer. Because nucleotide sequences have their own nature to make copies through DNA replication, there are many similar, evolutionarily closely related nucleotide sequences in INSD. We therefore developed new system to cluster similar sequences and make compressed sequence database consisting of representative sequences of those clusters. Each cluster consists of highly

homologous sequences, and they are presented as multiply-aligned form. We DDBJ [1] call those combined database as CAMUS (Compressed database And MUltiple aligned Sequence database). Its URL is http://wolf.genes.nig.ac.jp/camus/. For details, see ref. 14.

(9) Gene diversity of chimpanzee ABO blood group elucidated from intron 6 sequences

Takashi KITANO, Reiko NODA, Kenta SUMIYAMA, Robert E. FERRELL[1] and Naruya SAITOU ([1]Department of Human Genetics, University of Pittsburgh, USA)

The human and non-human primate ABO blood group gene shows relatively large numbers of nucleotide differences among the exon 7 region. In this study, we determined intron 6 sequences for 10 alleles of common chimpanzee and for 3 alleles of bonobo to estimate nucleotide diversities among them. Sequence length polymorphisms are observed in this region due to differences of repeat numbers from 1 to 5. From a phylogenetic network of intron 6 sequences of ABO blood group genes for human, common chimpanzee, and bonobo, effects of parallel substitutions and/or some kinds of convergent events are predicted in the chimpanzee lineage. We also estimated nucleotide diversities for common chimpanzee and bonobo ABO blood group genes, and these values were 0.219 and 0.208 percent, respectively. For details, see ref. 15.

(10) Evolution of the ABO blood group gene in Japanese macaque

Reiko NODA, Takashi KITANO, Osamu TAKENAKA[1] and Naruya SAITOU ([1]Primate Research Institute, Kyoto University, Inuyama)

We determined 5 sequences of Japanese macaque ABO blood group gene exon 7 (ca. 0.5kb) and 2 sequences for exon 5 and intron 6 (ca. 1.7kb). We compared those data with published sequences of other Old World monkey species, and the results suggest that alleles A and B were polymorphic in the ancestral species of macaques, and that B type allele evolved independently in macaque and baboon lineages. For details, see ref. 16.

(11) Evolutionary history of the Rh blood group-related genes in vertebrates

Takashi KITANO and Naruya SAITOU

Rh and its homologous Rh50 gene products are considered to form heterotetramer on erythrocyte membranes. Rh protein has Rh blood group antigen sites, while Rh50 protein does not have Rh blood group antigen sites, and is more conservative than Rh protein. We previously determined both Rh and Rh50 gene cDNA coding regions from mouse and rat, and carried out phylogenetic analyses. In this study, we determined Rh50 gene cDNA coding regions from African clawed frog and Japanese medaka fish, and examined the long-term evolution of the Rh blood group genes and their related genes. We constructed the phylogenetic tree from amino acid sequences. Rh50 genes of African clawed frog and Japanese medaka fish formed a cluster with mammalian Rh50 genes. The gene duplication time between Rh and Rh50 genes was estimated to be about 510 million years ago based on this tree. This period roughly corresponds to the Cambrian, before the divergence between jawless fish and jawed vertebrates. We also BLAST-searched amino acid sequence database, and the Rh blood group genes and their related genes were found to have homology with ammonium transporter genes of many organisms. Ammonium transporter genes can be classified into two major groups (amt a and amt b). Both groups contain genes from three domains (bacteria, archaea, and eukaryota). The Rh blood group genes and their related genes are separated from both amt a and b groups. For details, see ref. 17.

(12) Genetic structure of a 2500-year-old human population in China and its spatiotemporal changes

Li WANG[1,2], Hiroki OOTA[1], Naruya SAITOU, Feng JIN[2], Takayuki MATSUSHITA[3] and Shintaroh UEDA[1]([1]Graduate School of Science, University of Tokyo, Tokyo, [2]Institute of Genetics, Chinese Academy of Sciences, Beijing, China, [3]Doigahama Site Anthropological Museum, Houhoku)

To examine temporal changes in population genetic structure, we compared

the mitochondrial DNA (mtDNA) sequences of three populations that lived in the same location, Linzi of China, in different periods: 2500 years ago (the Spring-Autumn era), 2000 years ago (the Han era), and the present day. Two indices were used to compare the genetic differences: the frequency distributions of the radiating haplotype groups and the genetic distances among the populations. The results indicate that the genetic background of the three populations is distinct from each other. Inconsistent with the geographical distribution, the 2500-year-old Linzi population showed greater genetic similarity to present-day European populations than to present-day east Asian populations. The 2000 year-old Linzi population had features that were intermediate between the present-day European/2500-year-old Linzi populations and the present-day east Asian populations. These relationships suggest the occurrence of drastic spatiotemporal changes in genetic structure of Chinese people during the past 2500 years. For details, see ref. 18.

(13) Sequence variation in the ABO blood group gene exon 7 of chimpanzee and bonobo

Kenta SUMIYAMA, Takashi KITANO, Reiko NODA, Shintaroh UEDA, Robert E. FERRELL[1] and Naruya SAITOU ([1]Department of Human Genetics, University of Pittsburgh, USA)

Human and non-human primate ABO blood group genes show relatively large numbers of nucleotide differences. In this study, we determined exon 7 sequences for 10 individuals of common chimpanzee and for 4 individuals of bonobo to estimate nucleotide diversities among them. Sequence data showed the existence of chimpanzee specific 9 base deletion in the beginning of the exon 7 coding region. From a phylogenetic network of exon 7 sequences of ABO blood group genes for human, common chimpanzee, bonobo and gorilla, effects of parallel substitutions and/or some kinds of convergent events are inferred in the chimpanzee lineage. We also estimated nucleotide diversities for common chimpanzee and bonobo ABO blood group genes, and these values were 0.4 % and 0.2 % percent, respectively. These values are higher than that of most human genes. For details, see ref. 19.

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D-c. Division of Theoretical Genetics

(1) Autonomous formation of spatial pattern in development

Shigeru KONDOH

Most of biological phenomena occur as the result of complex interaction of the genes or molecules. As the number of the element increases, the relationships among them become terribly complex as the exponential function. Complex phenomena like morphogenesis or neural net formation, occurs as the chain reaction of the local interaction of cells. Therefore, it is quite difficult to imagine what really happens. In such case, computer simulation may help.

In our laboratory, using the skin pattern of zebra fish as a model system, we aim to develop a practical standard method to simulate complex phenomena

in biology.

From the observation with the skin pattern of Pomacanthus and other fish, we are sure that the skin pattern of fish is a "wave" generated by a putative mechanism called reaction-diffusion. However, there is no molecular information yet.

Several mutant lines that have altered skin pattern are isolated in the large scale screening experiment done in Tubingen. Cloning of the genes will be very helpful. On the other hand, knowing the skin structure as the field of wave formation is also very important. Our study is, now, concentrated on this subject.

From the electro-microscopic analysis and the transplantation experiment, followings are revealed.

(1)the skin of zebrafish is composed of the three layers, epithelium, mesenchyme and pigmented cell layer. Melanophore and iridophore are located in the pigment cell layer. However, xhantophore locates in the mesenchyme layer.

(2)From the transplantation experiment, the positional information exist in the mesenchyme layer.

(3)From the chimera experiment, we conclude that melanophore does not involved in the pattern formation, that is against the former anticipation.

Publication

None

E. Department of Integrated Genetics

E-a. Division of Human Genetics

(1) Genome analysis of the mouse 7F4/F5 imprinted domain

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

Genomic imprinting, an epigenetic gene marking phenomenon, causes parental-origin-specific monoallelic expression of a subset of mammalian genes. Imprinted genes tend to form clusters in specific regions of the genome, which may be related to the mechanism of imprinting or the reason for the evolution of imprinting. As a step to understand the structural and functional characteristics of the imprinted genome domains, we are studying an imprinted domain in mouse chromosome band 7F4/F5, which contains at least eight imprinted genes. This region is syntenic to human 11p15.5, which contains genetic loci responsible for Beckwith-Wiedemann syndrome and some types of childhood and adult tumors. YAC, BAC and cosmid contigs covering the 1-Mb region were constructed and the sequence of the entire imprinted domain has now been determined using selected BAC clones. Part of this sequence has been described in ref. 3. A more detailed analysis of the sequence is now ongoing to identify new imprinted genes and their regulatory elements. We have also started to map the nuclear matrix attachment regions (MARs) of the region, which might play an important role in the regulation of the imprinted domain.

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

Hiroyuki SASAKI, Ko ISHIHARA, Hiroyasu FURUUMI, Mizuki OHNO[1], Takayuki UEDA[2], Ryo KOMINAMI[3] and Akihiro UMEZAWA[4] ([1]Inst. of Genetic Information,

Kyushu Univ, [2]Inst. of Molecular Genetics and [3]Dept. of Biochemistry, Faculty of Medicine, Niigata Univ, [4]Dept. of Pathology, Faculty of Medicine, Keio Univ.)

The imprinted mouse 7F4/F5 domain contains two linked imprinted genes *Igf2* and *H19* near its centromeric boundary: *Igf2* is paternally expressed and *H19* maternally expressed. We have identified five evolutionarily conserved tissue-specific enhancers in the downstream region of *H19* which are potentially involved in the reciprocal expression patterns of the two genes (ref. 1). We also identified a conserved 39-bp element within the differentially methylated region (DMR) upstream of *H19* which is capable of forming complexes with specific nuclear factors including CTCF. Binding of one of the novel factors was inhibited by methylation of the CpG dinucleotides within the target sequence, just as was the case for CTCF (ref. 1). These complexes may contribute to the presumed boundary function of the unmethylated DMR on the maternal chromosome, which is proposed to insulate maternal *Igf2* from the enhancers (ref. 2). Paternal-specific methylation of the DMR is thought to be the primary imprint regulating the *Igf2/H19* imprinting. We found that this methylation is inherited from sperm (ref. 4 and 10) and that it is established in the gonocyte stage during fetal testis development (ref. 4). By using a novel allele-specific RNA-FISH technique, it was also found that the paternal-specific expression of *Igf2* begins in the blastocyst stage despite the presence of the methylation imprint in earlier pre-implantation embryos (ref. 7). Finally, we reported that the degree of chromatin packaging is different between the parental alleles of the imprinted genes (ref. 5).

(3) Role for *de novo* DNA methyltransferases *Dnmt3a/Dnmt3b* in genomic imprinting

Hiroyuki SASAKI, Naomi TSUJIMOTO, Masahiro KANEDA and Shoji TAJIMA[1]
([1]Inst. of Protein Biochemistry, Osaka Univ.)

DNA methylation works as an important gene marking mechanism to distinguish the parental alleles of imprinted genes. To understand how the primary imprints are established in the male and female germ cells, we are studying

the expression and localization of the *de novo* DNA methyltransferases *Dnmt3a/Dnmt3b* in the male and female gonads. We have also started to produce mice lacking *Dnmt3a* and/or *Dnmt3b* only in the germ cell lineage by using conditional gene knockout system.

(4) *de novo* DNA methyltransferases *Dnmt3a/Dnmt3b* and human disorders

Hiroyuki SASAKI, Hisao SHIROHZU, Shin-ich MIZUNO[1], Takeo KUBOTA[2] and Shoji TAJIMA[3] ([1]Inst. of Genetic Information, Kyushu Univ, [2]Natl. Center of Neurology and Psychiatry, [3]Inst. of Protein Biochemistry, Osaka Univ.)

It is known that tumor suppressor genes are often methylated and inactivated in a number of cancers. We have found that acute meyelogenous leukemia cases with methylated p15 tumor suppressor gene have higher levels of all three DNA methyltransferases *DNMT1*, *DNMT3A* and *DNMT3B* (ref. 6). We also studied three Japanese cases with ICF (immunodeficiency, centromeric instability, facial anomalies) syndrome, an autosomal recessive disorder caused by *DNMT3B* mutations, and identified three novel mutations.

(5) Studies on the possibilities of genomic imprinting and Z chromosome dosage compensation in chicken

Hiroyuki SASAKI, Takaaki YOKOMINE, Asato KUROIWA[1], Yoichi MATSUDA[1] and Masaoki TSUDUKI[2] ([1]Chromosome Research Unit, Hokkaido Univ, [2]Faculty of Animal Production, Hiroshima Univ.)

Although it is generally thought that genomic imprinting has evolved only in mammals, this has not been tested in other vertebrate species. We have asked whether the homologs of the mammalian imprinted genes show parental-origin-specific monoallelic expression in chicken. Using DNA polymorphisms identified between various breeds and strains, it was shown that both *IGF2* and *MPR1* are expressed biallelically in chicken embryos (ref. 9). We also found that chicken Z chromosome is not subject to inactivation for gene dosage compensation in ZZ males. We are currently studying the structure and function of

chicken DNA methyltransferases *DNMT3A/DNMT3B*, whose cDNAs have been cloned in our laboratory.

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

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

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

(7) Analysis of anti-sense RNA identified in the *Xist* region

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

X-chromosome inactivation is an important gene dosage compensation mechanism in female mammals. We have found several forms of processed RNAs transcribed from the anti-sense strand of *Xist*, an X-linked locus essential for X-chromosome inactivation. The anti-sense RNAs (*Tsix*) cover the whole transcription unit of *Xist* and do not code for a protein. To know whether these novel RNAs play a role in X-inactivation, we have disrupted *Tsix* in mice by targeted mutagenesis. The X chromosome with disrupted *Tsix* inappropriately expressed *Xist* and became inactivated, suggesting an important regulatory function of *Tsix* (ref. 8). This work was awarded with a Best Poster Prize in the 14th International Mouse Genome Conference held in Narita in November 2000.

(8) Human genome resources and their application to the human and primate genome analysis

Asao FUJIYAMA, Ayuko MOTOYAMA[1], Satoru YOSHIDA[1], Yoko KUROKI[2], Yutaka NAKAHORI[2], Tatsuo NAKAYAMA[3], Mieko KODAIRA[4], Norio TAKAHASHI[4] and Naruya SAITOU[5] ([1]Genome Science Center Riken, [2]Tokushima Univ, [3]Miyazaki Medical School, [4]Radiation Effect Research Foundation, [5]Evolution Genetics Div., National Institute of Genetics)

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), CM#9-12, #21, CM-Y fosmid libraries, and BAC library. In addition, we are in the process of constructing primate libraries including chimpanzee and gorilla. Using these resources, sequencing of the long arm of human chromosome 21 has been accomplished. A total of 33,546,361 bp of DNA, distributed over four contigs, were sequenced with very high accuracy. In order to carry out primate comparative genomic study, we have sequenced and mapped more than 10,000 BAC-ends on the human genome.

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

Yong-Sik BONG, Inaho DANJO[1], Nobuya OGAWA and Asao FUJIYAMA ([1]Cancer Center Research Institute)

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 micro-arrays of clones covering entire genome of the fission yeast.

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E-b. Division of Agricultural Genetics

(1) Developmental abnormalities induced by DNA hypomethylation mutation of Arabidopsis

Tetsuji KAKUTANI, Asuka MIURA and Koichi WATANABE

Substantial part of large genome of flowering plants and vertebrates are repetitive sequences, such as transposable elements and their derivatives. Genomic regions rich in repeated sequences tend to be inactive in transcription and recombination and have condensed chromatin (heterochromatin). In addition, cytosine residues in such regions are often methylated at high frequency. Although function of repeated sequences are largely unknown, uncontrolled activation of these sequences are presumed to be deleterious to genome stability. On the other hand, some of repetitive sequences such as centromeric repeats and telomeres are regarded as important for the chromosome function. We are studying control and biological function of repetitive sequences using DNA methylation mutants of Arabidopsis.

In Arabidopsis, genome sequencing has been completed for the first case in plants. In addition, many trans mutations affecting epigenetic states have

been isolated in this plant. Arabidopsis *ddm1* (*decrease in DNA methylation*) mutation results in decrease in methylation and transcriptional de-repression in genomic repeat sequences. The *DDM1* gene encodes a protein similar to the chromatin-remodeling factor SWI2/SNF2. The most striking feature of *ddm1* mutation is that it induces a variety of developmental abnormalities by causing heritable change in other loci. The molecular basis has been clarified in two of the loci directly causing the developmental abnormalities.

One of them, *clam*, is characterized by lack of elongation in leaves, roots and shoots. This phenotype is heritable, but somatic sectors with normal phenotype were occasionally observed. The size and frequency of the sector differ from plant to plant. The phenotype was stabilized in some of the progeny families; no reversion sector was observed in such family. We mapped the locus responsible for the *clam* phenotype at high resolution. By genotyping 926 chromosomes, we identified the gene responsible for the *clam* phenotype. It is *DWF4* gene, which involved in synthesis of brassinolide, a plant growth regulator necessary for cell elongation. The unstable *clam* phenotype was induced by insertion of a novel endogenous Arabidopsis transposon, which we named *CACTA1*. This transposon transposes and increases in the copy number specifically in *ddm1* mutant background. These results suggest that gene silencing associated with DNA methylation is important for suppression of transposons (in press).

Another developmental abnormality, late flowering trait, was induced by ectopic over expression of *FWA* gene associated with hypomethylation of tandem repeat upstream of the coding region. Interesting thing is that change in nucleotide sequence was also not observed in *fwa-1* and *fwa-2* alleles isolated by conventional mutagenesis. In both cases, over-expression associated with the hypomethylation resulted in the phenotypes. Combining *ddm1* mutation and linkage analysis is useful for identifying epigenetically regulated genes important for plant development.

(2) DNA hypomethylation mutation in rice

Tetsuji KAKUTANI, Koichi WATANABE and Asuka MIURA

Among plant species, Arabidopsis genome has extreme feature that it contains only small proportion of repeated sequences. We therefore extended our research by examining effect of de-repression of genomic repeated sequences in rice. Rice has more repeat sequences than Arabidopsis. We found a rice EST similar to Arabidopsis *DDM1* gene and generated a transgenic rice lines expressing that EST in antisense orientation. These transgenic lines shows reduced genomic DNA in centromeric repeats, repeat encoding rRNA and retroelement-like sequence Tos3.

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E-c. Division of Brain Function

(1) Origin and Migration of Guidepost Neurons in the Lateral Olfactory Tract

Naomi TOMIOKA[1], Noriko OSUMI[2], Yasufumi SATO, Hajime FUJISAWA[1] and Tatsumi HIRATA ([1]Nagoya University Graduate School, [2]Tohoku University)

The early-generated neurons designated as lot cells specifically mark the future site of the lateral olfactory tract (LOT) and guide LOT axons. We investigated the mechanism of how lot cells develop and get localized in the LOT position. Lot cells differentiated from neuroepithelial cells in all regions of the neocortex but not from those in the ganglionic eminence in culture. Cell tracing analyses demonstrated that lot cells generated from the neocortex subsequently followed a tangential migration stream ventrally toward the LOT position. Mutant mouse embryos lacking the function of transcription factor Gli3 showed disturbances of the migration stream, and translocation of lot cells in the

dorsal telencephalon. These results reveal a new type of neuronal migration in the telencephalon and introduce an unexpected dramatic feature of the earliest regionalization of the telencephalon.

(2) Short-Range and Long-Range Guidance of Olfactory Bulb Axons

Tatsumi HIRATA, Hajime FUJISAWA[1], Jane Y. WU[2] and Yi RAO[2] ([1]Nagoya University Graduate School, [2]Washington University School of Medicine)

During development, mitral cells, the major output neurons of the olfactory bulb, project their axons caudolaterally into the telencephalon and form the lateral olfactory tract (LOT). Two types of guidance cues have been suggested for this projection. Firstly, a long-range factor Slit, which is secreted from the septum repels mitral cell axons into a caudolateral direction. Secondly, the pathway of mitral cell axons contains a subset of neurons designated as lot cells, which guide the axons through short-range interactions. It is not clear how these two guidance cues relate to each other and how they share the physiological roles. We examined the behavior of mitral cell axons in organotypic culture upon ectopic application of Slit, and inhibition of endogenous Slit signaling. The results suggested that the short-range guidance cue in the LOT pathway functions independently from Slit. Furthermore, our results showed that removal of the septum and inhibition of Slit signaling did not affect the projection of mitral cell axons. Although the septum and exogenous Slit can repel olfactory bulb axons, our results cast doubts on the physiological relevance of the septum and endogenous Slit in guiding the projection of mitral cell axons.

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E-d. Division of Applied Genetics

(1) Analysis of genomic imprinting involved in murine X chromosome inactivation

Nobuo TAKAGI (Graduate School of Environmental Earth Science, Hokkaido University)

Mouse embryos having an additional maternally inherited X chromosome (X^M) invariably die before mid-gestation with the deficient extraembryonic ectoderm of the polar trophectoderm lineage, whereas postnatal mice having an additional paternally inherited X chromosome (X^P) survive beyond parturition. A cytogenetic study led us to hypothesize that abnormal development of such embryos disomic for X^M (DsX^M) is attributable to two doses of active X^M chromosome in extraembryonic tissues. To test the validity of this hypothesis, we examined the initial X chromosome inactivation pattern in embryos at the blastocyst stage by means of replication banding method as well as RNA FISH detecting *Xist* transcripts. X^P was the only asynchronously replicating X chromosome, if any, in $X^MX^MX^P$ blastocysts, and no such allocyclic X chromosome was ever detected in X^MX^MY embryos. In agreement with these findings, only one *Xist* paint signal was detected in 79 % of $X^MX^MX^P$ cells, whereas no such signal was found in X^MX^MY cells. Thus, two X chromosomes remaining active in the extraembryonic cell lineages due to the maternal imprinting explain the underdevelopment of extraembryonic structures and hence early postimplantation death of DsX^M embryos. We went on to further characterize the nature of imprinting on X^P making use of androgenetic embryos produced by pronuclear transfer. FISH analyses consistently revealed one and two *Xist* paint signals in X^PY and X^PX^P embryos, respectively from the 4-cell to morula stage. Furthermore, X^PX^P androgenones surviving to embryonic day (E) 7.5 achieved random X inactivation in all tissues including those derived from the trophectoderm and primitive endoderm characterized by X^P-inactivation in fertilized embryos. This finding supports the above hypothesis that X^M is rigidly imprinted to avoid inactivation, whereas X^P is free of such imprint in fertilized female embryos before implantation. It is likely that imprinted in-

activation was derived from random inactivation not the other way around.

(2) Developmental abnormalities in tetrasomy 11 mouse embryos

Nobuo TAKAGI (Graduate School of Environmental Earth Science, Hokkaido University)

In mammals, an excess copy of any autosomal pair invariably induces strict developmental abnormalities mostly resulting in spontaneous abortion or stillbirth, whereas effects of an increased copy of the X chromosome are much less severe. The dosage compensation mechanism silencing all X chromosomes in excess of one seems responsible for the alleviated phenotypic manifestation of X aneuploidy. This notion is substantiated by the observation that growth of female embryos defective in X-inactivation is severely impaired and they are absorbed immediately after implantation. This study was initiated to clarify whether X chromosome is involved in embryonic development disproportionately heavily than average autosomes. In this study we tried to analyze effects of two-fold increase in chromosome 11 (tetrasomy 11) on embryonic development taking advantage of high rate of nondisjunction in mice heterozygous for two Robertsonian translocations Rb(10.11)8Bnr and Rb(11.13)6Lub. About 5% of embryos recovered from heterozygous females mated with males carrying same translocations at E7.5 were tetrasomic for chromosome 11. Tetrasomy 11 embryos corresponding in size to E5.5 embryos showed no clear differentiation between embryonic and extraembryonic region. Furthermore, dead cells were abundant in the proamniotic cavity without mesoderm differentiation. These abnormalities are comparable in severity to those found in embryos having two copies of active X chromosomes. Thus, it seems probable that effects of four copies of chromosome 11 on embryonic development roughly correspond to those of two active copies of the X chromosome implying that X chromosome is not special if we assume that the level of activity is twice that of autosomes.

