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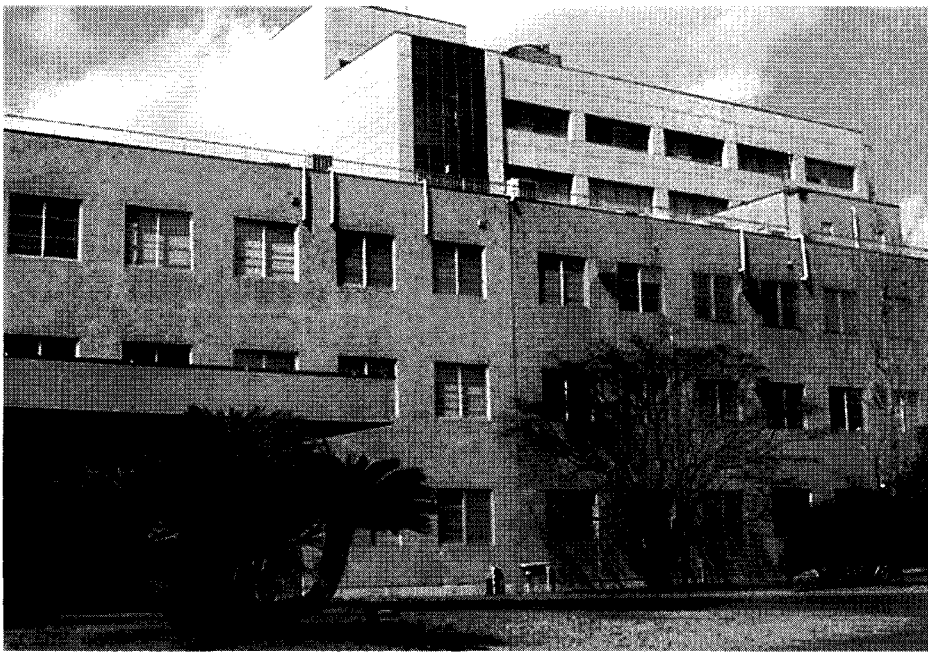

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GENERAL STATEMENT

National Institute of Genetics (NIG) was established 48 years ago as a center for genetics research. Major contribution of NIG in the research in genetics, in particular population genetics, plant genetics and molecular and developmental genetics, has 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 serve as Department of Genetics of the Graduate School of Life Science. We have at present about 30 graduate students and about ten special research students from other universities, including those from abroad. This year, 10 students obtained Ph.D. In addition, we have nine post-doctoral fellows of the center of excellence program. The steady flow of young scientists is important to constantly rejuvenating our research activities.

We have been carrying out several research-related services. The DNA Data Bank of Japan (DDBJ) is one of the three central data banks in the world that gather, annotate, store and distribute information on DNA sequences. Our institute also stores and distributes various organisms with genetically characterized traits. Among them, services with Escherichia coli, mice, rice and Drosophila are particularly significant. These service activities will continue to develop in the coming years. Recent development of new technology using various model organisms has enhanced the importance of genetics as bases of biology. Therefore, it is timely that we established Genetic Strains Research Center and Genetic Resource Information Center. Our institute is uniquely suited for pursuing cooperative work with scientists of various disciplines by sharing the genetic resources.

This year, we sponsored