(3) Molecular analysis of the NAC gene family in rice

Kazuhiro KIKUCHI[1], Minako UEKUCHI-TANAKA[2], Kaoru YOSHIDA[1], Yasuo NAGATO[1], Makoto MATSUOKA[2] and Hiroyuki HIRANO[1] ([1]Graduate School of Agricultural and Life Sciences, University of Tokyo, [2]BioScience Center, Nagoya University)

Genes that encode products containing a NAC domain, such as *NO APICAL MERISTEM* (*NAM*) in petunia, *CUP-SHAPED COTYLEDON2* (*CUC2*) and *NAP* in *Arabidopsis thaliana*, have crucial functions in plant development. We describe here molecular aspects of the *OsNAC* genes that encode proteins with NAC domains in rice (*Oryza sativa* L.). Sequence analysis revealed that the NAC genes in plants can be divided into several subfamilies, such as the NAM, ATAF, and OsNAC3 subfamilies. In rice, *OsNAC1* and *OsNAC2* are classified in the NAM subfamily, which includes *NAM* and *CUC2*, while *OsNAC5* and *OsNAC6* fall into the ATAF subfamily. In addition to the members of these subfamilies, the rice genome contains the NAC genes *OsNAC3*, *OsNAC4* (both in the *OsNAC3* subfamily), *OsNAC7*, and *OsNAC8*. These results and Southern analysis indicate that the *OsNAC* genes constitute a large gene family in the rice genome. Each *OsNAC* gene is expressed in a specific pattern in different organs, suggesting that this family has diverse and important roles in rice development. For details, see Ref. 5.

(4) Characterization of viviparous mutants in rice (*Oryza sativa* L.)

Kazumaru MIYOSHI, Eijirou NAKATA and Yasuo NAGATO (Graduate School of Agricultural and Life Sciences, University of Tokyo)

Four single recessive viviparous mutants of rice, *riv1-1*, *riv1-2*, *riv2* and *enl1*, were characterized. All *riv* mutants showed pre-harvest germination (vivipary) during seed development but no defects in the other traits. Vivipary was also observed in the *enl1* mutant that lacked endosperm in the seeds. Examination of the viviparous nature of the mutants under three rain conditions, no rain, artificial rain and natural rain, revealed that the induction of vivipary in

the *riv* mutants required a small amount of water. In contrast, precocious germination in the *enl1* mutant was caused by the absence of endosperm in the seeds, but not by the external signal of rain. In *riv* mutants, the precocious germination occurred simultaneously at late stages of seed development. The germination test in the presence of exogenous ABA revealed that sensitivity to ABA was gradually reduced from 15 through 35 days after pollination in the wild-type seeds, and was almost lost at 40 days after pollination. A similar tendency was observed in *riv1* and *riv2* seeds. However, at 15-35 days after pollination, *riv1-1* and *riv2* seeds had significantly lower sensitivity to ABA than the wild-type seeds. In addition, the three *riv* mutants lost the sensitivity to ABA at an earlier stage of seed development than the wild type. These results indicate that *riv1* and *riv2* have a lower sensitivity and shorter period of sensitivity to ABA. For details, see Ref. 6.

(5) *SHOOT ORGANIZATION* genes regulate shoot apical meristem organization and the pattern of leaf primordium initiation in rice

Jun-Ichi ITOH[1], Hidemi KITANO[2], Makoto MATSUOKA[3] and Yasuo NAGATO[1] ([1]Graduate School of Agricultural and Life Sciences, University of Tokyo, [2] Faculty of Agriculture, Nagoya University, [3]BioScience Center, Nagoya University)

The mechanism regulating the pattern of leaf initiation was analyzed using *shoot organization* (*sho*) mutants derived from three loci (*SHO1*, *SHO2* and *SHO3*). In the early vegetative phase, *sho* mutants show an increased rate of leaf production with random phyllotaxy. Resulting leaves are malformed, thread-like, or short and narrow. Their shoot apical meristems are relatively low and wide, i.e., flat in shape, although their shape and size are highly variable among plants of the same genotype. Statistical analysis reveals that the shape of the shoot meristem rather than its size is closely correlated with the variation of plastochron and phyllotaxy. Rapid and random leaf production in *sho* mutants are correlated with the frequent and disorganized cell divisions in the shoot meristem and with a reduction of expression domain of a rice homeobox gene, *OSH1*. These changes in the organization and behavior of the SAM suggest that *sho* mutants have a reduced number of indeterminate cells

and increased number of determinate cells, with many cells acting as leaf founder cells. Thus the *SHO* genes have an important role in maintaining the proper organization of shoot apical meristem which is essential for the normal initiation pattern of leaf primordia. For details, see Ref. 7.

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F. Genetic Strains Research Center

F-a. Mammalian Genetics Laboratory

(1) Molecular dissection of the critical region of a mouse preaxial polydactyly mutation, Hemimelic extratoes (*Hx*)

Tomoko SAGAI, Hiroshi MASUYA[1], Kunihiko SHIMIZU[2], Masaru TAMURA[3] and Toshihiko SHIROISHI ([1]Mouse Funct. Genomics Res. Group, RIKEN GSC, [2]Nihon Univ. Sch. of Dent. at Matsudo,[3]Kyoto Univ.)

As described in the previous report, we identified a possible candidate gene of a preaxial poydactyly (PPD) mutation, Hemimelic extra toes (*Hx*), by the positional cloning. This gene, *Lmbr1*, is a mouse homolog of human *c7orf2*, which was identified in the human syntenic region 7q36. In human, a genomic deletion including the exon4 of *c7orf2* was detected in the patients with bilateral congenital defect of the upper and lower extremities. However, their phenotypes are different from the typical PPD, and no coding sequence alterations or genomic rearrangements of *c7orf2* were detected in five independent families with PPD. In the mouse, it was reported that in *Hx* mouse, *Lmbr1* expression in the developing limb bud was dramatically altered. In opposition to the results in the report, we could not detect clear difference of *Lmbr1* expression between *Hx* and wild type by Northern blotting analysis, quantitative PCR and whole mount *in situ* hybridization. Thus, the evidence that *Lmbr1* is a causative gene for *Hx* is still circumstantial. Alternatively, *Hx* phenotype may be caused by alteration of a long-range cis-element of the sonic hedgehog gene (*shh*) that is localized to the down stream of the *Hx* mutation within a distance less than 1Mb, because *Hx* embryos indeed show an ectopic expression of *shh* in the anterior limb margin, like several other preaxial polydactyly mutants.

As another approach to search the mutation responsible for *Hx* phenotype, we carried out sequencing of the genome of the *Hx* critical region. *Lmbr1* comprises 17 exons and may span over a 150kb genomic region. Our physical

mapping narrowed down the *Hx* critical region to about a 100kb region that is covered by a single BAC clone. We have assembled the cosmid clones covering this region and sequenced them. Thus far we have read about 90% of this region.

(2) Analysis of a spontaneous mouse mutation, mesenchymal dysplasia (*mes*)

Shigeru MAKINO, Hiroshi MASUYA[1], Yukari YADA and Toshihiko SHIROISHI
([1] RIKEN GSC)

A recessive mouse mutation, mesenchymal dysplasia (*mes*), which arose spontaneously on Chromosome 13, causes excess skin, increased body weight and mild preaxial polydactyly. Fine gene mapping indicated that *mes* is tightly linked to patched (*ptc*) that encodes a transmembrane receptor protein for Shh, a key signaling molecule in vertebrate development. Molecular characterization of the *ptc* gene of the *mes* mutant and an allelism test using a *ptc* knockout allele (ptc^-) demonstrated that *mes* is caused by a deletion of the most C-terminal cytoplasmic domain of the *ptc* gene. Since *mes* homozygous embryos exhibit normal pattern formation and neural tube development as compared with ptc^- homozygotes, which die around 10 dpc with severe neural tube defects, the C-terminal cytoplasmic domain lost in *mes* mutation is dispensable for inhibition of Shh signaling in early embryogenesis. However, compound heterozygotes of ptc^- and *mes* alleles, which survive up to birth and die neonatally, have increased body weight and over-proliferation of mesenchymal cells in the dorsal trunk and the lung. These findings indicate that Ptc is a negative regulator of the proliferation of mesenchymal cells, and that the C-terminal cytoplasmic domain of Ptc is involved in its repressive action.

(3) Analysis of two preaxial polydactylous mouse mutations, X-linked polydactyly (*Xpl*) and luxate (*lx*)

Yukari YADA[1], Hiroshi MASUYA[2], Shigeru MAKINO and Toshihiko SHIROISHI
([1]Ochanomizu Univ, [2]RIKEN GSC)

The anteroposterior axis patterning on vertebral limb morphogenesis is controlled by the formation of the zone of polarizing activity (ZPA), located at the posterior margin of limb buds. The Sonic hedgehog (*Shh*) gene that is expressed at the posterior margin of limb buds mediates ZPA activity. However, regulation of the polarized expression of *Shh* in developing limb buds is poorly understood. X-linked polydactyly (*Xpl*) is a spontaneous mouse mutation, which exhibits preaxial polydactyly only on the hindfeet. An analysis by *in situ* hybridization revealed ectopic expression of *Shh* and *Fgf4* at the anterior margin of the hindlimb bud in the *Xpl* embryos. The trancription gene *Gli*, which is a downstream of *Shh*, was expressed at the anterior side of the *Xpl* hindlimb prior to the *Shh* expression. These data indicate that *Xpl* gene likely acts in the downstream or independent pathway of *Shh* signaling cascade. To isolate the *Xpl* gene, we performed the linkage analysis using 2,452 backcross progeny from cross between B6-*Xpl*/+ and wild mouse-derived strains, MSM, KJR and BLG2. The *Xpl* gene was mapped to a 0.89cM interval between the microsatellite markers, *DXMgc39* and *DXMit32*. Now, we are constructing BAC contig covering the causative gene of *Xpl*.

Another mouse mutation, luxate (*lx*), also exhibits preaxial polydactyly on the hindfeet. We found that *Fgf4* and *Gre* are expressed only in the anterior half of limb buds of the *lx* mutants. In spite of such anterior shift of *Fgf4* and *Gre*, endogenous expression of *Shh* was not altered. This observation indicates that the *Fgf4* expression is not directly involved in induction and maintenance of the endogenous *Shh* expression. In 11.5 day embryos of the *lx* mutants, *Fgf8* was expressed in the whole AER, but its expression domain was narrowed toward the anterior side prior to the *Shh* expression. These results suggest that the *Shh* expression is maintained by *Fgf8* in *lx* limb buds, and that the anterior shift of *Fgf4* and *Gre* is induced by the *Fgf8* signal. Furthermore, we observed anterior shift of the hindlimb position in the *lx* mutants. Thus, the *lx* gene is likely involved in positioning of hindlimb field and determing the *Fgf8* expression domain in normal limb development.

(4) Male sterility of a consomic strain B6.MSM-Chr.X and fine mapping of the X-linked gene responsible for the sterility

Ayako OKA, Nobuo TAKAGI[1], Kiyotaka TOSHIMORI[2], Akihiko MITA, Youichi MIZUSHINA, Noriko SAKURAI and Toshihiko SHIROISHI ([1]Res. Cent. For Mol. Genet. And Grad. Schl. Of Env. Earth Sci. Hokkaido Univ, [2]Department of Anatomy and Cell Biology, Miyazaki Medical College)

Hybrid sterility is a mechanism that safeguards the integrity of species by preventing a free flow of genetic information between related species. The infertility of inter-specific hybrids usually affects the heterogametic sex, which in the case of mammals is the male. In the mouse, male infertility has been found in hybrids between two species, *Mus spretus* and *Mus. Musculus*, from which most of the laboratory strains have been established. Understanding hybrid sterility might give us an insight into not only mechanism by which a new species is evolved, but also genetic regulation of gametogenesis in males.

Since several years ago, we have set out to construct a series of consomic strains that substitute each chromosomes of Japanese wild mouse-derived MSM strain (*Mus musuculus molossinus*) for the chromosomes of a laboratory strain C57BL/6J (*Mus musculus domesticus*). These inter-subspecific consomic strains would be of great use to study all kinds of multigenic traits. During the process of production of X-chromosome consomic strain, in which the X chromosome of C57BL/6J(B6) was replaced by the MSM-derived one, we noticed the lowered fertility of the males. It seemed attributable to the disturbance of coordination between gene(s) on the X chromosome of MSM ($X_{.MSM}$) and gene(s) on the B6 chromosomes. Those genes are thought to have been separated by a long period of evolution, approximately one million years. Previously, the severe reproductive disadvantage was reported in B6 strain that carries the X-chromosome of MSM strain (Takagi et al., 1994). The decline of the fertility was emphasized as backcross generations proceeded. Unlike $X_{.MSM}Y$ males, $X_{.B6}Y$ males in the same backcross generation were fully fertile. We observed that most epididymal spermatozoa from $X_{.MSM}Y$ males had shortened distal part of the head, while spermatozoa from B6 strain and $X_{.B6}Y$ males had well balanced hook-like head. To estimate the fertility of $X_{.MSM}Y$ males, *in vitro*

fertilization (IVF) was performed with their capacitated spermatozoa. Significant low fertility of IVF was observed in $X_{.MSM}Y$ males (6.1 ± 7.2%; N=6), though $X_{.B6}Y$ males retained high frequency (80.2 ± 23.3%). The frequency of pregnancy and the size of litters in natural mating were also evaluated. $X_{.MSM}Y$ males in N2 generation retained their fertility, i.e.4 out of 4 males were fertile, mean litter size 5.7 ± 3.5. Their fertility decreased with the progression of the backcross generations, and $X_{.MSM}Y$ males in N6 generation were fully sterile, while $X_{.B6}Y$ males in the same generation retained high fertility, i.e. 4 out of 4 were fertile, mean litter size 8.5 ± 1.7. In addition, the average of paired testicular weights were reduced in comparison with that of B6 males (B6; 102.7 ± 14.7mg, $X_{.MSM}Y$; 76.4 ± 13.5mg, N=8).

We intended to map gene(s) responsible for the morphological anomalies in their spermatozoa. QTL (quantitative trait loci) analysis demonstrated the most intense linkage with *DXMit166*, and the weaker linkage with an interval between *DXMit97* and *DXMit217*. It is of interest to note that the locus that controls the testis weight was mapped to an interval between *DXMit217* and *DXMit160*, which was possibly separated from the loci that control sperm morphology.

(5) Genetic modification of the phenotypes of a mouse progeria mutation, Klotho (*kl*)

Nobuyoshi JINNAI, Akihiko MITA and Toshihiko SHIROISHI

The klotho (*kl*) mouse was originally identified by insertional mutagenesis. The homozygous mutant mice show various phenotypes resembling human aging. The manifestations include short lifespan, atherosclerosis, gonadal atrophy, skin atrophy, emphysema, ataxia and ectopic calcification. They are used as model of human premature aging syndromes. Thus far, the klotho mutation has been introduced into various genetic backgrounds of the standard laboratory mouse strains. These mice didn't show remarkable difference in their phenotype in comparison with the klotho original stock, which was hybrid of C57BL/6J and C3H/J strains. By contrast, when the genetic background was replaced by MSM strain that was derived from Japanese wild

mice, we found that the mutant mice exhibited unique phenotypes different from the original stock. The klotho gene is expressed at the highest level in kidney. Nevertheless, no phenotype except for weak calcification has been observed in mice with genetic background of the standard laboratory strains. In this study, we observed severe pathological phenotype in the kidney of the mutant mice with the MSM background. On the other hand, abnormality in spermatogenesis, which is a representative phenotype of the original klotho mutation, is relieved at least at histological level. Furthermore, it is of interest to note that some of mutant mice homozygous for klotho exhibited phenotype indistinguishable from wild-type mice in the stage of early generations of the backcrossing to MSM, and they survived over about two years. The variation of phenotypes depending on genetic background suggests existence of modifier genes for the klotho phenotype and the presence of polymorphisms of the relevant gene(s) between the original stock and MSM strains. Now, we are conducting a linkage analysis to identify modifier genes interacting with the klotho gene.

(6) Mapping of Genes Responsible for the Performance in the Passive Avoidance Test Using Strains Derived from Wild Mice

Tsuyoshi KOIDE, Tamio FURUSE[1], Toshiyuki TAKANO, Kazuo MORIWAKI[2] and Toshihiko SHIROISHI ([1]Tokyo University of Agriculture and Technology, [2]Grad. Univ. Adv. Stud.)

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

BFM/2, KJR, SWN, MSM and C57BL/6, are the good learners in the passive avoidance test. In order to map the genes involved in the different performance between strains, we collected the progeny from a backcross, (C57BL/6 x BLG2)F1 x BLG2. The learning performance in the passive avoidance test was analyzed in each mouse. The tail DNAs were prepared for the following genetic study. The genome wide scanning using microsatellite markers was conducted using 282 mouse progeny from the cross. The typing data of genome wide scanning was analyzed by using the computer packages, MapManager QTX and QTL Cartographer. Linkage was observed on chromosomes 2, 7 and 18 at the suggestive level. Further genetic mapping is currently underway by increasing the number of mouse progeny in order to confirm the mapped data by statistical analysis. At the same time, we are on the way of making congenic strains by backcrossing C57BL/6 to BLG2 strain and selected the mice maintaining ability of good performance in the passive avoidance test. At the fifth generation of backcross, genome wide scanning was conducted. We found the persistence of the C57BL/6 alleles at the mapped loci on chromosomes 2, 7, and 18 in two of three congenic lines, which supported the idea that mapped loci are involved in performance in passive avoidance test.

(7) An analysis of sensitivity to capsaicin in the Mishima battery of mouse strains

Tamio FURUSE, David A. BLIZARD[1], Kazuo MORIWAKI[2], Yutaka MIURA[3], Kazumi YAGASAKI[1], Toshihiko SHIROISHI and Tsuyoshi KOIDE ([1]Tokyo University of Agriculture and Technology, [2]Center for Developmental and Health Genetics and Intercollege Graduate Program in Genetics, The Pennsylvania State University, [3]Graduate University of Advanced Studies)

Taste is usually classified into five classes: sweet, salty, sour, bitter, and umami. However, another type of pungent sensation produced by red peppers also plays an important role in the diet of different cultures. Red pepper is widely used as condiment of food in several cultures of the Central American, Asian, and African continents. Historically, red pepper has not been accepted as condiment for diet in European and North American continent. This differ-

ence is undoubtedly caused by both food culture and also may well be related to different levels of perception among people for pungent sensation that is controlled genetically. In order to approach the underlying genetic mechanism for diversity of preference for red pepper, we conducted a 12-hr, 1-bottle intake test of capsaicin solution using both male and female animals from the following inbred strains: 10 wild-derived inbred strains, PGN2, BFM/2, BLG2, NJL, CHD, HMI, SWN, KJR and MSM, 1 strain derived from the so-called fancy mouse, JF1, and 3 widely used domesticated strains, C57BL/6J, DBA/1J and BALB/cAnN. After two days of habituation, water intake was measured during the entire dark phase (20:00-8:00) for three days and the average volume of water intake was calculated. The concentration was then elevated successively each day as follows, 0.5, 1.0, 4.0, 7.0, 10.0 and 15.0 μ M. Fluid intake was calculated as percent of the control 3 day mean. Relative to baseline water intake, C57BL/6J and DBA/1J consumed 10 percent while KJR and MSM ingested approximately 60 percent of the 15 μ M capsaicin solution. In order to exclude the possibility that different levels of thirst affected intake of the capsaicin solution, we conducted a short-term, 3-hr, fluid intake test with some of the extreme strains from the first study. For measuring short-term fluid intake, the test was initiated just before the onset of the dark phase. Then, after three hours, the bottles were removed from the cages using a night-scope to avoid disturbing the animals in the dark phase of the circadian rhythm. Essentially the same result was obtained in that KJR and MSM consumed more capsaicin fluid than C57BL/6J and DBA/1J. In order to study whether this was caused by preference of capsaicin over water, a 2-bottle preference test was conducted on MSM and C57BL/6J strains. The mice were individually housed. For the first two days, two bottles both containing water were placed on the cage and the mice habituated to the test condition. From the third day, a bottle containing 0.5 μ M of capsaicin fluid was substituted for one of the water bottles. The positions of the tubes were alternated every 24 hours to control for side preferences. Fluid intake was measured every 24 hours for 10 days as previously described. In the 2-bottle test, however, both strains consumed a strikingly low level of capsaicin fluid relative to water with the preference ratio approximating 10% even at 0.5 μ M of concentration. There was no significant difference across strains. This data show that both

MSM and C57BL/6J strains are able to recognize the irritating sensation of capsaicin at low concentrations and avoid consuming capsaicin when the water is available. The pattern of strain differences of these sensitivities are similar to that displayed by these strains in the hot plate test that we had done previously. Because response to both heat and capsaicin are mediated by VR1 receptor, the similarity observed across hot plate and capsaicin intake tests, raises the possibility that the physiological pathway mediated by VR1 may be polymorphic among the strains. In addition to this possibility, further variations in the central pathways may be associated with the strain differences in capsaicin intake. In 1-bottle test, F1 progenies of KJR and C57BL/6 consumed capsaicin solution approximately same as KJR. This result indicates that the genes involved in capsaicin tolerance in KJR are dominant.

(8) Application of cryopreservation for wild mice embryos

Noriko SAKURAI[1], Youichi MIZUSHINA, Nobuyoshi JINNAI, Naomi NAKAGATA[2], Tsuyoshi KOIDE and Toshihiko SHIROISHI ([1]JAC, Inc., [2]Center for Animal Resources and Development, Kumamoto University)

Recently, the simple vitrification of mouse embryos was reported for preservation of the laboratory mouse strains. Although the method has been becoming popular, it is still not clear whether it is applicable to preservation of various mouse strains including wild stocks. In this study, we examined efficiency of the simple vitrification of embryos of the standard inbred strains and several wild-derived strains, and compared the efficiency with those obtained by the standard slow freezing methods. Embryos were obtained from *in vitro* fertilization, and 2cell embryos were cryopreservated by the two different methods. These embryos were thawed by the own methods, and morphologically normal embryos were transferred into presudopregnant females of B6C3F_1 mice. The pregnant mice were examined on day 18 of pregnancy to count the number of normal embryos. The result indicated that with most mouse strains there was no difference in the efficiency of the recovery from frozen embryos between two methods. However, efficiency by the simple vitrification with KJR/Ms and SWN/Ms strains was lower than that by the slow freezing method.

Contrary, with PGN2/Ms strain, we observed higher efficiency by the slow freezing method. With CHD/Ms stain, we could not obtain pregnant female at all by the both methods.

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F-b. Mammalian Development Laboratory

(1) Molecular mechanism of somite segmentation

Yumiko SAGA, Yu TAKAHASHI, Atsuya TAKAGI and Aya KITABAYASHI (National Institure of Health Sciences)

The somites are the first morphologically distinct segmental units formed in vertebrate embryo and give rise to metameric structures such as vertebrae, ribs and skeletal muscles. The somites are formed by segmentation from the anterior end of the presomitic mesoderm (PSM), which is unsegmented and posterior to the most recently formed somite. Each somite is subdivided into anterior (rostral) and posterior (caudal) compartments that differ in their properties and gene expression. The rostro-caudal polarity of a somite is established within the PSM prior to segmentation. However, molecular mechanisms underlying the formation of rostro-caudal polarity and the specification of segmental boundaries are totally unknown. Our aim in this study is to determine the mechanisms underlying the generation of a segmental pattern within the PSM.

Previously, we cloned *Mesp2*, a gene coding a novel bHLH transcription factor, MesP2. *Mesp2* is expressed in the rostral PSM. *Mesp2*-null mice exhibited defective somitogenesis due to the lack of rostral somitic compartment. *Notch1* expression is greatly reduced in *Mesp2*-null embryos, while *Mesp2* expression is downregulated in *Notch1*-, *Dll1*- or *RBP-Jk*-deficient embryos, indicating that MesP2 is in the feedback loop of the Notch signaling network. A membrane-bound protein presenilin-1(PS1), functions as a γ-secretase allowing the nuclear translocation of intracellular domains of Notch in both vertebrates and invertebrates. PS1-deficient mice exhibit defects in the segmentation as seen in Notch1 mutant mice. Based on the finding that, in mice, the lack either of two molecules involved in the Notch signaling pathway, MesP2 or Presenilin-1 (PS1) results in contrasting phenotypes, caudalized vs. rostralized vertebra, we adopted a genetic approach to analyze the molecular mechanism underlying the establishment of rostro-caudal polarity in somites. By focusing on the fact that expression of a Notch ligand, *Dll1* is critically important for prefigur-

ing somite identity, we found that MesP2 plays an important role to initiate establishment of rostro-caudal polarity by controlling two Notch signaling pathways. Initially, MesP2 may activate a PS1-independent Notch signaling cascade to suppress *Dll1* expression and specify the rostral half somite. While, PS1 mediated Notch signaling is required to induce *Dll1* expression in the caudal half somite. Therefore, MesP2- and PS1-dependent activation of Notch signaling pathways might differentially regulate *Dll1* expression resulting in the establishment of the rostro-caudal polarity of somites

(2) Function of Mesp1 and Mesp2 for heart formation

Yumiko SAGA and Satoshi KITAJIMA (National Institure of Health Sciences)

MesP1 and MesP2 are transcription factors containing an almost identical bHLH motif. *Mesp1* is expressed in the early mesoderm that is destined to become the extraembryonic and cranial-cardiac mesoderm. A lineage study using cre-lox system revealed that *Mesp1* is the earliest molecular marker expressed in the heart precursor cells. Previously, we generated MesP1- and MesP2-single-knockout mice to define their respective functions. Disruption of the *Mesp1* gene resulted in a morphogenetic abnormality of the heart, cardia bifida (Saga, 1998). *Mesp2*-single-knockout mice revealed the critical requirement of MesP2 for the normal somitogenesis (Saga et al., 1997). However, no notable defect was observed before somitogenesis in *Mesp2*-deficient mice, which was consistent with the apparent lack of expression of *Mesp2* during the early gastrulation stage. Analyses of *Mesp1*(-/-) embryos revealed that the defect is due to the delayed migratory activity of mesodermal cells. However, an abnormal heart was eventually generated in association with mesodermal migration (Saga et al., 1999). We found that this was the result of up-regulation of the *Mesp2* gene. Then we generated double-knockout (dKO) mice to define the precise functions of these two genes during gastrulation. Embryos deficient in both MesP1 and MesP2 lacked most of embryonic nonaxial mesoderm, and no posterior trunk structures developed. Furthermore, a chimera analysis revealed that dKO cells were scarcely observed in the anterior-cephalic and heart mesoderm, however, they did contribute to the formation of

the somites, notochord and gut. These results strongly indicate that the defect in the cranial-cardiac mesoderm is cell-autonomous, whereas the defect in the paraxial mesoderm is an non-cell-autonomous secondary consequence.

(3) Transcriptional regulation of Mesp1 and Mesp2 genes

Yumiko SAGA and Seiki HARAGUCHI (Shiga university of Medicine)

Mesp1 and *Mesp2* genes encode bHLH-type transcription factors, Mesp1 and Mesp2, respectively. The expression of both genes is observed in the nascent mesoderm, and subsequently in the rostral presomitic mesoderm. Our previous studies, however, suggested that *Mesp1* is predominantly expressed and plays a key role in gastrulation, while *Mesp2* is crucial for somitogenesis. To determine the regulatory mechanism for gene expression, we attempted to identify the enhancer elements by transgenic analysis. At least two enhancers which are responsible for the expression in the early mesoderm (early mesodermal enhancer: EME) and the presomitic mesoderm (PSM enhancer: PSME) and one suppressor, which is responsible for the rostrally restricted expression in the presomitic mesoderm, were identified. Furthermore, to determine whether each enhancer mediates the expression of *Mesp1* and *Mesp2* independently, we analyzed two types of knockout mice. When the *Mesp1* gene was deleted with EME (DE1), *Mesp2* expression was barely observed in the mesoderm of an embryo in the gastrulation stage. In contrast, in embryos that have targeted deletion of the *Mesp2* gene with PSME (p2-null), normal *Mesp1* expression was detected in the rostral presomitic mesoderm, suggesting the existence of an additional enhancer(s) that directs *Mesp1* expression in *Mesp2*-null mice. Therefore, EME is probably required for the expression of both *Mesp1* and *Mesp2* in the nascent mesoderm, while PSME may represent an enhancer specific to the *Mesp2* gene and is required for the expression during somitogenesis.

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F-c. Plant Genetics Laboratory

(1) Genetic dissection of embryogenesis, regeneration and gametogenesis of rice (*Oryza sativa*)

(1)-a. Regulation for expression of KNOX family class 1 homeobox genes of rice

Yukihiro ITO, Yasuo NIWA[1] and 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 five KNOX family class 1 homeobox genes and analysed their expression patterns during early embryogenesis and regeneration process by RT-PCR and *in situ* hybridization. We also found that constitutive expression of these genes are sufficient to maintain cells in a meristematic undifferentiated state. Since specific expression of KNOX family class 1 homeobox genes in a shoot meristem is essential for normal development of plant, we started to study its regulatory mechanisms. An *OSH1* promoter was shown to be ac-

tive in a leaf in addition to the shoot meristem, indicating that the promoter regeion is not sufficient to confer specific expression in the shoot meristem and other regions are necessary for its precise expression. Introduction of an *OSH1* cDNA into rice caused ectopic expression of endogeneous *OSH1* in the leaf and altered leaf morphology. These effects were independent of direction of the cDNA and presence or absence of the promoter. We obtained similar results for other genes such as *OSH6*, *OSH15* and *OSH71*. These results suggest that both the promoter regions and the exons are responsible for the specific expression of these genes in the shoot meristem.

(1)-b. Alternative splicing of KNOX family class 2 homeobox genes of rice

Yukihiro ITO, Hirohiko HIROCHIKA[1] and Nori KURATA ([1]Natl Inst. Agrobiol. Res.)

We previously identified three homeobox genes, *HOS58*, *HOS59* and *HOS66*, which were classified into KNOX family class 2. We detected alternative transcripts of these genes. The difference of the alternative transcripts was existence or absence of exon 1, which encodes an amino acid sequence with short stretches of alanin and glycin. Such characteristic sequences are suggested to modulate transcription or repression activities of these transcription factors. Because expression of each transcript of these genes was organ sepecifc, these gene products may have different activities depending on the organs. To identify loss-of-function mutants and to elucidate functions of these genes, we screened *Tos17*-insertional lines of rice. We identified a single line in which *Tos17* was inserted in a 3' untranslated region of *HOS59* and obtained a homozygous plant for the insertion. However, no difference of morphology and expression of *HOS59* were observed between the inserted line and wild type, suggesting that the *Tos17*- insertion gave no effect on *HOS59* activity in this line.

(1)-c. Isolation and sequence analysis of receptor-like protein kinase genes of rice

Kazuhiko TAKAYA, Yukihiro ITO and Nori KURATA

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* (*SERK*) of carrot and isolated its homologues from rice. We have cloned two genes and their cDNAs named *ORK1* and *ORK6*. The genome clone of *ORK1* showed unusual structure and detail analysis is in progress. *ORK6* encodes a protein with motifs characteristic to receptor-like protein kinases of plant. It contains, from N-terminus to C-terminus, a signal peptide, a receptor domain with leucin-rich repeats, a single transmembrane domain and a cytoplasmic protein kinase domain with serine/threonine specificity. The amino acid sequence of ORK6 is highly similar to plant receptor-like protein kinases such as SERK, CLAVATA1 and ERECTA of *Arabidopsis*, which are known to function in shoot meristem. Functional analysis of *ORK6* using transgenic rice is underway.

(1)-d. Functional analysis of *OsHAP3-1* using transgenic rice

Yukihiro ITO, Mitsugu EIGUCHI, Kazumaru MIYOSHI and Nori KURATA

LEC1 (*LEAFY COTYLEDON1*) gene was reported to be a central regulator of seed development in *Arabidopsis thaliana*. *LEC1* encodes HAP3 subunit protein, a component of transcriptional factor CBF (CCAAT-binding factor), which is ubiquitously present in many eukaryotes. We have isolated one of the HAP3 family genes, designated *OsHAP3-1* from rice and introduced *OsHAP3-1* cDNA driven by a CaMV 35S promoter in sense and antisense orientations into rice. Although the sense construct gave no effect on transgenic plants, the antisense construct caused pale green and short leaf phenotype on transgenic plants. Similar effects were also observed in transgenic rice with an RNAi construct. In rice there are a lot of mutants which show pale green phenotype, and mutations are mapped on chromosomes. To elucidate a possibility that *OsHAP3-1* corresponds to one of such mutations, we mapped *OsHAP3-1* on rice chromosome. *OsHAP3-1* was shown to locate between 125.5 cM and 140.5 cM of chromosome 1 in RI map and near this region *virescent6* is mapped (149 cM in CL map). We are now examining that *OsHAP3-1* is actually *virescent6* or not.

(1)-e. Analysis of genetic regulation of meiosis and gametogenesis in rice

Ken-Ichi NONOMURA, Kazumaru MIYOSHI, Hirohiko HIROCHIKA[1] and Nori KURATA ([1]Natl. Inst. Agr. Res.)

To elucidate genetic regulation of gametogenesis including meiosis and haploid gamete formation in rice, we screened sterile plants from mutants induced by tissue culture, in which transposition of rice endogenous retrotransposon Tos17 occurred to generate consequent insertional mutations. We selected forty lines of meiotic mutants and more than 60 mutants probably defective in gamete formation or pollination process. The meiotic mutations found in this study included the aberrations such as chromosome decondensation, asynapsis, desynapsis, meiocyte/ pollen immaturation, and so on. For cloning causal genes of the mutants, investigation about the linkage between meiotic or other sterile phenotypes and Tos17 transposition is underway.

We are also planning to utilize these meiotic mutants for cytological analyses of chromosome kinetics in rice meiosis.

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

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

Heterochronic mutations affecting the timing of developmental events may be of major significance in ontogeny and evolution. In plants, several heterochronic mutations that affect stepwise development of vegetative tissue and therefore alter shoot architecture have been identified. The recessive mutations at the rice *plastochron 1* (*pla1*) locus cause the short plastochron and ectopic expression of vegetative programs in the reproductive phase. To understand the molecular aspects of *Pla1* during plant development, we are trying to isolate the *pla1* gene by map-based cloning.

Following rough mapping of *pla1-1* allele on the short arm of chromosome 10 last year, we have carried out high resolution genetic and physical mapping

using 578 F2 segregants of *pla1-1* homozygous plants. A physical map encompassing the *pla1-1* locus was constructed by overlapping YAC and BAC clones through chromosome walking. The latest physical map showed that the *pla1-1* locus was localized in an interval of about 70 kb which corresponded to 0.17 cM genetic distance. The candidate BAC clone carrying the *pla1* gene have been sequenced almost entire length by the international rice sequencing consortiam and this would facilitate positional cloning of the gene.

(3) Analysis of centromere structure of rice chromosome 5 toward construction of plant artificial chromosome

Ken-Ichi NONOMURA, Tadzunu SUZUKI and Nori KURATA

The primary structure of the centromeric region of rice chromosome 5 was analyzed for long-range, as the first case in cereal species. The YAC and BAC contigs aligned on the centromere of the rice chromosome 5 (CEN5) covered the distance of more than 670-kbp long. The strong suppression of genetic recombination, one of the remarks for functional centromere, occurred along the contig region. The most remarkable nature of the CEN5 is the composition of the multiple repetitive elements. *Oryza*-specific short tandem repeats RCS2 clustered along less than 100-kbp long on the contig. At least fifteen copies of the conserved domain of 1.9-kbp RCE1 centromeric repeats, which are identical to the long terminal repeats (LTRs) of *gypsy* type retrotransposon *RIRE7*, were dispersed mainly in 320-kbp long next to RCS2 tandem clusters. Many copies of the LTR-like sequences of *RIRE3* and *RIRE8*, another *gypsy* type retrotransposon, were also found along all of the contig. On the other hand, *gag-pol* regions were less conserved in the contig. These results indicated that the rice centromere is composed of multiple repetitive sequences and RCS2 tandem clusters probably being situated as a core of the functional centromere in some hundreds kb to Mb length (see reference 1). Construction of rice artificial chromosomes with centromeric YAC and BAC clones selected in this study is now on the way and after then trails to transform rice cells with artificial chromosomes should be also carried out.

(4) A large scale isolation and characterization of rice nuclear protein genes

Kazuki MORIGUCHI and Nori KURATA

In many organisms, genes function in the nucleus are expected to be a 10-20% of total genes of the organism. The proteins working in the nucleus; chromosomal proteins, nuclear matrix-associated proteins, transcription regulators, proteins related to replication and transcription machineries, should have significant roles in the nucleus. However, little is known about biological and biochemical status of nuclear proteins in respect to developmental stages and physiological conditions of the plants.

The primary aim of this theme is to comprehensively isolate and structualy analyse nuclear protein genes using rice as a monocot plant model. The second aim is to make clear what events and factors are necessary for organizing and maintaining genome structure and functions in the nucleus by observing the localization, movement and expression specificity of the isolated nuclear proteins. For the isolation of nuclear protein genes, we use yeast nuclear transport trap (NTT) system, which can specifically trap NLS coding cDNAs (Ueki et al., 1998. Nat. Biotechnol., **16**: 1338-1342.). The two cDNA libraries from panicles, which contain cells of early embryogenesis and meiotic stages, has been used. In 2000, we started sequencing analysis of trapped cDNA clones. At present, we trapped more than 100 independent clones and found a tendency that the clones encoding transcription factors are more efficiently trapped than the results obtained in the mouse. On the other hand, the cDNA clones encoding non-nuclear proteins have been also trapped, yet they were low efficiency. The population of non-nuclear proteins trapped by the system would be needed to clarify.

In 2001, to inspect the statistical reliability of NTT system for the isolation of plant nuclear proteins, we start to check the nuclear localization of trapped genes by expressing cDNA-GFP fusion proteins. After the inspection, we are planning to trap and analyze their nucleotide sequences of additional 1000 independent clones.

(5) Genome-wide analysis of reproductive barriers in the intra-specific rice hybrids and positional cloning of one of the barriers

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

Yoshiaki HARUSHIMA and 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. The distortions of allele frequencies from Mendelian expectation in progeny of inter- or intra- species hybrid due to hybrid sterility genes, hybrid breakdown genes and gametophytic competition genes have been often observed. We have developed a new method for detecting the map location and gene action of loci that contribute to the distortions of allele frequencies from Mendelian expectation by regression analysis of allele frequencies of markers covering an entire genome (submitted). Asian rice cultivars, *Oryza sativa*, can be classified into two main types, Japonica and Indica, based on several characteristics. To clarify the state of Japonica-Indica differentiation, all reproductive barriers causing allele frequency distortions from Mendelian expectation in F_2 populations were mapped and compared among three different Japonica-Indica crosses. The number of reproductive barriers in the three crosses was similar, however most of the barriers were mapped at different loci. Therefore, these reproductive barriers formed after Japonica-Indica differentiation. In the three Japonica-Indica cross combinations, the genetic variations within both Japonica and Indica are small as shown as high similarity of the restriction fragment length of RFLP markers. Considering the high genetic similarity within Japonica cultivars and Indica cultivars, the differences in the reproductive barriers on each cross were unexpectedly numerous. The reproductive barriers of Japonica-Indica hybrids likely evolved more rapidly than other genetic elements (submitted).

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

Yoshiaki HARUSHIMA and Nori KURATA

The aim of this study is isolation of the most prominent barrier on chromosome 3 detected in F2 of Nipponbare-Kasalath hybrid by positional cloning, and elucidation of the molecular nature of the individual reproductive barriers. In the last year, we have clarified the aimed gene was a male gametophyte gene that interact with maternal locus on chromosome 6. In other words, the pollen with Kasalath genotype at the gametophyte gene preferentially fertilized by 94% probability in maternal plant that is heterozygote or Kasalath homozygote at the interactive locus on chromosome 6. For detailed mapping the gametophyte gene, we have selected plants with recombination in 1.9cM interval from 1000 F2 and 473 backcross plants and retrieved their genomic DNA of the selected plants from bulked young leaves of their selfed seedling. Considering the genotypes of the interactive locus, the genetic map of the male gametophyte gene is being constructed by the dosage analysis using the selected population. The physical map of this region is also being constructed using Nipponbare BACs. Nine BACs were selected by the closest RFLP marker, C582, that was mapped 0.13cM away from the male gametophyte gene.

(6) Generation of enhancer trap lines of rice

Yukihiro ITO, Mitsugu EIGUCHI and 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 named *Ds-GUS* that harbours a GUS coding region with a CaMV 35S minimal promoter and a hygromycin resistance gene. *Ds-GUS* was flanked by the 35S promoter and a coding region of a selectable chlorsulfuron resistance gene, so that excision of *Ds-GUS* causes

connection of the 35S promoter and the coding region and can confer chlorsulfuron resistance. We also use a 35S-Ac transposes (AcTPase) gene together with a bialaphos resistance gene to supply transposase which is essential and sufficient for transposition of *Ds-GUS*. We generated forty-two transgenic rice lines with a single copy of *Ds-GUS* and six lines with 35S-AcTPase. We crossed four lines of *Ds-GUS* with six lines of 35S-AcTPase and obtained F1 seeds from seventeen combinations. The exision of *Ds-GUS* in leaves of the F1 plants was observed in eight combinations out of seventeen. We screened germinal transposants at F2 generation by PCR. Out of 10,524 examined in total, 675 (6%) were judged to be transposants. Since the transposants showed the chlorsulfuron resistance as expected from the structure of the vector, we further screened transposants by the chlorsulfuron and the hygromycin resistance and 1,564 were judged to be transposants. Frequency of the germinal transposition was different from plant to plant and further panicle to panicle even in the same plant in both screenings. This suggests that the transposition of *Ds-GUS* took place during panicle development and that the transposants obtained from different panicles were independent. To obtain a large number of transposants we crossed another twenty-two *Ds-GUS* lines with the 35S-AcTPase line. Screening of transposants will be carried out in 2001. We will also conduct mapping of *Ds-GUS* insertion sites on rice chromosomes to select chromosome specific *Ds-GUS* lines. Combinations with the AcTPase lines and one AcTPase line could not excise the *Ds* in any combinations with the *Ds* lines. This result indicates that this enhancer trap system is functional in rice. Screening of germinal transposants is now in progress.

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plasmid indicates its chimeric structure and evolutionary relationship between tumor-inducing (Ti) and symbiotic (Sym) plasmids in *Rhizobiaceae*, J. Mol. Biol., in press.
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F-d. Microbial Genetics Laboratory

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

Akiko NISHIMURA

We found previously that *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 missence 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.We also identified novel Ap4A binding protein A (AbpA) and analyzed N terminal sequence of amino acid. Conditional null mutation of *abpA* induced delayed cell division, and overproduction of *abpA* induced Cfc phenotype.

(2) HscA is involved in the dynamics of FtsZ-ring formation in *Escherichia coli* K12

Tsuyoshi UEHARA, Hiroshi MATSUZAWA[1] and Akiko NISHIMURA ([1]Department of Bioscience and Biotechnology, Aomori University)

FtsZ, a universal and essential protein for cell division in prokaryotes, possesses GTP-binding and GTPase activity. This protein is a structural and functional homologue of eucaryotic tubulin, capable of dynamic assembly in the presence of GTP. Depolymerization of FtsZ consumes GTP *in vitro*. FtsZ proteins are found scattered in the cytoplasm during most of the cell cycle, but re-localize in the early stages of septum apparatus construction to the middle of the cell. There, these proteins assemble into a cytokinetic ring on the inner membrane; FtsZ proteins maintain a position at the leading edge of the invaginating septum, followed by the assembly of other proteins, such as FtsA, FtsI, FtsK, FtsL, FtsN, FtsQ, FtsW, and ZipA, and constriction of the FtsZ-ring. FtsZ-rings are therefore observed at potential division sites, even in the filaments of these cell division mutants. After completion of cell division, the FtsZ proteins again re-localize throughout the cytoplasm of the daughter cells. The molecular mechanism governing the dynamic localization of FtsZ proteins remains unknown; no factors involved in the dynamics of FtsZ-ring formation have previously been discovered.

JE10715 mutant bacteria form multinucleated filaments at 42℃. In immunofluorescence analysis of the filaments, FtsZ-ring was highly reduced at potential division sites. Mapping of this mutation by P1-phage mediated transduction, complementation analysis, and sequencing of genomic DNA demon-

strated that JE10715 phenotype results from a missense mutation in the *hscA* gene, corresponding to a single alanine to valine substitution at position 192 in the ATPase domain. *hscA* conditional knockout of wild type strain exhibited an abnormal localization of FtsZ and reduced growth, although grew producing longer rod cells, at non permissive conditions. Overexpression of *dnaK* partially complemented the JE10715 mutant. *In vitro* experiments demonstrated that the ATPase activity of HscA715 was reduced to 63% of wild-type levels. HscA cosedimented with FtsZ-polymers in the presence of GTP. 90° angle light scattering revealed that HscA715 inhibited the polymerization of FtsZ in the presence of GTP at 36℃. We conclude that HscA is involved in FtsZ-ring formation, interacting with FtsZ; defects in *hscA*, however, are partially compensated for by the redundant genes including the wild type *dnaK*.

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

Shinsuke NAKADE, Kimiko SAKA, Kunio MATSUMOTO, Masanari KITAGAWA, Hirotada MORI[1] and Akiko NISHIMURA ([1]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 cell division. To isolate a whole set of cell division genes and to analyze the hierarchy and network responses in expression of cell division genes as a model case, we have selected 334 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 by complementation using *E. coli* gene bank. Furthermore, to assign a corresponding ORF to each *fts* mutation by genetic analysis, vectors were constructed and cloning has been performed for 2,500 ORFs. Using 1,000 ORF clones, complementation test was done for 21 fts mutants; as the results, 11 fts mutants were identified.

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2. NISHIMORI, K., TAKAGI, H., WACHI, M., FUJISHIMA, H., KAWABATA, T., NISHIKAWA, K. and NISHIMURA, A.: (2001) The *kdsA* mutations affect the FtsZ-ring formation in *Escherichia coli*.(submitted).

F-e. Invertebrate Genetics Laboratory

(1) Control of tracheal tubulogenesis by Wingless signaling

Takahiro CHIHARA and Shigeo HAYASHI

The tubular epithelium of the Drosophila tracheal system forms a network with a stereotyped pattern consisting of cells and branches with distinct identity. The tracheal primordium undergoes primary branching due to an induction by the FGF homolog Branchless, differentiates cells with specialized function such as fusion cells that perform target recognition and adhesion during branch fusion, and extends branches toward specific targets. Specification of an unique identity for each primary branch is essential for directed migration, as a defect in either the EGFR or Dpp pathways leads to a loss of branch identity and the misguidance of tracheal cell migration. Here, we investigate the role of Wingless signaling in the specification of cell and branch identity in the tracheal system. Wingless and its intracellular signal transducer, Armadillo, have multiple functions, one specifying dorsal trunk through activation of Spalt expression and the other inducing differentiation of fusion cells in all fusion branches. Moreover, we show that Wingless signaling regulates Notch signaling by stimulating Delta expression at the tip of primary branches. Those activities of Wg signaling are combined to specify the shape of dorsal trunk and other fusion branches. For detail, see ref. 2.

(2) EGF receptor attenuates Dpp signaling and helps to distinguish the wing and leg cell fates in Drosophila

Kazumasa KUBOTA[1], Satoshi GOTO, Kazuhiro ETO[1] and Shigeo HAYASHI ([1]Department of Molecular Craniofacial Embryology, Graduate School of Tokyo Medical and Dental University)

Wing and leg precursors of Drosophila are recruited from a common pool of ectodermal cells expressing the homeobox gene Dll. Induction by Dpp promotes this cell fate decision toward the wing and proximal leg. We report here that the receptor tyrosine kinase EGFR antagonizes the wing-promoting function of Dpp and allows recruitment of leg precursor cells from uncommitted ectodermal cells. By monitoring the spatial distribution of cells responding to Dpp and EGFR, we show that nuclear transduction of the two signals peaks at different position along the dorsoventral axis when the fates of wing and leg discs are specified and that the balance of the two signals assessed within the nucleus determines the number of cells recruited to the wing. Differential activation of the two signals and the cross talk between them critically affect this cell fate choice. For detail, see ref.1.

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

Hitashi MATAKATSU, D BRENTRUP[1] and Shigeo HAYASHI ([1]Max Plank Institute for Biophysics, Getchingen, Germany)

The wing of Drosophila is separated into several sectors by the wing veins. Vein primordia are specified by the positional information provided by hedgehog and decapentaplegic in the wing imaginal disc and express the key regulatory gene rhomboid. We have previously cloned the gene *plexus* involved in the repression of rhomboid transcription. *plexus* was found to encode a novel nuclear matrix protein. To understand molecular functions of *plexus*, we studied the function of *net* that appears to act together with *plexus* to repress vein differentiation. Brentrup et al have shown that *net* encodes a basic helix-loop-helix protein expressed in the intervein territories where *rho* transcription is re-

pressed. We have shown that *plexus* and *net* act synergistically to repress *rho* transcription. Plexus affects nuclear localization of Net. Those results together suggest a hypothesis that the function of plexus is to tether Net protein to specific location of the nucleus to facilitate repression of specific target genes such as *rho*. This idea is being tested by genetic and biochemical analyses.

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

Satoshi GOTO, Misako TANIGUCHI, Yukiko SADO and Shigeo HAYASHI

To identify novel genes and gene functions in the pattern formation of the imaginal disc and the trachea, we are conducting an enhancer trap screen using the Gal4-UAS system. About 4500 lines were established in collaboration with groups in Japan and were examined for the activity of enhancers flanking the inserts in embryos, larvae and adults. This year we sequenced genomic DNA flanking the P-element insertion points of all the lines, and successfully mapped about 3000 of them onto unique locations in the annotated complete genomic sequence of Drosophila. Insertions were grouped into 1722 clusters, most of which lie close to 5' ends of transcription units. Those data should be of great value for the use of Gal4 strains in manipulating gene expression patterns, studying P-element insertion specificity in the genome, and the comprehensive analysis of gene functions.

Publications

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2. CHIHARA, T. and HAYASHI, S.: Control of tracheal tubulogenesis by Wingless signaling. Development, **127**, 4433-4442, 2000.

G. Center for Genetic Resource

G-a. Genetic Informatics Laboratory

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

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

The Oryzabase is a comprehensive rice science database established in the collaboration with rice researcher's committee in Japan.

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

The Oryzabase map represents the integration of seven different maps from classical linkage map to the latest physical map provided by Rice Genome Project.

The gene dictionary has been constantly updated by the specialists coupled with the activity of Rice Gene Cooperative.

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

We are planning to do more extensive cross-referencing of Oryzabase to the sequence database, literature database and other rice databases in the world. Oryzabase is available from http://www.shigen.nig.ac.jp/rice/oryzabase/.

We are constructing the Kyushu Rice Genetic Resource DB and the NIG Rice Genetic Resource DB separately from the Oryzabase because of the discrepancy of data sets.

These databases are enriched with rather agronomical data.
Oryzabase architecture should be updated to integrate these data.

(2) PEC : Profiling of Escherichia coli Chromosome

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

The Profiling of Escherichia coli chromosome (PEC) database has been constructed to compile any relevant information that could help to characterize the E. coli genome, especially with respect to discovering the function of each gene. The database is intended to provide an interface comprehensible to most experimental researchers. The E. coli genetic resource committee of Japan supports the construction and maintenance of this database.

The database consists of 8 parts, (1) Basic information about each gene, (2) The essentiality of each gene for cell growth, classified as essential, non-essential, or unknown based on information from research reports and deletion mutation studies. The criteria for classification are described below. References, on which the classification is based, are listed in the format "Medline (PMID)" in the section for each gene, (3) The names of the strains related to each gene, which are stored in the Stock center of the Natl. Inst. Genet, Japan, (4) Results of similarity searches for each gene product (BLAST, PSI-BLAST, FASTA), (5) Structural features (domain, motif, etc.) for each gene product, (6) Results of comparative analyses of each gene (homologues in other sequenced bacteria, etc.), (7) Information from rather long deletion mutations, and (8) Locations of Kohara clones.

PEC provides the Genomic-, Linear- and Motif overviews.

In the genomic overview the E. coli genome is displayed as a circle, on which the locations of the tRNA genes have been marked. Essential genes, non-essential genes, and unknown genes are painted in different colors, allowing easy visualization of their distribution on the genome. In the linear view, the genome is linearly displayed. Each gene is displayed in a linear manner along the genome together with its name, direction, size, location, and class (essential, non-essential, unknown). Also, the regions deleted in the deletion mutant(s) for each gene are shown. This view also shows classical markers, whose exact locations remain unknown, contig information, and the location of

the Kohara clones. As the Kohara clones are derived from the strain W3110, there positions were assigned by doing a homology search on the MG1655 genome. Structural domains and motifs of a gene product are displayed graphically along with those of other genes having the same domains or motifs in the motif overview.

PEC is available at http://www.shigen.nig.ac.jp/ecoli/pec/.

Publications

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2. YAMAZAKI, Y., YOSHIMURA, A., NAGATO, Y. and KURATA, N.: ORYZABASE--INTEGRATED RICE SCIENCE DATABASE, Fourth International Rice Genetics Symposium, Laguna, Philippines, October.

G-b. Genome Biology Laboratory

(1) NEXTDB: The nematode expression pattern database

Tadasu SHIN-I and Yuji KOHARA

We are updating "NEXTDB" that integrates all the information of ESTs, gene expression patterns and gene functions of *C.elegans* which are being produced and analyzed in this laboratory, and, are preparing to make it open by the worm meeting. Images of whole mount *in situ* hybridization for mRNA were taken by CCD cameras equipped on Zeiss Axioplans, loaded to the Sun workstation to process and arrange them properly in the database. Then, they were annotated with respect to developmental stages and expression patterns on the database. Images of immunostaining taken on Zeiss LSM510 confocal microscope, and images and descriptions of RNAi phenotypes, were also stored in the database. New version of NEXTDB contains about 12,000 unique cDNA groups, in situ images of about 7,600 cDNA groups, RNAi phenotype images of 160 groups, and immunostaining images of 60 groups. The new version will be demonstrated and will be available over the Internet. URL: <http://helix.genes.nig.ac.jp/db/index.html>

(2) The worm transcriptome project

Jean THIERRY-MIEG, Danielle THIERRY-MIEG, Yutaka SUZUKI[2], Sumio SUGANO[2], Kazuko OISHI, Masako SANO, Hisayo NOMOTO, Shinobu HAGA, Satoko NISHIZAKA, Hiroko HAYASHI, Fumiko OHTA, Sachiko MIURA, Hiroko UESUGI, Michel POTDEVIN[3], Yann THIERRY-MIEG[1], Vahan SIMONYA[1], Adam LOWE[1], Tadasu SHIN-I and Yuji KOHARA ([1]NCBI, NIH, [2]Institute of Medical Sciences, U. of Tokyo, [3]CRBM, CNRS)

As of March 2001, 16175 yk clones have been sent to researchers around the world, in answer to 3463 requests. The main progress since the last worm meeting has been the isolation of full length cDNA libraries, selected by the cap and stage specific. All 83,000 cDNA clones and 9000 sequences collected from other sources including public databases were coaligned using the Acembly program on the 99.4 Mb of genome sequence (many thanks to the Consortium). We confirm the very high quality of the genome and estimate, from the unaligned clone proportion, that we are now missing only 0.7 to 1.2 Mb of genome. The basecall of the cDNAs was edited, and cloning artifacts, such as mosaic clones, internal deletions or inversions, internal oligo dT priming or unspliced RNA / DNA, were flagged and treated with appropriate care: these amounted to 1% of the clones from the first yk libraries, 5% of the capped clones and 2% of the clones in Genbank.

As of today, we have cDNAs in Acembly/AceView for just over half of the *C.elegans* genes: 10126 genes produce 14154 transcripts through alternative splicing or alternative polyadenylation. We have started to annotate the proteins and to submit the results to the public databases. We will submit in priority the genes you ask for. We use names, such as 1K18 (mec-8), that contain chromosome, megabase (letter), kilobase number and strandedness (even/odd) and allow easy distinction from the predictions.

Indeed, contrary to the outstanding quality of the genome sequence, the Wormpep de novo predictions are usually incorrect: in a sample of 111 newly hit genes, only 33% were exactly correctly predicted: 7% were not predicted, the first and last exons were incorrect in 35% and 28% cases respectively (in particular 9% of genes touch two predictions), and finally, in the common region,

33% of the genes had at least one internal exon incorrectly predicted. Gene prediction remains a difficult problem.

The new capped libraries are a huge step forward for the transcriptome project. More than 80% of the 12000 capped clones actually contain the entire mRNA, from 5' cap to polyA. Transpliced leaders are present in up to 65% of the genes, the remaining 35% genes are not transpliced (for example, collagens or surface proteins). We identify 12 types of transpliced leaders, possibly encoded by 30 SL genes clustered in 15 loci: each SL gene is followed in the genome by a strong donor site. Among the genes transpliced, 76% are transpliced to SL1 exclusively, the remaining 24% are transpliced to SL2/SL12. We confirm Tom Blumenthal et al 's hypothesis relating minor SLs to operons: the 700 genes with SL2/12 are most often located less than 300 bp downstream of an expressed gene in cis. The proportion of pure SL1 genes with such a close neighbor is 20 times lower, and, for genes not transpliced, 10 times lower. The specific transpliced leader used appears to be stage dependent: in particular, usage of the minor leaders increases from 5% in embryos to more than 25% in adults. In a compact genome such as C.elegans', close genes tend to be cotranscribed. Such physical constraints for coexpression of close genes during development could be tuned by controlled availability of minor leaders.

(3) Open-reading-frame sequence tags (OSTs) support the existence of at least 17,300 genes in *C. elegans*

Jerome REBOUL[1], Philippe VAGLIO[1], Jean FRANCOIS RUAL[1], Nicolas THIERRY-MIEG[2], Troy MOORE[3], Cindy JACKSON[3], Tadasu SHIN-I, Yuji KOHARA, Danielle THIERRY-MIEG[4], Jean THIERRY-MIEG[4], Hongmei LEE[5], Joseph HITTI[5], Lynn DOUCETTE-STAMM[5], James L. HARTLEY[6], Gary F. TEMPLE[6], Michael A. BRASCH[6], Jean VANDENHAUTE[7], Philippe E. LAMESCH[7], David E. HILL[1] and Marc VIDAL[1] ([1]Dana-Farber Cancer Institute and Harvard Medical School, [2]Laboratoire LSR-IMAG, [3]Research Genetics, [4]NCBI, NIH, [5]Genome Therapeutics Corp., [6]Life Technologies Inc., [7]Universitaires Notre-Dame de la Paix)

The genome sequences of *Caenorhabditis elegans*, *Drosophila melanogaster* and *Arabidopsis thaliana* have been predicted to contain 19,000, 13,600 and

25,500 genes, respectively. Before this information can be fully used for evolutionary and functional studies, several issues need to be addressed. First, the gene number estimates obtained in silico and not yet supported by any experimental data need to be verified. For example, it seems biologically paradoxical that *C. elegans* would have 50% more genes than *D. melanogaster*. Second, intron/exon predictions need to be experimentally tested. Third, complete sets of open reading frames (ORFs), or ORFeomes, need to be cloned into various expression vectors. To simultaneously address these issues, we have designed and applied to *C. elegans* the following strategy. Predicted ORFs are amplified by PCR from a highly representative cDNA library using ORF-specific primers, cloned by Gateway recombination cloning, and then sequenced to generate ORF sequence tags (OSTs), as a way to verify identity and splicing. In a sample (n=1,222) of the nearly 10,000 genes predicted ab initio (that is, for which no expressed sequence tag (EST) is available so far), at least 70% were verified by OSTs. We also observed that 27% of these experimentally confirmed genes have a different structure from that predicted by GeneFinder. We now have experimental evidence that supports the existence of at least 17,300 genes in *C. elegans*. Hence we suggest that gene counts based primarily upon ESTs may underestimate the number of genes, in human and in other organisms.

(4) Genome-wide functional analysis by RNAi-by-soaking with a non-redundant cDNA set

Ikuma MAEDA[1], Atsuko MINAMIDA[1], Masayuki YAMAMOTO[1], Yuji KOHARA and Asako SUGIMOTO[1] ([1]Dept. of Biophysics and Biochemistry, University of Tokyo)

The genome of *C. elegans* consists of approximately 19,000 genes, and the function(s) of many of them are still unknown. We have been performing a systematic functional analysis of the expressed genes by the RNAi-by-soaking method 1. In this method, L4 worms are soaked in dsRNA solutions for RNA delivery, and their F1 phenotypes as well as P0 germline phenotypes are examined. As templates for in vitro dsRNA synthesis, a set of tag-sequenced non-redundant cDNAs corresponding to ~10,000 genes (representing half of the predicted genes) has been used. Because the size of the cDNA set is compa-

rable to the number of detectable genes by a whole-genome DNA microarray, this cDNA set is likely to contain most genes expressed in the development of *C. elegans*.

We examined the RNAi phenotypes for ~2,800 genes (15% of the predicted genes) to date, and 27% of them showed detectable developmental phenotypes under a dissection microscope. The ratio of the cDNA clones that caused phenotypes was apparently higher than that of the predicted ORFs (13~14%)3,4, consistent with the finding by Fraser et al.(2000) that ORFs having EST are more likely to give RNAi phenotypes. Thus, the RNAi screen using the non-redundant cDNA set is an efficient way to comprehensively identifying essential genes for development. The general RNAi phenotypes observed were; F1 embryonic lethality (14% of the examined clones), F1 post-embryonic lethality (4.5%), P0 sterility (7%), F1 sterility (1%), and morphological abnormality (0.7%). Of these, we analyzed the phenotypes of F1 sterility in detail with DIC microscopy and DAPI staining, and have identified 31 genes that might play important roles in germline development. Each cDNA clone affected a distinct process, such as proliferation of germ cells, germline sex determination, gonadogenesis, gametogenesis, and fertilization.

The genes corresponding to the cDNA clones we used distribute almost evenly to all chromosomes. The incidence of the essential genes was comparable among the autosomes (26% ~ 34%), however, that on the X chromosome was significantly lower (16%). Because a mutation in the essential genes on the X chromosome would directly lead to the lethality/sterility of males, they appear to be removed from the X chromosome in the process of evolution.

We aim to complete the RNAi screen of the whole cDNA set. The functional information obtained in this work will provide a starting point for further analysis of each gene.

(5) Expression based RNAi reveals the function of proteasome in oocyte maturation and fertilization in *C. elegans*

Keiko HIRONO, Kyoko NAKATA, Yumiko UETA, Mina IWATA, Masahiro ITO and Yuji KOHARA

Our cDNA/expression pattern project has provided unique sets of limited numbers of genes that possibly participate in specific stages, lineages, tissues and so on. Such sets of genes are suitable to systematic analysis to elucidate the molecular mechanisms governing the biological processes. Since we are particularly interested in early embryogenesis, we have focused on a set of genes whose mRNA are maternally supplied and disappear or localize to specific cells during early stages (2-cell~early gastrulation). We found out 477 (about 10%) such genes when the expression analysis of about 5,000 genes was finished, and have done RNAi (RNA mediated interference) analysis on the 477 genes.

We microinjected double-stranded RNA into the gonads of N2 worms. 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, and so on. Out of the 477 genes, 5% showed the reduction of the number of F1, 33% showed F1 embryonic lethality, 27% showed one or various phenotypes with only escapers and 35% did not show any phenotype. Interestingly, genes in X chromosome showed much higher ratio of no-phenotype than those in autosomes (X chromosome: 42/71, autosomes: 125/404).

We further analyzed early cell division using the 4D microscope for the genes that showed more than 50% embryonic lethality (61 genes) in the RNAi screening. Out of them, 12 genes showed the arrest at fertilized egg and no division, 29 genes showed aberrant process until 8-cell stage and 20 genes showed no phenotype until 8-cell stage.

Interestingly, 5 out of the 12 genes of the severest phenotype (arrest at fertilized egg) related to proteasome; 2 genes are proteasome components and 3 genes are proteasome regulatory subunits. Proteasome is known to participate in the regulation of meiotic maturation and fertilization in fish and frog

oocytes. Thus, we examined all possible proteasome genes. In *C.elegans*, 14 genes for proteasome components are predicted. cDNA are available in our collection for 13 genes including the 2 genes in the above analysis. Their mRNA were detected in gonads (12/13), strongly detected in extreme early stage embryo and disappear by early gastrulation (6/13) or localize to specific cells (6/13). We did RNAi experiments for these genes, and they showed high F1 embryonic lethality (12/13) and strong reduction of the number of F1 (12/13). The affected embryos did not show any cell divisions and seemed to be arrested during meiosis like the previous ones. Immunostaining for one of the genes showed strong staining at nuclei of oocytes. These results indicate that proteasome plays important roles during meiosis in *C.elegans*. The experiments on the genes of proteasome regulatory subunits are in progress. Some of them show similar results to that of proteasome components.

(6) Translational control of maternal *glp-1* mRNA by POS-1 and its interacting protein PIP-1

Ken-ichi OGURA, Shohei MITANI[1], Keiko GENGYO-ANDO[1] and Yuji KOHARA ([1]Tokyo Women's Medical University School of Medicine)

A maternal factor POS-1 regulates cell fate determination of early embryos (Tabara et al., Development, **126**, 1-11, 1999). POS-1 has two TIS11 type (CCCH) zinc finger motifs, and is present in cytoplasm of posterior blastomeres, being a temporary component of P granules. Previously, we reported that POS-1 binds to PIP-1 that has an RNP type RNA binding motif. PIP-1 is present in cytoplasm of oocytes and early embryos, also being a temporary component of P granules. RNAi analysis showed that PIP-1 is also required for cell fate determination of early embryos.

This time, we have isolated a deletion null mutant *pip-1(tm291)* and found that the phenotypes are identical to those of RNAi, and that POS-1 and PIP-1 regulate maternal glp-1 mRNA translation. Translation of the maternal *glp-1* mRNA is temporally and spatially regulated by the 3' UTR (Evans et al., Cell, 77, 183-194, 1994). In wild type embryos, GLP-1 begins to be detected in anterior AB blastomeres at two-cell stage. At four-cell stage, GLP-1 is only

detected in anterior blastomeres ABa and ABp, but not in posterior EMS and P2. We found that GLP-1 was not detected in *pip-1* embryos, and that GLP-1 was ectopically detected in the posterior blastomeres in *pos-1* embryos. At four-cell stage embryos of *pos-1*, GLP-1 was detected in posterior EMS and P2 in addition to anterior ABa and ABp. GLP-1 was not detected in *pos-1* oocytes and one-cell stage embryos, suggesting that the temporal regulation is normal. We examined POS-1 localization in *pip-1*, and PIP-1 localization in *pos-1*, and found that their localizations were unchanged. These results suggest that PIP-1 is a positive regulator and POS-1 is a negative regulator of the translation of maternal *glp-1* mRNA. In *pos-1; pip-1* double RNAi embryos, the GLP-1 localization was identical to that of *pos-1* embryos, suggesting that *pos-1* is genetically epistatic. Finally, by using the three hybrid system, we found that POS-1 specifically interacted with spatial control region (SCR) identified in the 3' UTR of *glp-1* mRNA (Rudel and Kimble, Genetics, 157, 639-654, 2001), but PIP-1 did not. We will discuss possible mechanisms of the translational regulation of maternal *glp-1* mRNA.

(7) Computer Simulation of Early Cleavage of *C. elegans* Embryo

Atsushi KAJITA, Masayuki YAMAMURA[1] and Yuji KOHARA ([1]Tokyo Institute of Technology)

Cellular arrangements are important for development. In *C. elegans*, cell-cell interactions are essential for cell-fate determination in early embryos. The arrangement of the cells in embryo is largely restricted by physical conditions including the force and direction of cell division, the existence of hard egg shell, the properties of cell adhesion, membrane, cytoskeleton, and so on.

Here we present a computer simulation of early cleavage of *C. elegans* embryo, which is constructed to provide a platform to examine the relationships among various physical parameters and early cleavages by comparing the results of simulation with real embryos of wild type and various mutants. Currently this simulator is based solely on dynamic model of cells and, therefore, includes neither chemical reaction networks nor gene-regulated networks within the cells, in order to make the model as simple as possible. For this

purpose, we implemented cells, eggshell and cell division, and we expressed a cell by a mesh bag (= membrane) containing soft balls (= cytoplasm). The unit particles of the cell parts are connected by springs and dampers (=shock absorbers). Eggshell is approximated by a quadric function. Timing of cell division was given and cell division was carried out by contraction of a contractile ring in the middle of the cells.

Using the simulator, we performed several simulations of early cleavages up to 4-cell stage. The results were that the movement and arrangement of the cells were almost the same as the real embryo, except that the cell arrangement of 3-cell stage was slightly different. This means that the restriction of cell movements by eggshell and the repulsive forces of cell division were reconstructed successfully but the lack of cytoskeleton may cause the discrepancy at 3-cell stage. In the simulation, the cleavage of P1 is temporally a bit behind that of AB, which is the normal pattern of wild type. In another simulation, we set the cleavage timing of P1 ahead that of AB. The results were striking; the resulting 4 cells were arranged not in the usual diamond shape but in T shape in which ABx can not contact with P2. Interestingly, this T shape, to some extent, looks like the embryo of A. nanus in which the cleavage of P1 is much ahead that of AB (Wiegner and Schierenberg, Dev. Biol. **204**, 3-14 (1998)). We are currently improving the simulator by adding attractive forces between the particles of cytoplasm and implementing other phenomena in early cleavages of the embryo.

(8) PGL-1, PGL-2 and PGL-3, a family of P-granule proteins, function redundantly to ensure fertility in both sexes of C. elegans

Ichiro KAWASAKI, Anahita AMIRI[1], Yuan FAN[1], Takeshi KARASHIMA[2], Yuji KOHARA and Susan STROME[1] ([1]Dept. of Biology, Indiana University, [2]Dept. of Biophysics and Biochemistry, University of Tokyo)

P granules are distinctive ribonucleoprotein complexes observed specifically in the cytoplasm of germ cells throughout development. We previously identified PGL-1 as being a constitutive protein component of P granules. The presence of an RGG box predicts that PGL-1 is an RNA-binding component of P

granules. *pgl-1* mutants contain defective P granules and are sterile, due to defects in proliferation and gametogenesis. Interestingly, the sterility caused by null alleles of *pgl-1* is highly sensitive to temperature. Our identification and analysis of two additional *pgl-1*-related genes, termed *pgl-2* and *pgl-3*, demonstrate that the PGL proteins function redundantly, and at low temperature, PGL-2 and PGL-3 are sufficient for fertility.

PGL-2 has 34% identity with PGL-1 in its N-terminal region. PGL-3 has 62% identity with PGL-1 throughout its length and contains an RGG box. Based on yeast two-hybrid and GST pull-down results, the three PGL proteins interact with each other. Furthermore, PGL-1 and PGL-3 are co-immunoprecipitated from both embryo and oocyte extracts, indicating that they are indeed in the same protein complex in vivo. Immunofluorescence analysis has demonstrated that in wild-type worms PGL-3 is associated with P granules at most stages of development, like PGL-1, but interestingly, PGL-2 is associated with P granules only during postembryonic development. Based on molecular epistasis results, each PGL protein associates with P granules independently of the other two.

To address whether PGL-3 functions redundantly with PGL-1, we isolated a *pgl-3* deletion allele. We found that *pgl-1; pgl-3* double mutant hermaphrodites and males show significantly enhanced (but not 100%) sterility at low temperature, compared to either single mutant. Double mutant hermaphrodites contain a severely underproliferated germline, indicating that the primary defect is in proliferation. Depletion of *pgl-2* by RNAi did not enhance sterility further.

Our findings suggest that both PGL-2 and PGL-3 are components of P granules that interact with PGL-1 and that at least PGL-3 functions redundantly with PGL-1 to ensure fertility in both sexes during *C. elegans* germline development.

(9) Identification of inducible innate immune defences in *C. elegans*

Gustavo MALLO[1], C. Leopold KURZ[1], Samuel GRANJEAUD[1], Yuji KOHARA and Jonathan EWBANK[1] ([1]Centre d'Immunologie de Marseille-Luminy)

Until now, it has not been clear whether *C. elegans* possesses an inducible system of defence or not. We have used high-density cDNA arrays to address this issue. We have found that following infection with the Gram-negative bacterium Serratia a number of genes are induced. We found 14 genes that showed a greater than 2-fold induction after both 24 h and 48 h of infection. Among them, other than genes for which no known homologues exist, we have identified genes encoding lectins and lysozymes, known to be involved in immune responses in other organisms. By *in situ* hybridisation, we have shown that the majority of these are expressed in the intestinal cells. Studies using invertebrates have significantly contributed to our current understanding of vertebrate innate immunity. Our findings thus open a new avenue for the investigation of evolutionary conserved mechanisms of innate immunity.

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

Yoshiki ANDACHI

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

mality of body-wall muscle.

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

Publication

None

H. Structural Biology center

H-a. Biological Macromolecules Laboratory

(1) Single Molecule Imaging in cells

Makio TOKUNAGA

Imaging of single fluorescent molecules *in vitro* has been achieved in a relatively simple manner using objective-type total internal reflection fluorescence microscopy (TIRFM). It is also found that single molecule imaging *in vivo* can be realized using the objective-type TIRFM. However, the technique is applied only when fluorescent molecules are from the glass-medium or glass-cell surface to a depth of less than about 150 nm. I have further developed this single-molecule technique by refining the way of laser-beam illumination. The new microscopy allowed visualization of single molecules in cells up to the depth of about 10 μ m from the glass-medium interface.

(2) Single molecule imaging of nucleocytoplasmic transport

Makio TOKUNAGA and Naoko IMAMOTO[1] ([1]Gene Network Laboratoy)

The above new microscopy was applied to imaging of single nuclear pores and single molecules involved in nucleocytoplasmic transport. Recent years have seen considerable progress in researches of macromolecule import into the nucleus and export from the nucleus. In contrast to detailed knowledge of soluble factors, molecular mechanisms of the transport and the interaction between nuclear pore complex (NPC) and molecules have been remained to be solved.

We examined behavior of green fluorescent protein (GFP)-tagged importin beta and GFP-tagged cargo in digitonin permeablilized cells. Fluorescence images of importin beta or cargo molecules bound to the nuclear rim were

obtained. Images were composed of many fluorescent spots, which represented single NPC's. Furthermore, imaging of single molecules of GFP-importin beta on the NPC has been also achieved. Both imaging of single pores and imaging of single molecules enabled us to analyze images quantitatively at the molecular level and to obtain kinetic parameters in cells, i.e., retention time at the pore, number of molecules bound to pores, and binding constant. Thus, single molecule imaging has opened a new way to obtain quantitative information on kinetics of molecular interactions in cells.

(3) Characterization of Xp105-Xp65 complex gathered around the centrosome in *Xenopus*

Nobuyuki SHIINA, Kazumi SHINKURA and Makio TOKUNAGA

The centrosome is nucleating center of microtubules and plays key roles to establish and maintain the intracellular polarity. We identified a novel protein Xp105 as one of molecules gathered around the centrosome in *Xenopus*. Xp105 was localized on granules with a diameter of 60-70 nm bordered with electron-dense coat. We found and identified a novel protein Xp65 as a binding protein of Xp105. Expression of Xp65-GFP fusion protein in cultured *Xenopus* A6 cells induced formation of the granules around centrosomes. The amino acid sequence of Xp65 showed a little homology to that of a retrotransposable element of insects and the Xp65-GFP-induced granules resembled VLP (virus-like particles) formed by retrotransposable elements, indicating that Xp65 is a retrotransposable element in *Xenopus*. In contrast to Xp65, Xp105 is a gene product of host cells and highly conserved from insects to mammals. So Xp105 should be responsible for some function(s) in/for host cells, although it was found originally as a molecule gathered around the centrosome together with the VLP. To get a clue to the function of Xp105 in host cells, we observed the behavior of Xp105-GFP fusion protein in cultured cells. Expression of Xp105-GFP in cultured cells induced electron-dense amorphous structures in the cytoplasm, in which beta-COP was colocalized. This novel structure was totally distinct from COP I-coated vesicles or endosomes, where beta-COP has been reported to be localized. Dynamic movement of Xp105-GFP at the cell periph-

ery was found and is being investigated in further detail.

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

Michio HIROSHIMA and Makio TOKUNAGA

We have developed intermolecular force microscopy (IMF) by refining atomic force microscopy (AFM) which is able to measure interaction forces between biomolecules through a gap distance of nanometers and to measure intramolecular forces of single molecule. This microscopy has achieved the force resolution of subpiconewton, which is over 100-fold more sensitive than that of conventional AFM. A new system using a laser radiation pressure was incorporated to reduce thermal fluctuation of the cantilever and to control its position. We have further refined the IMF on the following points: 1) expansion of force ranges up to 200 pN, 2) development of a new horizontal scanning system in which cantilever moves horizontally and horizontal positions are controlled by the feedback system.

Use of a new laser for optical control reduced thermal fluctuations upto 0.8nm (r.m.s.) and expanded the force range to more than 200 pN. In the horizontal scanning system, thermal fluctuations were reduced to 0.7nm (r.m.s.) and the force range was 90 pN. These performances are sufficient to measure inter- or intramolecular forces at the single molecule level.

(5) Single molecule measurement of protein folding by intermolecular force microscopy

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

Mechanical unfolding events of single protein molecules are measured with the intermolecular force microscope. Using single molecule measurements, we expect to obtain new information on protein folding mechanism, which would not be obtained by conventional methods. Staphylococcal Nuclease (SNase) is

a small globular protein, and is one of the proteins most generally used for folding researches. Its folding kinetics is well characterized, and also its atomic structure is available. Therefore, SNase is suitable for single molecule experiments. Cysteines were substituted at the both N- and C-termini of Staphylococcal Nuclease. It was attached to gold substrates by the cysteinyl residues. Single protein molecules of SNase were stretched mechanically with the intermolecular force microscope. Force curves, relations between force and length of single molecules, showed that full-stretched length of a molecule was 87 nm. On the assumption of the molecule as a dimmer, this result corresponds with the length of 0.29nm per amino acid, which agrees well with previously reported results. We are carrying out measurements with higher resolution to obtain detailed information on kinetics of proteins unfolding.

(6) Single molecule measurement of nucleocytoplasmic transport at nuclear pores

Atsuhito OKONOGI, Michio HIROSHIMA, Nobuyuki SHIINA, Shingo KOSE[1], Naoko IMAMOTO[1] and Makio TOKUNAGA ([1]Gene Network Laboratoy)

To ravel out the molecular mechanism of nucleocytoplasmic transport, it is essential to obtain detailed knowledge of the interaction between nuclear pore complex (NPC) and proteins involved in transport. We are trying to carry out single molecule measurement of nucleocytoplasmic transport, using subpiconewton intermolecular force microscopy. The inner radius of the nuclear pore is thought to be approximately 20-30 nm, so we have tried to make cantilevers, probes for scanning probe microscopy, with very sharp-pointed tips. Carbon nanotubes, whose diameter is approximately 10 nm, were used as the tip of the catilevers. *In vitro* assay system of nuclear transport is also essential for the single molecule measurement. Nuclear envelope were formed *in vitro* using extracts made from *Xenopus laevis* frog eggs.

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H-b. Molecular Biomechanism Laboratory

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

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

The conventional model for the mechanism of transcription initiation is composed of four sequential steps: the binding of RNA polymerase and necessary factors to promoter, formation of open complex that has partially melted duplex, formation of initiation ternary complex that retain short transcript, and promoter clearance that means dissociation of RNA polymerase from a promoter. The alternative model, which we proposed in 1996, allows the promoter-RNA polymerase complex to follow two pathways, productive and arrested pathways. In productive pathway, RNA polymerase is committed to synthesize long mature RNA, whereas in arrested pathway the enzyme forms moribund complex that synthesize only short abortive transcripts and then converted into dead-end complex that has no elongation activity (Nuc. Acids Res. **24**, 1380-1381 (1997): J. Mol. Biol. **256**, 449-457 (1996)). We observed the formation of the nonproductive complexes at several promoters, but no such complexes are found at T7A1and *rrnB*P1 promoters, indicating the existence of two classes of promoter.

DNA and protein footprinting analyses showed that the dead-end complex is backtracked from the promoter, lacks the strand opening and has more exposed conserved region 3 of α^{70} (Sen et al. J. Biol. **275**, 10899-10904 (2000).

This inactivation was mitigated by a α^{70} factor with S506F mutation in region 3 (SEN et al., J. Biol. Chem. **273**, 9872-9877(1998)) or the Gre factors (GreA and GreB: Sen et al., Gene. Cell. in press). These factors introduce reversibility between productive and non-productive pathways so that moribund complex can be converted into productive complex at a high concentration of the initiating nucleotide. This result indicates that the behavior of T7A1 and *rrnB*P1 promoters can be explained as reversible promoters on the basis of the branched pathway mechanism. We are on the way to prove the universality of the branched mechanism.

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

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

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

The function of ω subunit of *E. coli* RNA polymerase has not been identified for decades, and a disruption of its gene rpoZ does not have any clear phenotype. We have purified RNA polymerase core enzyme from a *rpoZ* disruptant and found the enzyme is tightly bound to GroEL shaperon. The core enzyme can bind to σ^{70} and active. It is inactivated, however, with a loss of binding activity to σ^{70}. If the associated GroEL is removed with a dye-affinity column or denaturation by urea. Reconstitution of activity is greatly stimulated by the presence of ω (Mukherjee et al., Eur. J. Biochem. **266**, 228-235 (1999)). These results show ω is structurally required for maturation of core enzyme. The lack of any phenotype of *rpoZ* mutant may be due to the functional suppression

by *groEL*

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

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

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

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

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

Takashi KINEBUCHI[1], Kumiko SOGAWA[1], Hiroyuki KABATA[1,2], Nobuo SHIMAMOTO[1],

Osamu KUROSAWA[2,3], Hironori ARAMAKI[4] and Masao WASHIZU[2] ([1]Structural Biology Center, National Institute of Genetics, [2]Department of Mechanical Engineering, Kyoto University, [3]Advance Co., [4]Department of Molecular and Life Science, Daiichi College of Pharmaceutical Science)

We have showed the existence of a sliding motion of protein along DNA through direct visualization of single molecules of *E. coli* RNA polymerase (Science **262**, 1561-1563 (1993)). To check the generality of sliding, we applied similar single molecule dynamics to a bacterial repressor protein, *P. putida* CamR, which was observed to slide along DNA. In the absence of its inducer, d-camphor, CamR molecules were trapped at three positions on a λ DNA, one was its cognate operator cloned in λ DNA, and the other two are likely to be homologous to the operator. All trapping occurred at specific sites, and only sliding complexes were observed at non-specific sites. This observation indicates that the non-specific complex is the sliding complex itself.

The most distinct difference between the movements of RNA polymerase and CamR was the pathway of dissociation from their specific sites. RNA polymerase slides out of its specific site into nonspecific sites and then dissociates 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*

Akane OISHI, Takeshi ISHIHARA and Isao KATSURA

Fluoride-resistant (*flr*) mutations of *C. elegans* are recessive and grouped into two categories: class 1 mutations, which map in *flr-1*, *flr-3* and *flr-4*, and class 2 mutations, which map in *flr-2* and *flr-5* (Katsura, I. *et al.*: Genetics **136**, 145-154, 1994). Class 1 *flr* mutations show many phenotypes: slow growth, short defecation cycle periods, frequent skip of the expulsion step of defecation, and synthetic abnormality in dauer formation (See (2) below), besides strong resistance to fluoride ion. The *flr-1* gene encodes an ion channel belonging to the DEG/ENaC (*C. elegans* degenerins and mammalian amiloride-sensitive epithelial sodium channels) superfamily, while *flr-4* and *flr-3* code for a

novel Ser/Thr protein kinase and a kinase-like molecule, respectively, both having a hydrophobic domain on the carboxyl-terminal side. A functional *flr-1*::GFP fusion gene is expressed only in the intestinal cells from the comma stage of embryos to the adult stage, while *flr-4*::GFP is expressed in the intestinal cells from the 1.5-fold stage, in the isthmus of the pharynx from the 3-fold stage and in a pair of head neurons called AUA from L1 larvae to adults. Moreover, the expression of *flr-3*::*lacZ* is detected mainly in the intestine. We therefore think that class 1 *flr* genes constitute a regulatory system that acts in the intestine and that controls many foodrelated functions. Class 2 *flr* mutations, which confer weak resistance to fluoride ion, suppress the slow growth and dauer formation abnormality but not the defecation abnormality and strong fluoride-resistance of class 1 *flr* mutations. Hence, it seems that the regulatory pathway bifurcates after class 1 genes, and that class 2 genes control only one of the two branches. Of the class 2 genes, *flr-2* encodes a secreted protein belonging to the gremlin/DAN/cerberous family. Although most proteins of this family are TGF-β antagonists, there is so far no evidence showing that FLR-2 interacts with any of the four TGF-β proteins in *C. elegans*. In the year 2000, we studied the expression of *flr-2* gene. Although we could not detect the fluorescence of the functional *flr-2*::GFP fusion gene that we made, we detected the staining with anti-GFP antibodies in a small number of neurons in the pharynx, head and tail. We also detected the product of the endogenous *flr-2* gene with antibodies against recombinant FLR-2 protein. A small number of neurons in the head, tail and pharynx were stained with the antibodies. The neuronal expression of FLR-2 may be a clue to solving the mystery: how the class 1 *flr* genes, which act in the intestine, can control sensory signals.

(2) Analysis of Synthetic Dauer-constitutive Mutations

Kouji MIYAHARA, Kiyotaka OHKURA, Tomoko YABE, 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, to identify genes acting in neural functions, 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, but most of the remaining 36 mutations, which map in at least 13 genes, seem to be alleles of novel genes. Of these mutants, that in *sdf-1* gene (1 allele) avoids benzaldehyde, isoamyl alcohol, butanone, NaCl, and lysine, which are attractants of wild-type animals. It behaved as thermophilic in thermotaxis. Mutants in *sdf-13* gene (2 alleles) showed defects in adaptation of chemotaxis to odorants sensed by AWC neurons (benzaldehyde, isoamyl alcohol and butanone), although they showed normal chemotaxis to these odorants. This gene encodes a homolog of mammalian Tbx2 and *Drosophila* Omb, a transcription factor containing the T-box domain.

The following results were obtained in 2000.

The cells in which *sdf-13* gene is expressed and the candidates of the cells in which *sdf-13* gene acts for olfactory adaptation were identified. We made an *sdf-13*::GFP fusion gene in which GFP cDNA is connected to the end of the *sdf-13* coding region. This GFP fusion gene rescued the mutant phenotypes (Sdf

and adaptation defects) and was expressed in the pharyngeal neurons M2 and I5 from the late embryonic to the adult stage. However, since the fluorescence was very weak, we also studied the expression of *sdf-13* gene with antibodies against the N-terminal 86 amino acid residues of SDF-13, which was synthesized in *E. coli* cells. The antibodies stained the amphid neurons AWB, AWC, ASJ and the pharyngeal neurons I1, I3, I5, M1, M2, M5, NSM. Furthermore, when the *sdf-13* cDNA was expressed in AWB, AWC and I1 by connecting to the *gcy-10* promoter, it rescued the olfactory adaptation defects but not the Sdf phenotypes of *sdf-13* mutants. The results is consistent with the idea that *sdf-13* acts cell-autonomously. To prove this, we plan to test whether the olfactory adaptation defects are rescued by expression of *sdf-13* in AWC neurons and the Sdf phenotypes in ASJ neurons.

In *sdf-9* gene, five mutant alleles have been obtained, which complement all the dauer-related mutations that map around the locus. We found that nearly 100% of *sdf-9* mutants animals become dauer larvae and spontaneously recover from dauer to L4 larvae, if the promoter of *daf-7* gene (a TGF- β gene that acts in dauer regulation) is introduced into the mutants. However, *daf-7*::GFP was expressed in *sdf-9* mutants like in wild type animals. We mapped *sdf-9* mutations to the right end of chromosome V (right side of *rol-9*) by three-factor cross with known mutations and by using single nucleotide polymorphism. Presently, we are trying to clone *sdf-9* gene by introducing DNA fragments of this region into the mutants and checking the rescue of the Sdf phenotype. Studies of this gene may be useful for analyzing dauer formation and dauer recovery separately.

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

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

Animals receive environmental cues with sensory cells, select and integrate many pieces of necessary information, and make proper responses, which can be modified by former experience or memory. In *C. elegans*, analyses of behav-

ioral mutants have been performed, which lead to the molecular mechanisms of sensation of odorants and temperature, for instance. On the basis on these results, we are analyzing mutants on learning and the selection (evaluation) of sensory signals, to elucidate novel mechanisms of higher-order sensory signal processing.

The nematode *C. elegans* shows avoidance of copper ion and chemotaxis to odorants by perceiving them with different sensory neurons in the head. We devised a behavioral assay method for the interaction between the two responses to learn a possible role of interneurons. Wild-type animals change their preference between the responses, depending on the relative concentration of copper ion and odorants. This suggests that the two sensory signals interact with each other in a neural circuit consisting of about 10 neurons, on the basis of the neural circuitry of *C. elegans* and the identity of the sensory neurons that act in these behaviors.

To elucidate the mechanism of the complex behaviors mentioned above, we are isolating and analyzing mutants that show abnormality in this assay. The mutant *ut236* has a tendency to choose avoidance of copper ion rather than chemotaxis to odorants, although it shows no abnormality in each behavior in case only one of the stimuli is given. The result shows that *ut236* is abnormal in the interaction between the two behaviors. Furthermore, *ut236* shows abnormality in the conditioning (learning) by the paired presentation of starvation and NaCl. While wild-type animals are normally attracted by NaCl, they avoid it after the conditioning. In contrast, the paired presentation has only very small effect on the *ut236* mutant animals. Since the mutant shows normal behavioral responses to starvation alone, we think that it has abnormality in the learning process.

We identified the gene for *ut236* by positional cloning. It encoded a novel secretory protein having an LDL receptor ligand-binding domain. Using antibodies against its recombinant protein, we found that the gene product is localized in the axon of each one pair of sensory and inter-neurons. The localization in the axon was abolished in *unc-104* (kinesin KIF1A homolog) mutants, which show defects in the transport of synaptic vesicles. Expression studies with various transcriptional promoters showed that this gene acts cell-non-autonomously in the nervous system. Studies using a heat-shock pro-

moter showed that the wild-type phenotype is recovered if it is expressed after the mature neural circuitry is formed, but not if expressed during the development of the nervous system. These results indicate that the gene product is a novel neuromodulatory factor that acts in diverse functions such as the modification of sensory information and various types of learning.

In the above experiments, the assay of selection between two behaviors was carried out with well-fed animals. When it was conducted with animals starved for 5 hours, the animals showed a stronger preference for odorants, because they responded more weakly to copper ion. This starvation effect was not detected in the presence of serotonin, which is considered to induce a well-fed state. The behavioral change upon starvation seems appropriate, because starved worms can look for food over a wider area.

We isolated a mutant, *ut235*, which lacks the effect of starvation in this assay. This mutant behaved essentially normally in many other responses to starvation such as the strarvation-induced increase of locomotion speed. Interestingly, the double-mutant *ut235; ut236* showed a preference to avoidance of copper ion, regardless of starvation. To clone the gene for the *ut235* mutation, we mapped it precisely by using SNP between two wild-type strains, N2 and CB4856.

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H-d. Biomolecular Structure Laboratory

(1) Crystallographic Study of F1-ATPase

Yasuo SHIRAKIHARA and Aya SHIRATORI

F1-ATPase, with a subunit composition of $\alpha 3 \beta 3 \gamma \delta \varepsilon$, is a catalytic sector of the membrane bound ATP synthase. The ATP synthase plays a central role in energy conversion in mitochondria, chloroplasts and bacteria, generating ATP from ADP and inorganic phosphate using energy derived from a transmembrane electro-chemical potential. The rotational catalysis mechanism of F1 is accompanied by rotation of the rod-like γ subunit, which is thought to control the conformations of the three catalytic β subunits in a cyclic manner. We previously solved the structure of the $\alpha 3 \beta 3$ subassembly of F1-ATPase from a thermophilic bacterium *Bacillus* PS3. We have been extending the structural study to $\alpha 3 \beta 3 \gamma$ and $\alpha 3 \beta 3 \gamma \varepsilon$ sub-assemblies, both of which exhibit kinetic properties very similar to those of F1. In this extension, we aim to detect structural changes caused by different mode of nucleotide binding, which should provide with structural basis for understanding the rotational catalysis mechanism.

As reported lat year, extensive studies on purification and crystallization had been done to get crystals of $\alpha 3 \beta 3 \gamma$ sub-assembly. Currently, however, crystals are not large enough for in-house diffraction study, due to their weak diffracting capability. Usual crystals allowed us to record to resolutions of only about 40-20A in house. After examining freezing conditions for data collection at synchrotron, we found that those crystals diffracted to resolutions of 15-10A at SPring8. Although the values were poor, they were twice better than values for resolution limit obtained with laboratory x-ray source. Crystals exhibited high mosaicity(2.5 degree). The unit cell parameters were a=190.1A, b=194.0A, c=254.6A, α =111.7, β =111.6, γ =91.0. Further efforts are being made to extend the resolution of the crystals and to find appropriate conditions for cooling crystals.

The $\alpha 3 \beta 3 \gamma \varepsilon$ sub-assembly crystals have been found to be much better than the $\alpha 3 \beta 3 \gamma$ sub-assembly crystals described above. The $\alpha 3 \beta 3 \gamma \varepsilon$

sub-assembly crystals, when experimented with rotating-anode source, diffracts to 4.5 A resolution at room temperature and 7 A at 100K. With SPring8 beam, we collected a data set to resolution of 4.5 A at 100K. After trials on various aspects on cryo-cooling, we found the best way for preparing the cooled crystals was to combine the rapid cooling with liquid propane and the subsequent annealing in the cold nitrogen gas flow. The crystals needed to be grown in the presence of 20% ethylene glycol. This cooling procedure was successful only for medium sized crystals, like 0.2x0.2x0.1mm. The unit cell parameters were a=225.3A, b=225.7A, c=224.6A, α =94.1, β =117.5, γ =117.8, and the unit cell contains 8 of the sub-assembly. The data were merged with Rmerge of 12.2% at 4.5 A resolution. Structural analysis by molecular replacement is in progress.

These structure studies were done in collaboration with Toshiharu Suzuki and Masasuke Yoshida, at Research Laboratory of Resource Utilization, Tokyo Institute of technology.

(2) X-ray crystallographic analysis of repressor protein CamR

Katsumi MAENAKA and Yasuo SHIRAKIHARA

The Pseudomonas putida cam repressor (CamR) is a homodimeric protein that binds to the operator DNA (camO) to inhibit transcription of cytochrome P450cam operon camDCAB. This repression is relieved by binding of the inducer D-camphor. The unique feature in the molecular recognition of CamR are: (1) CamR has typical two domain structure, one of which is a DNA binding one and the other binds the inducer D-camphor, (2) Two molecules of D-camphor can bind to one molecule of homodimeric CamR in a negative cooperative manner, and (3) similar amino acid sequence responsible for camphor binding exists in three proteins of cytochrome P450cam operon, CamR, cytochrome P450 and purtidaredoxin reductase. Our structural study aims to clarify these unique characteristics of CamR.

The selenomethionyl derivative CamR (SeMet-CamR) has been overproduced in *Escherichia coli* and purified by the slightly modified method for the wild-type CamR. Preliminary crystallization trials were conducted at 297 K using

Natrix (Hampton Research). The SeMet-CamR protein was crystallized in the slightly modified condition of Natrix condition 15, 10% MPD, 25mM Na-cacodylate, 20mM MgCl2, pH6at 15℃. Crystals reached maximum size (0.3x0.3x0.5mm) after 1 week. MAD data using the SeMet-CamR crystal were collected at SPring8. The collected data reveal the space group to be orthorhombic (P222) and the diffraction reached beyond 3 Å resolution. A full structure determination is in progress.

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

(3) Crystallographic Study of the Transcription Activator, PhoB

Kazuyasu SHINDO, Katsumi MAENAKA and Yasuo SHIRAKIHARA

PhoB Protein is a positive transcriptional activator for the genes in the phosphate (*pho*) regulon of E. coli, such as *phoA* and *pstS*, that are induced by phosphate starvation. PhoB acts by binding to the *pho* box in the promoter region, which is the consensus sequence shared by the regulatory regions of *phoA*, *phoB*, *phoE* and *PstS*. The activity of PhoB is regulated by phosphorylation by PhoR. The N-terminal domain of PhoB is responsible for this regulatory role, whereas the C-terminal domain (spanning from a residue 125 to the C-terminus) has a DNA binding ability.

The selenomethionyl derivative of DNA-binding C-terminal domain has been crystallized by the micro batch method to solve its structure employing the MAD (Multi-wavelength Anomalous Dispersion) method. Diffraction data to 2.5 Å resolution were collected at SPring8 for MAD analysis. The crystals belong to the space group $P2_1$ with unit cell dimensions a=30.63 Å , b=37.54 Å , c=44.37 Å , and β =109.42. Data analysis has been in progress.

This work has been done in collaboration with Hideyasu Okamura, Yoshifumi Nishimura, Yokohama City University and Kozo Makino, Research Institute for Microbial Diseases, Osaka University.

(4) Crystallization of *E.coli* nucleoid proteins

Yasuo SHIRAKIHARA and Aya SHIRATORI

The genome of E.coli is composed of a single molecule of circular DNA and the 10 major DNA binding proteins, together forming the nucleoid. These DNA binding proteins play not only a structural role, but also pleiotropic regulatory roles in global regulation of gene transcription. Three of those proteins, IciA (Inhibitor of Chromosome Initiation A), Lrp(Leucine-Responsive regulatory Protein) and StpA(Suppressor of td-Phenotype A) were subjected to a crystallization experiment. IciA formed needle-shaped small crystals formed from ammonium sulfate solution, and Lrp formed plate crystals from phosphate solution. Lrp crystals diffracted to 4 Å resolution.

This work has been done in collaboration with Akira Ishihama and Talukder A. Azam in the Department of Molecular Genetics.

(5) Crystallization of the *Drosophila* GAGA factor

Katsumi MAENAKA and Yasuo SHIRAKIHARA

The Drosophila GAGA factor specifically binds the GAGA-related DNA sequences in the promoter regions of a variety of genes including *fushi tarazu* and *Ultrabithorax* to induce the chromatin remodeling in the GAGA binding promoter region and the transcriptional activation. The GAGA factor is a 519 amino acid protein and consist of several functional domains including BTB/POZ, Glutamine-rich, and DNA binding domains. Since the GAGA factor tends to form a multimer, we also focus on each functional domain for crystallographic analysis. We established the *E.coli* expression system for the whole GAGA factor and some of these domains. The purification procedure of each recombinant protein suitable for crystallization is now being examined.

This work has been done in collaboration with Susumu Hirose and Takahiro Nakayama, National Institute of Genetics.

(6) Structural and functional studies of immunoglobulin(Ig)-like receptors in immune system

Katsumi MAENAKA and Yasuo SHIRAKIHARA

Among the cell surface receptors in immune system, the most common domain type, about 50% of total, is immunoglobulin(Ig) superfamily including fibronectin type III. Therefore it is important to reveal the generality and diversity of the molecular recognition of Ig-like cell surface receptors. By using surface plasmon resonance (SPR), we have studied the kinetic and thermodynamic analysis of two Ig-like receptors, human killer cell Ig-like receptors (KIRs) and Fc γ receptors (Fc γ R), whose structures have similar unique topology (intermediate between I set and C2 set) revealed by several structural studies of KIRs and FcgRs including ours.

A repertoire of KIRs with two or three tandem Ig domains in their extracellular regions is expressed on human natural killer (NK) cells. These KIRs activate or inhibit NK cell cytotoxicity following recognition of the MHC class I molecules on target cells. Different two domain KIRs (KIR2Ds) recognise distinct subsets of HLA-C alleles. SPR analysis showed that the ectodomain of KIR2DL3 exhibited the allele- and peptide-specific recognition to HLA-Cw7 with fast kinetics (an affinity of ~10^{-6}M) and a favourable binding entropy, similar to other cell-cell recognition. Although the T cell receptors (TCRs) and KIRs both show allele- and peptide-specific MHC recognition, the TCR-MHC interactions has slow kinetics and alarge unfavourable binding entropy, which is in contrast with those of KIR-MHC interactions.

On the other hand, Fc γ receptors (Fc γ R) are expressed on immunologically active cells and bind the Fc portion of IgG and are besides other functions responsible for the clearance of immune complexes. SPR analysis for the interactions of the ectodomain of all human low-affinity FcyRs (Fc γ RIIa, Fc γ RIIb and Fc γ RIII), which consist of two Ig-like domains, toward Fc showed the fast kinetics with similar low affinity (~10^{-6}M) as observed in other cell surface receptors including KIRs. These observations suggest that Fc γ Rs recognize the immune complex in similar way of cell-cell interactions of cell surface receptors in terms of kinetics.

We further determined the crystal structure of human MHC class I molecule, HLA-B51, which is a ligand for KIR3DL1. The HLA-B51 structure clearly exhibited the electrostatic differences on the predicted KIR binding site from other MHC class I molecules, suggesting that these electrostatic differences may determine the specificity of KIR binding.

This work has been done in collaboration with Yvonne Jones, David Stuart, Taeko Maenaka, Anton van der Merwe (University of Oxford), Peter Sondermann (Max-Planck-Institut für Biochemie, Martinsried) and Izumi Kumagai, Kouhei Tsumoto, Mitsunori Shiroishi (Tohoku University).

(7) 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 a crystallization experiment. The crystals were formed by the crystallization condition using 27% PEG4000, 0.2 M $LiSO_4$, 10 mM Tris-HCl, pH 7.5, 10% glycerol. In this condition, we obtained several crystal forms, plate-like, coffine-like, cubic, and needle-like forms. High resolution was obtained using the plate-like crystals. The symmetry was C_2, the lattice constants were 241.6, 141.1, 239.1 Å , and the resolution was 2.4 Å. We are now analysing the diffraction pattern. This work has been done in collaboration with Toshiaki Hosaka, Toshiyuki Meguro and Ichiro Yamato, Science University of Tokyo.

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

Yasuo SHIRAKIHARA

D-aminoacylase catalyzes hydrolysis oh N-acylderivatives of various neutral D-amino acids to D-amino acids and fatty acids. The enzyme is expected to have a unique active site structure that distinguishes from abundant L-amino

acids, but also to have industrial relevance with a potential to produce large amount of D-amino acids. The enzyme from *Alcaligenes xylosoxydans subsp. xylosoxydans A-6* has been crystallized from MPD or isopropanol solutions, but the crystallization was not reproducible due to varied purity of the preparations. This year, after a solid preparation procedure having been established, the crystallization condition search has been repeated with grid screen and crystal screen from Hampton.

A related enzyme, glutaminase from a marine bacterium *Micrococcus luteus K-3*, has been crystallized from a citrate solution. The crystals diffracted to at least 3 Å resolution, and had a hexagonal lattice with a=b=110.5 Å, c=210.5 Å.

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

(9) X-ray crystallographic analysis of the anti-tumor enzyme, arginine deiminase from Mycoplasma argini

Katsumi MAENAKA and Yasuo SHIRAKIHARA

Arginie deiminase (AD) catalyzes the hydrolysis of L-arginine to L-citrulline and ammonia, and is involved in the arginine metabolism pathway in various microorganisms, which is the main energy source for mycoplasma. The recombinant Mycoplasma argini arginine deiminase showed a strong inhibition of the growth of various kinds of human and mouse tumor cells by depleting L-arginine. We are trying to address the structural basis of the ariginine hydrolysis mechanism of AD, which could provide useful information to improve the AD function as a potential anti-tumor drug.

Previously it was established that the crystals of the recombinant AD obtained from a solution containing PEG6000 and NaCl, diffracted to 3.0 Å resolution. Using the currently available preparations, crystallization conditions were again searched employing Crystal Screen I (Hampton research), to find the condition of 25% PEG6000, 0.2M Ammonium Acetate, 0.1M Tris-Cl (pH8) at 297K. AD has also been crystallized in this condition but with added 20% glycerol, which is suitable for cryo-experiment. The crystals diffracted to

3.0Å resolution at Spring8. Preliminary characterization of the crystals indicated a P1 space group. The heavy atom derivative search is being performed.

This work has been done in collaboration with Satoru Misawa, Japan Energy Corporation.

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H-e. Gene Network Laboratory

Nucleocytoplasmic exchange of macromolecules

Naoko IMAMOTO

Nucleocytoplasmic exchange is a very dynamic activity, in which vast number of molecules enter and exit the nucleus in a rapid, accurate, and often regulated manner. This exchange of molecules is important, in order for cells to maintain their homeostasis, and adapt to their extracellular environment.

Since the identification of the first transport factor, referred to as importin α and β, which mediates nuclear import of classical basic nuclear localization signal (NLS)-containing substrates, significant progress has been achieved toward our understanding of the mechanism of nucleocytoplasmic transport, as well as the diversity of nucleocytoplasmic transport pathways. The presence of so many transport pathways obliges us to raise a naive but fundamental question: What is the benefit to having such a complexity of nuclear trans-

port pathways leading to regulatory changes of gene expression in vivo? In order to understand basic mechanism of transport and biological significance of diversity of transport pathways, our present effort focuses on the understanding of the function of nuclear pore complex, and identification of transport pathways and factors that function under different cellular conditions.

(1) Single molecule imaging of nucleocytoplasmic transport at nuclear pores

Naoko IMAMOTO and Makio TOKUNAGA[1] ([1]National Institute of Genetics, Biological Macromolecules Laboratory)

A central mediator of nucleocytoplasmic traffic is the nuclear pore complex (NPC), a large supramolecular structure of protein complex consisting of about 50-100 different polypeptides with an estimated molecular mass of 125 megadaltons in vertebrates. More than one million macromolecules are estimated to pass through the nuclear envelope every minute in dividing cells. In contrast to our growing knowledge of soluble factors involved in transport, function of nuclear pore complex (NPC), as well as mechanism of translocation step of transport is very poorly understood.

As one approach to investigate the function of NPC, we are trying to visualize behavior of transport factors and transport substrates at NPC, using modified objective-type total internal reflection fluorescence microscopy (TIRFM). This method allows us single molecule imaging in vivo. In the digitonin permeabilized cell-free transport assay, we examined a behavior of green fluorescent (GFP)-tagged importin β. Through the imaging of single nuclear pore complexes, and visualizing single GFP-importin β molecules at NPCs during transport, a quantitative information on kinetics of NPC-translocation of importin β can be obtained.

(2) Analysis of crystal structure of the uncomplexed form of importin β

Soo Jae LEE[1], Naoko IMAMOTO, Hiroaki SAKAI[1], Atsushi NAKAGAWA[1], Shingo KOSE, Makiko KOIKE, Masaki YAMAMOTO[3], Takashi KUMASAKA[3], Yoshihiro YONEDA[2] and Tomitake TSUKIHARA[1] ([1]Institute for Protein Research, Osaka University, [2]Department of Cell Biology and Neuroscience, Graduate School of Medicine, Osaka University, [3]The Institute of Physical and Chemical Research (RIKEN))

Importin β is a nuclear transport factor which mediates the nuclear import of various nuclear proteins. The N-terminal 1-449 residue fragment of mouse importin β (imp β 449) possesses the ability to bidirectionally translocate through the nuclear pore complex (NPC), and to bind RanGTP. The structure of imp β 449 has been solved at a 2.6 Å resolution by X-ray crystallography. It consists of ten copies of the tandemly arrayed HEAT repeat and exhibits conformational flexibility, which is involved in protein-protein interaction for nuclear transport. The overall conformation of the HEAT repeats shows that a twisted motion produces a significantly varied superhelical architecture from the previously reported structure of RanGTP-bound importin β. These conformational changes appear to be the sum of small conformational changes throughout the polypeptide. Such a flexibility, which resides in the stacked HEAT repeats, is essential for interaction with RanGTP or with NPCs. Furthermore, it was found that imp β 449 has a structural similarity with another nuclear migrating protein, namely β-catenin, which is composed of another type of helix-repeated structure of ARM repeats. Interestingly, the essential regions for NPC translocation for both importin β and β-catenin are spatially well overlapped with one another. This strongly indicates the importance of helix stacking of the HEAT or ARM repeats for NPC-passage (see publications 1 and 2).

(3) Mechanism of nucleocytoplasmic translocation of importin- β

Hitomi OZAWA[1], Shingo KOSE, Jun KATAHIRA[1], Taro TACHIBANA[1], Miki HIEDA[1], Hiroaki SAKAI[2], Tomitake TSUKIHARA[2], Naoko IMAMOTO and Yoshihiro YONEDA[1]

([1]Department of Cell Biology and Neuroscience, Graduate School of Medicine, Osaka University, [2]Division of Protein Crystallography, Institute for Protein Research, Osaka University)

Importin β is a nuclear transport factor which mediates the nuclear import of various nuclear proteins. We have found that importin β has an intrinsic activity to translocate through the nuclear pore complex (NPC), and this activity requires interactions with nucleoporins.

To understand the underlying mechanism of nucleocytoplasmic migration of importin β, we made various importin β mutants, based on crystal structure of uncomplexed form of importin β. These mutants harbor amino acid substitutions within the NPC binding domain, which we have shown to be necessary and sufficient to exhibit the translocational activity of importin β through NPC. By transient transfection experiments, we observed that a mutant showed inefficient accumulation to the nuclear envelope and the nucleoplasm, while the other localized at the nucleus more efficiently than wild type.

The activities of these mutants to translocate through NPC, as well as their abilities to support nuclear import of NLS-bearing substrates and the interaction with various nucleoporins are now under investigation.

(4) Analysis of nuclear export of importin β through nuclear pores

Shingo KOSE, Yoshihiro YONEDA[1] and Naoko IMAMOTO. ([1]Department of Cell Biology and Neuroscience, Graduate School of Medicine, Osaka University)

Nucleocytoplasmic transport occurs through the nuclear pore complex (NPC). Small molecules (<20-40 kDa) move passively through the NPC, but larger molecules are selectively transported by nucleocytoplasmic shuttling receptors of the importin β family. We have previously reported that importin β, which functions as a carrier of classical nuclear localization signal (NLS)-mediated nuclear import, shuttles rapidly between the nucleus and the cytoplasm by direct interaction between importin β and components of the NPC (nucleoporins). However, the mechanism for a directional movement of importin β at the NPC remains unclear.

In chilled or ATP-depleted cells, importin β was transported into the nucleus, while the nuclear export of importin β was inhibited. Further, it was found that the nuclear export inhibition of importin β is not due to nuclear retention via binding to nucleoporins or nuclear importin α. These data show that the nuclear export of importin β involves energy-requiring step(s) in living cells.

In vitro transport assay revealed that an energy-dependent nuclear export of importin β requires soluble factors. To address the issue of how the energy is utilized during the NPC-passage of importin β, we have now attempted to purify an energy-dependent nuclear export factor of importin β from mouse Ehrlich cytosolic extract.

(5) Analysis of nuclear export of β-catenin

Makiko KOIKE and Naoko IMAMOTO

β-catenin plays a key role in Wnt signaling pathway. Upon activation, β-catenin accumulates and enters the nucleus, where it modulates the activation of the target gene expression. Therefore, it is important to understand the mechanism of nucleocytoplasmic transport of β-catenin. Our previous studies indicate that β-catenin can enter the nucleus on its own, and shuttles between the cytoplasm and nucleus. The export of β-catenin examined in living cells showed that this protein exit the nucleus in CRM1-independent and Ran-independent manner. Moreover, we found that deletion mutant containing Arm repeats 10-12 and C-terminus of β-catenin efficiently entered the nucleus on its own like full length β-catenin, and that this region is also necessary and sufficient for β-catenin to exit the nucleus. In order to investigate export of β-catenin, we examined the export of β-catenin using digitonin-permeabilized cell-free transport assay. We found that β-catenin are capable to exit the nucleus on its own, but export of β-catenin are often regulated by factor(s) present in cytosol in the presence of ATP. A nature of factor that affect β-catenin export is now under investigation.

(6) Stress mediated nuclear import of 70kDa heat shock protein

Maiko FURUTA[1], Shingo KOSE and Naoko IMAMOTO ([1]Department of Cell Biology and Neuroscience, Graduate School of Medicine, Osaka University)

Heat-shock induces a strong stress response that drastically alter gene expression in cells. Heat-shock response may provide us an ideal system to examine whether specific transport pathway or transport factors function under different cellular conditions. To start with, we are examining heat-shock stress-mediated nuclear import of 70kDa heat-shock protein (hsp70/hsc70). Both hsp70 and hsc70 efficiently migrates into the nucleus under heat-shock condition, but this protein is largely cytoplasmic under normal condition.

In order to elucidate a mechanism of stress-mediated nuclear import of hsp70/hsc70, we have developed transport assay to examine stress-mediated nuclear import of hsp70/hsc70 both in living mammalian cells and in the in vitro system using digitonin-permeabilized cells. Our present results indicate that nuclear accumulation of hsc70 requires soluble factors and ATP, and its nuclear accumulation is importin α / β or transportin independent. Crude cytosolic extracts, for example, prepared from Ehrlich ascites tumor cells or Xenopus oocytes that efficiently support nuclear import of classical NLS-containing substrates does not support nuclear accumulation of hsc70 efficiently, indicating that some unique mechanism is involved in stress-mediated nuclear import hsc70.

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I. Center for Information Biology

I-a. Laboratory for DNA Data Analysis

(1) *In silico* chromosome staining: Reconstruction of Giemsa bands from the whole human genome sequence

Yoshihito NIIMURA and Takashi GOJOBORI

Giemsa staining has been used for identifying individual human chromosomes for over 30 years, though the molecular basis of these banding patterns has remained elusive. The banding patterns of Giemsa-dark (G) and Giemsa-light (R) bands are known to reflect the regional differences in chromatin structures and functions. In this study, we developed an "*in silico* staining" method and reconstructed Giemsa bands computationally from the draft genome sequence of human. We found that G bands are the regions where the GC content is lower than that of the flanking regions. By a two-window analysis, we demonstrate that Giemsa bands are strongly correlated with the *difference* in GC content between windows of 2.5 Mb and 9.3 Mb for almost all of 43 chromosomal arms. Our findings suggest that isochore structures, the large-scale variation in GC content, has evolved by a selective pressure towards AT-richness in G-band regions to maintain compact chromatin structures.

(2) The evolutionary origin of brain structure characterized by gene expression profile: A cytoarchitectonic map of the planarian brain defined by cDNA microarray

Masumi NAKAZAWA, Francesc CEBRIA[1,2], Katsuhiko MINETA, Kazuho IKEO, Kiyokazu AGATA[1,2] and Takashi GOJOBORI ([1]Department of Biology, Faculty of Science, Okayama University, [2]RIKEN Center for Developmental Biology)

The origin of the brain remains a challenging problem in evolutionary studies. To understand when and how the structural and functional brain emerged,

we analyzed the CNS of a lower invertebrate, planarian. We conducted a large-scale screening of the head part-specific genes in the planarian by constructing a cDNA microarray. We created a microarray of 1,640 nonredundant genes, randomly chosen from 9,000 EST sequences. Competitive hybridization of cDNAs between a head portion and the other body portion of planarians revealed 205 genes with head part-specific spikes, including homologues of *synapsin, synaptotagmin* and *arrestin* genes essential in the vertebrate nervous system. The expression patterns of the top 30 genes showing the strongest spikes implied that the planarian brain has undergone functional regionalization. Here, we demonstrate the complex cytoarchitecture of the planarian brain, despite its simple superficiality of the morphology. These data indicate that a highly organized brain had already emerged by the time of planarian divergence.

(3) SNP profiles of the human subgenomic regions

Michihiro OGASAWARA, Silvana GAUDIERI[1], Tadashi IMANISHI and Takashi GOJOBORI ([1]The University of Western Australia)

To investigate frequencies and distributions of single-nucleotide polymorphisms (SNPs) in the human genome, we have previously constructed a SNP profile of the MHC region, showing that nucleotide diversities between different haplotypes at non-HLA sites were unexpectedly high (>10%) (Gaudieri, S. *et al.* (2000) Genome Research **10**: 1579-1586), up to 80 times greater than elsewhere in the genome (0.08-0.2%). In the present study, we extended our analysis of SNP profiles to other regions of the human genome. And we reported characteristic features of SNP profiles in several subgenomic regions, such as the beta-globin region and others in the chromosomes 7, X and Y, by comparing between two different haplotype sequences chosen from the public databases. In particular, we found a total of 523 SNPs in 596.62kb surveyed (one SNP in 1141 nucleotides on average, namely 0.088%)when the MHC region was excluded. The mean frequency of SNPs obtained in our study is lower than those of other previous reports (Wang, D.G. *et al.* (1998) Science **280**: 1077-1082; Yamada, R. *et al.* (2000) Hum Genet **106**: 293-297). And our obser-

vations indicate that the skewness and complexity of SNP profiles depend heavily upon subgenomic regions.

(4) Study of molecular evolution in *vsx* gene families

Yoshiko UCHIYAMA, Kazuho IKEO and Takashi GOJOBORI

Vsx genes are involved in the differentiation of retina, spinal cord, and nervous system of vertebrates. It is known that vertebrate *vsx* genes are classified into two subtypes, *vsx* 1 and *vsx* 2. *Vsx* genes have two domains, a paired-like homeodomain and a typical CVC domain. The relationship between these domains and the restricted expression patterns is unclear from the viewpoint of molecular evolution. Thus, we focus on the evolution of *vsx* genes having pairedlike homeodomains. In this study, we performed evolutionary phylogenetic analysis by using 45 paired-like genes including *vsx* genes. It revealed that the duplication of *vsx* genes into two subtypes had occurred specifically in the lineage of vertebrates. It also showed that the duplication occurred about 600 million years ago, the appearance time of vertebrates. This result suggests that *vsx* genes would have played an important role for the formation of spinal cord. More detailed studies on the molecular evolution of *vsx* genes is conducted using experimental approaches.

(5) Evolutionary features of the gene expression profile of planarian brain

Katsuhiko MINETA, Kazuho IKEO and Takashi GOJOBORI

Planarian is considered to possess the primitive central nervous system (CNS). Therefore, planarian can be a useful model for understanding the evolutionary process of the CNS. Our purpose of the present study is to identify the planarian genes related to CNS, to observe their expression profiles, and to compare them with those of other species. We sequenced over 8,000 redundant expressed sequence tag (EST) clones from the cDNA library derived from the head portions of planarians (*Dugesia japonica*). As a result, we obtained a total of 3,101 non-redundant EST clones. According to a frequency analysis,

77% of 3,101 clones appeared only once. This means that most of genes were rarely expressed. Conducting the homology search, we found that 45% of 3,101 clones had significant similarities with proteins whose functions were known. Among these genes, we found that at least 71 genes were involved in CNS. Comparing these 71 planarian genes with the genes of human, mouse, fruit fry and nematode, we found that 68 genes were well-conserved and 3 genes were not conserved very much. This feature of gene conservation can be considered as a reflection of the diversification of CNS during evolution, supporting our idea that a comparative approach based on the planarian ESTs is useful for elucidating the evolutionary process of CNS.

(6) Establishment of integrated human microsatellite database

Takaho ENDO and Takashi GOJOBORI

We have established microsatellite database of human genomic sequences. We cooperate with medical laboratory of Tokai University (Dr. Inoko) and made computer programs which find and analyze microsatellite regions in human chromosomes.

Microsatellites are expected as genetic markers to detect disease candidate genes because of their high polymorphism. In isolating disease genes, we would conduct association analysis with many polymorphic markers such as single nucleotide polymorphisms (SNPs), variable number tandem repeat (VNTR), and microsatellites. Microsatellites are known as highly polymorphic region in the repeat number and show higher linkage (about 100kb) than SNPs (SNPs is thought to be associated in the range of 10kb).

We have acquired microsatellite markers by some experimental methods (in wet laboratory) so far. Today we can utilize the database from human genome project and we have scanned all microsatellites in it. Our database integrates the primer designing program and it provides specific markers (avoiding repetitive sequences) suit for various experimental conditions. We have constructed more than 10,000 polymorphic markers through my database and we believe they should become useful tool for isolation of disease genes in the genome-era.

(7) Higher evolutionary rates in the contact regions between catecohlamine receptors and G proteins

Hisakazu IWAMA and Takashi GOJOBORI

The catecholamine (CA) neurotransmission was recently reported to play a central role of the emotional control in humans. The CAs are the bioamines that are constituted of dopamine and adrenalin. The receptors (Rcs) of CAs are G protein (GP)-coupled Rcs (GPCRs). In particular, the function of GP_ subunit (G_) works as a connecter between an outer ligand-Rc system and a system of inner second messenger pathways (SMPs).

In order to understand how functionally important those receptors are, we examined the evolutionary conservation of the receptor genes involved with the CA neurotransmission system. First, we made alignments of amino acid sequences of CA Rc and G_, separately. For the alignment of CA Rc, we divided it into two parts; ligand-binding and GP-contact regions. For the alignment of G_, we also divided it into two parts; the Rc-contact region and the switch region in which the SMP is activated. The rate (_) of amino acid substitutions was computed for each of the four regions.

As a result, we found that each of the four regions showed different values of _; for the ligand-binding region, 1.01 _ 0.01 substitutions/year/site, for GP-contact region 1.26 _ 0.09, for Rc-contact region 1.18 _ 0.03, and for the switch region 0.68 _ 0.02. We also found that the difference in those substitution rates was statistically significant based on Welch's test. Thus, we concluded that the GP-contact and Rc-contact regions have substitution rates higher than those of both ligand-Rc and the switch regions, implying that the connecting regions of the two systems have evolved under less functional constraints.

(8) Does apoptosis play an active role in the regeneration of planarian?

Jung Shan HWANG and Takashi GOJOBORI

Regeneration, a mechanism for repairing or replacing damaged or lost body parts. It is found in two primitive organisms, hydra and planarian, but re-

stricted in higher organisms. Vertebrate regeneration is merely found in amphibian limbs and lens, mammalian liver and muscle tissue. This raises the questions of why the regenerative ability is limited only to some primitive, selected organisms and further that in what extent the evolution lead to the loss of regeneration capability? We used the freshwater planarian, which belongs to the platyhelminthes family and possess three tissue layers, bilateral symmetrical body plan as a model to study molecular mechanisms and evolution significance underlying its remarkable regenerative ability. One of the physiological mechanisms that likely takes part in the regeneration of planarian is the apoptosis since one of its central role is to regulate the precise development of cell function and cell population.

Five caspase homologs have been isolated from the cDNA library of planarian and the homology search of their ORFs has been carried out using the BLAST programs. Among the caspase family, caspase 3 has the highest homology to all the isolated clones, followed by caspases 7 and 8. Sequence alignment of each amino acid sequence was generated using the CLUSTAL X multiple alignment program. Alignments indicated that all clones contain caspase conserved sequence and a very short amino-terminal domain. Furthermore, Northern Blotting has been performed to one of the clone and the result showed that its mRNA level decreases during the regeneration. It seems that apoptosis probably plays a role in the regenerative process of planarian, however further evidence is required to ensure the Northern blot result and to determine the role of apoptotic cascade in the processes of reproducing the whole body, injury and wound healing.

(9) The base compositional localisation of genes in the human genome sequence

T. Daniel Andrews and Takashi GOJOBORI

We have analysed the distribution of genes within the finished portion of the human genome sequence to look for correlations between the presence of genes and the base composition of the isochore in which they reside. A window analysis was employed to divide the approximately 650Mb of finished non-redun-

dant human genome sequence into contiguous 100Kb regions in which base composition was calculated. A new partial annotation method that uses non-redundant EST or cDNA sequence information was then applied to gain relative measures of the density of coding sequences within each window. Hence, the relationship between gene density and isochore base composition was directly appraised. Previous studies have found high concentrations of genes in the highest G+C regions of the human genome, but we have found that the gene distribution is actually far more uniform. While genes are rare in the most A+T rich regions of the genome, there is comparatively less than a four-fold enrichment of genes in the G+C richest isochores. Our findings predict that only around 26% of all genes are to be found in the approximately 12% of the human genome that represents the highest G+C isochore families. These results are supported by the recently published full draft of the human genome sequence obtained by the public genome sequencing effort, and by the sequence analysis conducted by Celera Genomics. These findings have strong implications for our current understanding of the processes of isochore formation in warm-blooded vertebrates.

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I-b. Laboratory for Gene-Product Informatics

(1) Structural/functional assignment of bacteriophage T4 unknown proteins by iterative database searches

Takeshi KAWABATA, Fumio ARISAKA[1] and Ken NISHIKAWA ([1]Department of Molecular and Cellular Assembly, Tokyo Institute of Technology)

Among the total of 274 orfs within bacteriophage T4, only half have been reasonably well characterized, and the functions of the rest have remained obscure. In order to predict the molecular functions of the orfs, a position-specific iterated (PSI)-BLAST search of bacteriophage T4 against the sequence database of known 3D structures was carried out. PSI-BLAST is one of the most powerful iterative sequence search methods using multiple sequence alignment, with the ability to detect many more proteins with distant homology than standard pairwise methods. The 3D structures of proteins are considered to be better preserved than the sequences, and the detected distantly homologous proteins are likely to possess highly similar 3D structures. Thirteen orfs of phage T4, whose homologues were not detected by standard pairwise methods, were found to have significantly homologous counterparts by this

method. The plausibility of the results was confirmed by checking whether important residues at substrate/ ligand-binding sites were conserved. Among them, two orfs, *vs.1* and *e.1*, which are similar to *Escherichia coli* lytic enzyme and *Mut T* protein, respectively, had not been studied previously. Also, gp *rIIA*, a rapid lysis protein, whose gene structure had been intensively studied during the development of molecular biology in the 1950s and yet whose molecular function remains unknown, has an N-terminal domain that is significantly similar to the N-terminal region of the heat shock protein Hsp90. See Ref. 1 for the details.

(2) Protein structure comparison using the Markov transition matrix of evolution

Takeshi KAWABATA and Ken NISHIKAWA

A number of automatic protein structure comparison methods have been proposed; however, their similarity score functions are often decided by the researchers intuition and trial-and-error, and not by theoretical background. We propose a novel theory to evaluate protein structure similarity, which is based on the Markov transition model of evolution. Our similarity score between structures i and j is defined as log $P(j \rightarrow I)/P(i)$, where $P(j \rightarrow i)$ is the probability that structure j changes to structure i during the evolutionary process, and $P(i)$ is the probability that structure i appears by chance, this is a reasonable definition of structure similarity, especially for finding evolutionarily related (homologous) similarity. The probability $P(j \rightarrow i)$ is estimated by the Markov transition model, which is similar to the Dayhoff's substitution model between amino acids. To estimate the parameters of the model, homologous protein structure pairs are collected using sequence similarity, and the numbers of structure transitions within the pairs are counted. Next those numbers are transformed to a transition probability matrix of the Markov transition. Transition probabilities for longer time are obtained by multiplying the probability matrix by itself several times. In this study, we generated three types of structure similarity scores: an environment score, a residue-residue distance score, and a secondary structure elements (SSE) score. Using

these score, we developed the structure comparison program, MATRAS (MArkovian TRAnsition of protein Structure). It employs a hierarchical alignment algorithm, in which a rough alignment is first obtained by SSEs, and then is improved with more detailed functions. We attempted an all-versus-all comparison of the SCOP database, and evaluated its ability to recognize a superfamily relationship, which was manually assigned to be homologous in the SCOP database. A comparison with the FSSP database shows that our program can recognize more homologous similarity than FSSP. We also discuss the reliability of our method, by studying the disagreement between structural classifications by MATRAS and SCOP. See Ref. 2 for the details.

(3) Redesign of artificial globins: Effects of residue replacements at hydrophobic sites on the structural properties

Yasuhiro ISOGAI[1], Anna ISHII[2], Tetsuo FUJISAWA[3], Motonori OTA and Ken NISHIKAWA
([1]RIKEN at Wako, [2]Gakushuin Univ., [3]RIKEN Harima Inst.)

Artifical sequences of the 153 amino acids have been designed to fit the main-chain framework of the sperm whale myoglobin (Mb) structure based on a knowledge-based 3D-1D compatibility method. The previously designed artificial globin (DG1) folded into a monomeric, compact, highly helical and globular from with overall dimensions similar to those of the target structure, but it lacked structural uniqueness at the side-chain level [Isogai *et al.*, (1999) *Biochemistry* **38**, 7431-7443]. In this study, we redesigned hydrophobic sites of DG1 to improve the structural specificity. Several Leu and Met residues in DG1 were replaced with β-branches amino acids, Ile and Val, referring to the 3D profile of DG1 to produce three redesigned globins, DG2-4. These residue replacements resulted in no significant changes of their compactness and α-helical contents in the absence of denaturant, whereas they significantly affected the dependence of the secondary structure on the concentration of guanidine hydrochloride. The analyses of the denaturation curves revealed higher global stabilities of the designed globins than that of natural apoMb. Among DG1-4, DG3, in which 11 Leu residues of DG1 and replaced with seven Ile and four Val residues, and one Met residue is replaced with Val, displayed the

lowest stability but the most cooperative folding-unfolding transition and the most dispersed NMR spectrum with the smallest line width. The present results indicate that the replacements of Leu (Met) with the β-branched amino acids at appropriate sites reduce the freedom of side-chain conformation and improve the structural specificity at the expense of stability. See Refs. 3 and 4 for the details.

(4) The genomic DNA sequences of various species are distinctively distributed in nucleotide composition space

Hiroshi NAKASHIMA[1] and Ken NISHIKAWA ([1]Kanazawa University)

The composition of di-, tri- through to hexa-nucleotides was analyzed in genomic sequence data from 18 organisms, including the complete genomes of 13 microorganisms, two complete chromosomes from protozoa and partial genome sequences from humans, *Arabidopsis thaliana* and *Caenorhabditis elegans*. Double-stranded DNA from each genome was chopped into fragments of a fixed length, and the oligonucleotide composition of these fragments was examined in multi-dimensional composition space, defined by axes corresponding to individual oligonucleotide components. Each DNA fragment was plotted at one point n this composition space. When DNA fragments of several different organisms were plotted at once, they clustered separately as clearly shown by a projection on a two dimensional plane. We examined the extent to which DNA fragments from the 18 organisms could be correctly assigned to a species purely in terms of nucleotide composition. The species discrimination accuracy increased not only as the length of oligonucleotide was increased from di- to hexa-nucleotide, but also as the length of fragmented DNA was increased from 0.1 to 10 kbp. Using the symmetrical trinucleotide frequencies, 82% of 43,045 DNA fragments of 1 kbp long from 18 organisms could be correctly identified. This accuracy rose to 96% when DNA length was increased to 10 kbp. These results indicated that the characteristic nucleotide compositions specific to individual organisms exist throughout the genome. In addition, the codon-usage frequencies of protein-encoding sequences (i.e, genes) were compared with the trinucleotide frequencies of non-coding part of a genome. The

results strongly suggest that the well-known, species-specific codon usage bias might be caused by the same factor that maintains a characteristic nucleotide composition for the entire genome of each organism. See Ref. 5 for the details.

(5) Physico-chemical evaluation of protein folds predicted by threading

Akira KINJO and Ken NISHIKAWA

Protein structure prediction remains an unsolved problem. Since prediction of the native structure seems very difficult, one usually tries to predict the correct fold of a protein. Here the fold is defined by the approximate backbone structure of the protein. However, physicochemical factors that determine the correct fold are not well understood. It has recently been reported that molecular mechanics energy functions combined with effective solvent terms can discriminate the native structures from misfolded ones. Using such a physicochemical energy function, we studied the factors necessary for discrimination of correct and incorrect folds. We first correct selected and incorrect folds by a conventional threading method. Then, all-atom models of those folds were constructed by simply minimizing the atomic overlaps. The constructed correct model representing the native fold has almost the same backbone structure as the native structure but differs in side-chain packing. Finally, the energy values of the constructed models were compared with that of the experimentally determined native structure. The correct model as well as the native structure showed lower energy than misfolded models. However, a large energy gap was found between the native structure and the correct model. By decomposing the energy values into their components, it was found that solvent effects such as the hydrophobic interaction or solvent shielding and the Born energy stabilized the correct model rather than the native structure. The large energetic stabilization of the native structure was attained by specific side-chain packing. The stabilization by solvent effects is small compared to that by side-chain packing. Therefore, it is suggested that in order to confidently predict the correct fold of a protein, it is also necessary to predict side-chain packing. See Ref. 7 for the details.

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2. KAWABATA, T. and NISHIKAWA, K.: Protein structure comparison using the Markov transition matrix of evolution. PROTEINS, **41**, 108-122, 2000.
3. ISOGAI, Y., ISHII, A., FUJISAWA, T., OTA, M. and NISHIKAWA, K.: Redesign of artificial globins: Effects of residue replacements at hydrophobic sites on the structural properties. Biochemistry, **39**, 5683-5690, 2000.
4. ISOGAI, Y., ISHII, A., ISHIDA, M., MUKAI, M., OTA, M., NISHIKAWA, K. and IIZUKA, T.: Structural and functional properties of designed globins. Proc. Indian Acad. Sci. (Chem. Sci.), **112**, 215-221, 2000.
5. NAKASHIMA, H. and NISHIKAWA, K.: The genomic DNA sequences of various species are distinctively distributed in nucleotide composition space. Res. Comm. In Biochem., Cell & Mol. Biol., **4**, 25-45, 2000.
6. YUSUF ALI, M., KHAN, M. L. A., SHAKIR, M. A., FUKAMI-KOBAYASHI, K., NISHIKAWA, K. and SIDDIQUI, S.S.: Expression and cDNA cloning of klp-12 gene encoding an ortholog of the chicken chromokinesin, mediating chromosome segregation in C. elegans. J. Biochem. Mol. Biol., **33**, 138-146, 2000.
7. KINJO, A.R., KIDERA, A., NAKAMURA, H. and NISHIKAWA, K.: Physico-chemical evaluation of protein folds predicted by threading. Eur. Biophys. J., in press.

I-c. Laboratory for Gene Function Research

(1) Evolution of the HLA class I region in human genome

Yoshio TATENO, Hidetoshi INOKO[1] and Masaaki YAMAZAKI[2] ([1]Tokai University, [2]Fujiya Bioscience Institute)

HLA class I region is occupied mainly by A, B and C loci, where B and C loci are located in duplicated genome fragments respectively. We found several defective LINE sequences in both fragments, and found that they were homologous between the fragments. We estimated the evolutionary origin of B and C loci by analyzing the LINE sequences. It is known that these loci have a great number of alleles. Investigation into their origin led us to the suggestion that some of them originated from a common ancestor of human and chimpanzee,

supporting the trans-species polymorphism that J. Klein has proposed. This would be an interesting case where neutral LINE sequences and a gene being subject to typical positive selection co-exist and co-evolve in a unit genome fragment.

(2) Evolution of protein structure in the periplasmic binding protein (PLBP) superfamily

Kaoru FUKAMI-KOBAYASHI, Yoshio TATENO and Ken NISHIKAWA

In the previous study (Fukami-Kobayashi et al, 1999), we have shown that one of the type I PLBPs is most likely to resemble a primordial PLBP, and that a type II PLBP arose from the type I PLBPs to evolve into various type II PLBPs. Recently, the crystal structures of another type of PLBPs have been reported. The topological arrangements of the type III PLBPs are different from either type. To find a clue to the evolutionary origin of the type III, we constructed phylogenetic trees for PLBPs and their partner ABC proteins, and obtained a preliminary result that the type III was closer to the type II.

Some of bacterial transcription factors such as lactose repressor (LacI) and purine repressor (PurR) are homologous to periplasmic binding proteins (PLBPs) in their C-terminal ligand binding domains. Phylogenetic analysis of repressors and their PLBP homologues revealed that the attachment of the DNA-binding domain was originated first, and substrate specificity was gained in evolution thereafter. The tree also shows that the emergence of the attachment predates the divergence of major lineages of eubacteria.

(3) Compensatory covariation in protein evolution

Kaoru FUKAMI-KOBAYASHI and Steven A. BENNER[1] ([1]Florida University)

Compensatory covariation arises in divergent evolution of protein sequences, when two amino acid sites have particular mutations that work together to maintain a global property of a protein (net charge, for example), while each mutation alone does not. We are now finding such covariations by examining branches of an evolutionary tree of a specified group of proteins and recon-

structing ancestral sequences in it.

(4) DNA Data Bank of Japan (DDBJ) in collaboration with mass sequencing teams

Yoshio TATENO, Satoru MIYAZAKI, Motonori OTA, Hideaki SUGAWARA and Takashi GOJOBORI

We at DDBJ (http://www.ddbj.nig.ac.jp)process and publicise the massive amounts of data submitted mainly by Japanese genome projects and sequencing teams. It is emphasised that the collaboration between data producing teams and the data bank is crucial in carrying out these processes smoothly. The amount of data submitted in 1999 is so large that it alone exceeds the total amount submitted in the preceding 10 years. To cope with this situation, we have developed tools not only for processing such massive amounts of data but also for efficiently retrieving data on demand.

Publications

1. TATENO, Y., MIYAZAKI, S., OTA, M., SUGAWARA, H. and GOJOBORI, T. (2000) DNA Data Bank of Japan in collaboration with mass sequencing teams. Nucleic Acids Res **28**: 24-26.
2. MIYAZAKI, S., HASHIMOTO, H., SHIMADA, A., TATENO, Y. and SUGAWARA, H. (2000) A new file format and tools for the large-scale data submission to DNA Data Bank of Japan (DDBJ). In Currents in Computational Molecular Biology, pp 60 - 61, S. Miyano, R. Shamir and T. Takagi ed., Universal Academy Press, Tokyo.
3. ABOLA, E.E., BAIROCH, A., BAKER, W.C., BECK, S., BENSON, D.A., BERMAN, H., CAMERON, G., CANTOR, C., DOUBET, S., HUBBBARD, T.J.P., JONES, T.A., KLEYWEGT, G.J. KOLASKAR, A.S., KUIK, Van A., LESK, A.M., MEWS, H.-W., NEUHAUS, D., PFEIFFER, F., TENEYCK, L.F., SIMPSON, R.J., STOSSER, G., SUSSMAN, J.L., TATENO, Y., TSUGITA, H., ULRICH, E.L. and VIEGENTHART, J.F.G. (2000) Quality control in data banks for molecular biology. BioEssays **22**: 1024-1034.
4. YUSUF ALI, M., KHAN, M. L. A., SHAKIR, M. A., FUKAMI-KOBAYASHI, K., NISHIKAWA, K. and SIDDUQUI, S. S. (2000) Expression and cDNA cloning of klp-12 gene encoding an ortholog of the chichen chromokinesin, mediatingchromosome segregation in Caenorhabditis elegans. J. Biochem. Mol. Biol. **33**: 138-146.

I-d. Laboratory for Molecular Classification

(1) Research and Development for DDBJ

(1)-a. From YAMATOII to TSUNAMI

The laboratory is in charge of the development and management of databases for DDBJ. Since 1998, DDBJ has developed a data submission system with parser in order to process massive data originated from genome projects and a study on biodiversity. The development was in time for the completion of the draft sequencing of human genome in June 2000. In the meantime, the system could also cope with a number of cDNA sequences of human, mice and other organisms.

DDBJ expects that the draft sequences will be updated, especially by improvement of annotation. It requires streamline of the system for review and release of data. Therefore, DDBJ looked through the existing review system (YAMATOII) and the mass submission system resulting in planning a new data processing system. Development of the new system is named TSUNAMI project and is underway.

(1)-b. Expansion of Genome Information Broker (GIB)

GIB was originally created for *E. coli* genome for the retrieval and analysis of genomic information in a set. We implemented microbial genome data into GIB whenever genome sequencing were completed and the data is made open to the public. At the GIB Web page (http://www.ddbj.nig.ac.jp/), key word search, homology search, links to DBGET, KEGG and GTOP and visualization of the data are available for 42 organisms as of February 2001.

We have utilized XML and CORBA to integrate distributed computational resources in order to cope with the explosion of microbial genome information.

(1)-c. Application of XML to DDBJ

Interoperability of databases is still an issue in bioinformatics. XML is a

powerful yet simple tool that may improve the interoperability. We defined a data type definition (DTD) for DDBJ entries. The XML will firstly implanted into the DDBJ retrieval system "getentry". It will make it possible to integrate automatically a private database and a subset of DDBJ based on DTD.

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

(2)-a. WDCM

WFCC and MIRCEN stand for World Federation for Culture Collections and Microbial Resource Centers network respectively. The laboratory is a host of WDCM that is the data center of WFCC and MIRCEN. We maintain the world directory of 500 culture collections in 60 countries in databases of "CCINFO" and "STRAIN" that are accessible at http://wdcm.nig.ac.jp/.

(2)-b. Development of a Workbench for Biological Classification and Identification (InforBIO)

We have been interested in introduction and development new Internet technologies. We introduced the World Wide Web (WWW) as one of the first ten WWW servers. We also developed a simultaneous search system of distributed system named Agent for Hunting Microbial Information in Internet (AHMMI). We now develop the digital workbench named InforBIO.

In InforBIO, users are able to integrate databases and analytical tools that are distributed in the Internet including their own resources. We aim at an open system by use of JAVA, XML, and tools of CORBA and a relational database management system in the public domain. We will distribute the prototype of InforBIO in CD-ROM this year.

(3) Others

(3)-a. Data processing system for patent sequences

DDBJ has received patent sequences from Japan Patent Office (JPO) and

disseminate them to the public. Therefore DDBJ and JPO have discussed about a system for data transfer from JPO to DDBJ.

This year, we cooperated a survey by Japan Bioindustry Association (JBA) on data processing system in European Patent Office (EPO) and United States Trademark and Patent Office (USTPO). It is to be noted that EPO has already uses the EMBL data system that is outside EPO. USTPO also look into the possibility to use computer resources outside. The primary reason of the outsourcing is to realize efficient data processing while sequence data increase exponentially.

(3)-b. Biological Resources Centres (BRC)

The Working Party for Biotechnology (WPB) of OECD set up a task force on BRC in February1999 to develop a policy guidance to support BRCs. Professor Hideaki Sugawara is the chair of the task force and compiled a report entitled "Biological Resource Centres: Underpinning the future of life sciences and biotechnology". The report calls for actions in OECD countries and beyond. The report and a follow-up task force were approved by WPB on 16 February 2001.

(3)-c. Global Biodiversity Information Facility (GBIF)

GBIF is a scheme that was created by a Biological Informatics working group in Megascience Forum of OECD. GBIF will improve the accessibility of all kinds of data of all kinds of organisms on the globe. One of the staffs (HS) participated in the discussion of the working group for three years and it is expected that the laboratory will contribute GBIF when it becomes reality. The GBIF HomePage is at "http://www.gbif.org/".

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1. GOTO, Y., MIYAZAKI, S. and SUGAWARA, H.: Journal of Information and Knowledge Vol. **10**. No.4 (2001)
2. MIYAZAKI, S. and SUGAWARA, H.: Development of DDBJ-XML and Its Application to a Database of cDNA,Genome Informatics 2000,Universal Academy Press, Inc (Tokyo), 380-381, 2000.

3. SUGAWARA, H.: Microbial Resource Centers and Data Activities (In Japanese), Biological Resources Centers and Data Banks for Life Science (ed. Nakatsuji, N.), Kyoritsu-Shuppan (Tokyo), 130-135, 2000.
4. MORI, H., SUGAWARA, H. and UCHIYAMA, I.: Escherichia coli genome (In Japanese), Biological Resources Centers and Data Banks for Life Science (ed. Nakatsuji, N.), Kyoritsu-Shuppan (Tokyo), 118-124, 2000.
5. MIYAZAKI, S., SUGAWARA, H. and GOJOBORI, T.: Genome Informatics (In Japanese), Saishin-Igaku, **56**(1), 64-71, 2000.

Oral presentation

1. AMANO, M., EZAKI, T., KAWAMURA, Y. and SUGAWARA, H.: Utilization of DNA chip for the Identification of Bacteria (In Japanese), 7th Annual Coneference of Japan Society for Culture Collections (Sendai), 8 June 2000.
2. SUGAWARA, H. and MIYAZAKI, S.: Issues of Microbial Information Network (In Japanese), 7th Annual Coneference of Japan Society for Culture Collections (Sendai), 8 June 2000.
3. SUGAWARA, H.: Biological Resources Centers In the New Age - a report by OECD-(In Japanese), Workshop on Biological Resources Centers (Tokyo), 14 June 2000.
4. MIYAZAKI, S. and SUGAWARA, H.: WFCC World Data Centre for Microorganisms (WDCM): Its activities and the utilization of information technology for culture collections and microbiology, The 9th International Congress for Culture Collections, 22-27 July 2000, Brisbane.
5. SUGAWARA, H. and MIYAZAKI, S.: Linking microbial genetic resources in culture collections with nucleic acid sequences, The 9th International Congress for Culture Collections, 22-27 July 2000, Brisbane.
6. SALOMON, W., DOYLE, A. and SUGAWARA, H.: OECD Task Force on strategy for the long-term development and sustainability of Biological Resource Centres, The 9th International Congress for Culture Collections, 22-27 July 2000, Brisbane.
7. SUGAWARA, H.: Bioinformatics, Management and Operation of Culture Collections Training Course of the 9th International Congress for Culture Collections, 29-30 July 2000, Briabane.
8. SUGAWARA, H.: Roles and Value of Public Databases in the time of Genomics and its Industrialization, Proceedings of BIO JAPAN 2000 (Tokyo), 83-86, 27 Sep 2000.

J. Radioisotope Center

(1) Expresion and Purification of *Escherichia coli* Transcription Factors

Katsunori YATA and Akira ISHIHAMA

Transcription regulation is a major cellular process by which the exression of a large number of genes on the genome are controlled. The RNA polymerase holoenzyme of *Escherichia coli* is composed of the core enzyme with the subunit composition $\alpha_2 \beta \beta'$ and one of seven molecular species of the σ subunit. The function of RNA polymerase holoenzyme is further modulated through interplay with one or two of about 100-150 transcription factors. The intracellular concentrations of transcription factors are therefore considered to be a major determinant of the expression level of most inducible genes. In order to determine the intracellular levels of various transcription factors in *E. coli*, we expressed as many transcription factors as possible in His-tagged forms using the respective cloned genes. So far more than 25 molecular species of the *E. coli* transcription factor have been purified to apparent homogeneity by affinity chromatography on Ni^{2+}-NTA columns, including AcrR, AhpC, AhpF, ArcA, DeoR, DnaK, Fur, GroEL, KdpE, IclR, IlvY, InfB, LacI, LeoO, MerR, NusA, OxyR, PhoP, RpsB, RpsF, PspF, SdiA, SoxR, SoxS, ThiI, and TufB. Polyclonal antibodies were raised in rabbits against the purified transcription factors. The antibodies are being used, in Division of Molecular Genetics, for detemination of the transcription factor level in cell lysates by quantitative Western blot.

The purified transcription factors will also be used for identification of their contact sites on RNA polymerase subunits by mapping of the protein-protein contact-dependent clevaga sites by FeBABE (iron-*p*-bromoacetamidovenzyl EDTA) tethered to each transcription factor.

Publications

1. ISHIHAMA, A.: Functional modulation of *Escherichia coil* RNA polymerase. *Ann. Rev. Microbiol.* **54**, 499-518 (2000)
2. ISHIHAMA, A.: Molecular anatomy of RNA polymerase using protein-conjugated metal probes with nuclease and protease activities. *Chem. Commun.* **2000**, 1091-1094 (2000)

K. Experimenntal Farm

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

Ken-Ichi NONOMURA, Mitsugu EIGUCHI, Toshie MIYABAYASHI, Yukihiro ITO 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 rice. 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.: The centromere composition of multiple repetitive sequences on rice chromosome 5. Chromosoma, 2001(in press).

ABSTRACTS OF DIARY FOR 2000

Biological Symposium

Jan. 7 Detection of DNA-looping by single-molecule manipulation (Kumiko Sogawa)

Jan. 13 Partitioning of DNA in bacteria - Intracellular localization of DNA depending on cell division cycle (Hironori Niki)

Jan. 19 Molecular mechanism for determining dorso-ventral axis in zebra fish (Masahiko Hibi)

Jan. 20 Schrodinger's dream over 50 years - Complex electron microscopy and gene direct-reading (Kuniaki Nagayama)

Jan. 25 Control mechanism of cell cycle in higher organism cells using warts and fzr/cdh1 (Hideyuki Saya)

Jan. 28 Transcription of yeast rDNA, RNA polymerase specificity, rDNA repeats and nucleolar structures (Masayasu Nomura)

Feb. 4 Function and regulation of the transcription factor Oct-4 in the mammalian germline (Hans R. Schoeler)

Feb. 15 Research papers in an age of electronic media and problems in Japan (Hideaki Tanaka)

Feb. 17 A 4-dimensional analysis of the embryogenesis of C. elegans : migrations, regions, linesge and binary specification (Ralf Schnabel)

E.coli FIS controls DNA architecture and promoter activity (Georgi Muskhelishvili)

Feb. 18 Control of morphogenesis and cell proliferation by mammalian polycomb group (Haruhiko Koseki)

Mar. 1 Molecular mechanisms of Wnt signal and TGF-beta signal (Koji Shibuya)

Mar. 2 Role of Ras-MAP kinase signal transduction system in olfactory sense of C. elegans (Yuichi Iino)

Mar. 7 Gene Regulation by Peptide Nucleic Acid (PNA) (Peter E. Nielsen)

Mar. 8 Protein transport in secretory organella - Protein transport to

vacuolar of higher plants and transport apparatus of budding yeast - (Ken Matsuoka)

Mar. 13 Activity dependent plasticity : new insights into functional andmorphological changes on the synaptic level (Tobias Bonhoeffer)

Mechanism for transporting functional molecules between nucleus and cytoplasm (Naoko Imamoto)

Mar. 14 Mechanisms of activity-dependent plasticity in the mammalian visual cortex (Michael P. Stryker)

Development of new bacterial chromosome partitioning technique (Hironori Niki)

Mar. 15 Function of LAP2 on nuclear envelope breakdown and reconsitution (Kazuhiro Furukawa)

Apr. 10 Cerebral function of speech - from physics to linguistics - (Kuniyoshi Sakai)

Connections among transcription, chromatin, and mRNA processing (Stephen Buratowski)

Apr. 12 Mechanisms underlying induction and progression of a neurogenic wave in the developing zebrafish retina (Ichiro Masai)

A multiprotein mediator complex is required for the stimulation oftranscription by acitvators in human cells (Arnold J. Berk)

Apr. 17 Development of selective techniques for visualizing synaptic neural pathways using WGA transgene and application thereof (Yoshihiro Yoshiwara)

Apr. 24 Loose coupling of molecular mechanism (Fumio Ohsawa)

Apr. 26 Isolation and analysis of DL gene that regulates the flower development of rice - Is ABC mode applicable to monocotyledon? - (Hiroyuki Hirao)

May 1 Post-transcriscriptional and post-transnational control in bacteriophage lambda lysis-lysogeny decision (Prof. Amos Oppenheim)

May 16 Signal transduction and formation of neural cells : polarity and mechanism of forming axon (Naoyuki Inagaki)

May 29 Functions of transcription factors Mesp1 and Mesp2 : Early me-

	soderm embryogenesis and segmentation (Yumiko Saga)
	Role of transcription factor Gli in controlling the expression the sonic hedgehog target gene (Hiroshi Sasaki)
July 6	Search for molecular mechanism for the developmental determination of plant cells (Kiyotaka Okada)
July 10	Check-point control by Chk1 and chk2 (Shuhei Matsuoka)
July 27	Gene group for controlling neuroaxonal induction and cell migration : approach using C. elegans (Takehiro Kawano)
Aug. 1	1. Reverse transport of protein found in membrane protein degradation, 2. Identification and analysis of different PI3KVps34p complexes that are effective for autophagy and protein transport (Akio Kihara)
Sep. 8	Attenuation of Influenza Viruses via Genetic Engineering : Modification of the Interferon Antagonist (Prof. Peter Palese)
Sep. 20	Molecular Dissection of Central Spindle Assembly and Cytokinesis (Dr. Michael Glotzer)
Sep. 27	Intracellular signal transfer mechanism of neuronal growth cone: approach of cell-localized selective molecular targeting with CALI method (Kotaro Takei)
Sep. 29	Predicting the future course of human influenza virus evolution (Dr. Walter Fitch)
	What determines the risk of inflammatory disease in HTLV-I infection? (Dr. Charles Bangham)
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Oct. 12	Sensing and responding to DNA damage (Noel Lowndes)
Oct. 17	Mechanisms of cell fate determination during embryogenesis of ascidian by maternal localized mRNA and cellular interaction (Hiroki Nishida)
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Nov. 2	Gene identification from genome sequence (Tetsushi Yada)

Nov. 6 Function analysis of a new bHLH family, hesr-1, -2 and -3 : potential regulators of vertebrate simitogenesis (Hiroki Kokubo)

Nov. 10 Germ Line, Stem Cella and Genomic Imprinting (M. Azim Surani)
DNA Methylation in Cancer Tissue (Peter A. Jones)

Nov. 13 Novel homeobox gene isolated by EST to be dominantly expressed in female germ cell during sex determination of mouse (Nobuka Takasaki)
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Nov. 14 Kinesin I is required for organelle transport in axons and for anterior-posterior axis determination (William M. Saxton)
MES PROTEINS AND MATERNAL COTROL OF GERMLINE DEVELOPMENT IN C. ELEGANS (Susan Strome)

Nov. 15 Rat Genome Database : A Platform for Rat, Mouse and Human Comparative Genomics (Jian Lu)

Nov. 30 Molecular genetics of murine dorsl-vemtral limb and mid-hindbrain development (Dr. Randy L. Johnson)

Dec. 19 The Dynamic End : Silencing and Replocation of Telomeres in Yeast (Daniel Gottschling)
Ubiquitin-like protein SUMO-1 and control of chromosomal DNA homologous recombination and cell growth (Hisahito Saito)
Codon bias evolution in Drosophila : Detecting the "footprint" of weak selection at silent sites in DNA (Dr. Hiroshi Akashi)

Dec. 20 X-chromosome specific localization mechanism of drosophila male-specific lethal complex (Yuji Kageyama)
The role of the corepressor Groucho in the early embryonic function of Even-skipped (Masatomo Kobayashi)

FOREIGN VISITORS IN 2000

Jan. 7-8	Dr. Alisa S W shum, Department of Anatomy The Chinese University of Hong kong, Hong kong
Jan. 27-28	Dr. Masayasu Nomura, Department of Biological Chemistry University of California, Irvine
Feb. 14-15	Hans R. Scholer, University of Pennsylvania New Bolton Center, Center for Animal Transgenesis and Germ Cell Reseach
Feb. 17-18	Ralf Schnabel, Institut fur Genetik, TU Braunschweig Pat Nolan, MRC Mouse Genome Center and Mammalian Genetics Unit, Harwell
Mar. 7-8	Peter E. Nielsen, University of Copenhagen, IMBG, Laboratory B, The Panum Institute, Blegdamsvej3, DK-2200 Copenhagen N, Denmark
Mar. 13-14	Michael P. Stryker, Department of Physiology and W. M. Keck Foundation Center for Integrative Neuroscience, UCSF Tobias Bonhoeffer, Max-Planck-Institut fur Neurobiology, Munchen-Martinaried
Apr. 10	Stephen Buratowski, Biological Chemistry and Molecular Pharmacology, harvard Medical School
Apr. 11-12	Dr. Avante Paabo, Director, Max-Planck-Institute of Evolutionary Anthropology Leipzig, Germany
Apr. 12-13	Arnold J. Berk, Molecular Biology Institute, University of california, Los Angeles
May 1-2	Amos Oppenheim, The Hebrew University-Hadassah Medical School Department of Molecular Genetics and Biotechnology Jerusalem, Israel
July 10	Matsuoka Shuhei, Baylar College of Medicine, U.S.A.
July 27-28	Dr. Tekehiro Kawano, Samuel Lunenfeld Research Institute of Mount Sinai Hospital, Toront
Sep. 7-8	Prof. Peter Palese, Mount Sinai School of Medicine Department of Microbiology NEW York, NY 10029, U.S.A.
Sep. 20-21	Dr. Michael Glotzer, Research Institute of Moleculer Pa-

	thology Vienna, Austria
Sep. 28-29	Dr. Charles Bangham, Department of Immunology Imperial college School of Medicine St Mary's Hospital, U.K.
Oct. 3-4	Mr. Neil J. Campbell, Senior Director Celera Genomics, U.S.A.
Oct. 12-13	Noel Lowndes, ICRF, Clare Hall Laboratories, U.K.
Oct. 23-24	Harumi Kasamatsu, Moleculer Biology Institute, University of California, Los Angeles, U.S.A.
Nov. 1-2	Masaki Okano, Cardiovascular Research Center, Massachusetts General Hospital, Harvard Medical School
Nov. 6-7	Hiroki Kokubo, University of Texas, MD. Anderson Cancer Center Department of Biochemistry and Moleculer Biology
Nov. 10-11	M. Azim Surani, Wellcome/CRC Institute of Cancer and Developmental Biology, University of Cambridge Peter A. Jones, Norris Comprehensive Cancer Center, University of Southern California
Nov. 13-14	Dr. Nobuyosi Takasaki, Laboratory of Cellular and Development Biology, NIDDK, NIH Willian M. Saxton, Department of Biology, indiana University
Nov. 15-16	Mr. Jian Lu, Bioinformatics Research Center Medical College of Wisconsin, U.S.A.
Nov. 30-Dec. 1	Randy L. Johnson, MD Anderson Cancer Center, Houston
Dec. 18-19	Dr. Yoshiyuki Imai, Department of Development Biology Stanford University School of Medicine
Dec. 19	Dr. Hiroshi Akasi, Dept. of Biology, Institute of Moleculer Evolutionary Genetics Pennsylvania State University, U.S.A.
Dec. 19-20	Dr. Hisato Saitoh, Picower Institute, N.Y., U.S.A.
Dec. 20-21	Yuji Kageyama, Howard Hughes Medical Institute, Baylor College of Medicine Dr. Masatomo Kobayasi, kimmel Cancer Institute, Thomas Jefferson University

AUTHOR INDEX

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Issued by Director-General : Yoshiki Hotta, D.Med.
National Institute of Genetics
Yata 1,111 Mishima, Shizuoka-Ken, 411-8540 Japan

Tel. 0559-81-6718
Fax. 0559-81-6715

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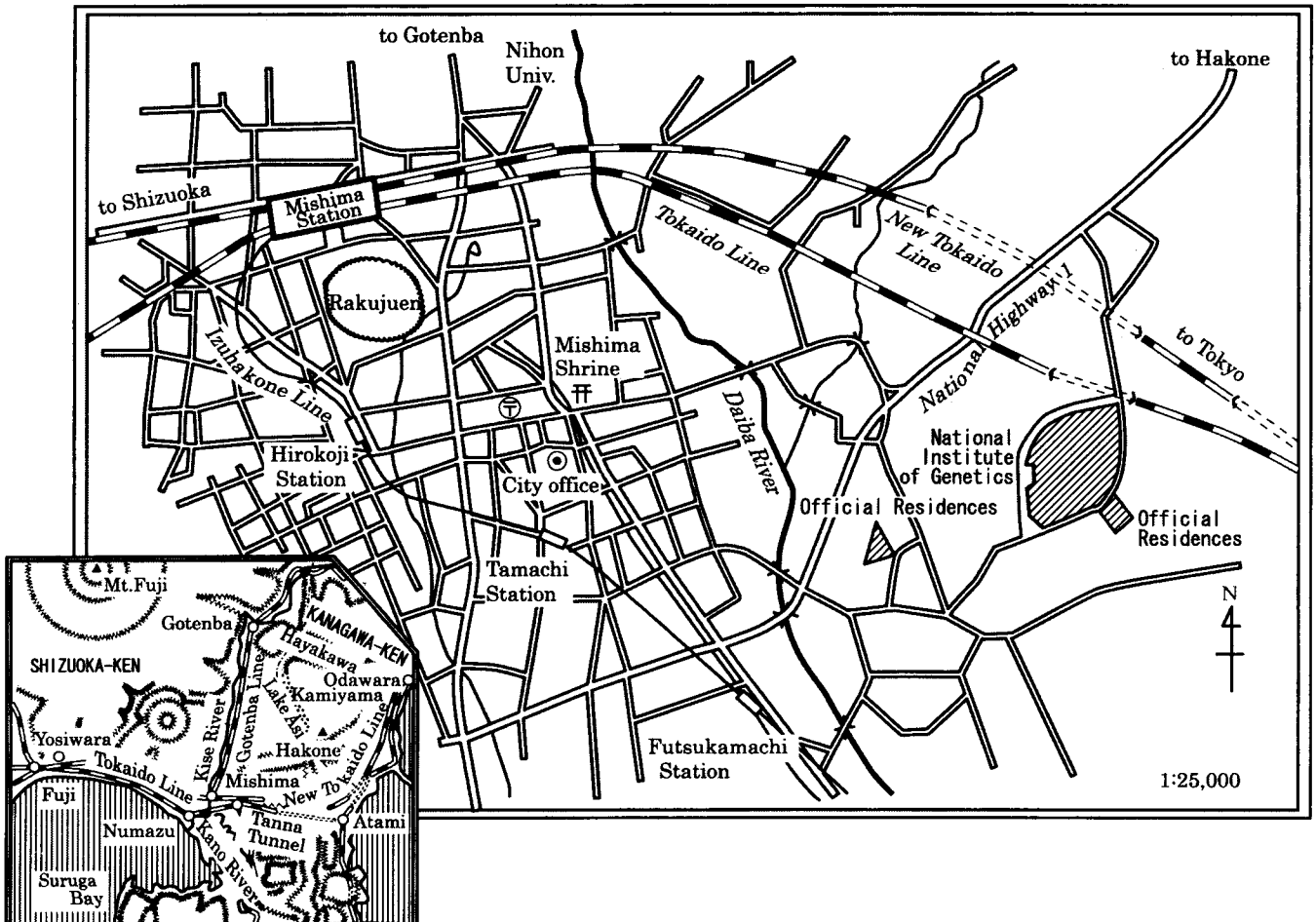
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Official Residences
Tamachi Station
N
Futsukamachi Station
1:25,000
Mt.Fuji
Gotenba
KANAGAWA-KEN
Hayakawa
SHIZUOKA-KEN
Odawara
Kamiyama
Gotenba Line
Lake Ashi
Yosiwara
Kise River
Hakone
Tokaido Line
New Tokaido Line
Mishima
Fuji
Tanna Tunnel
Atami
Numazu
Kano River
Suruga Bay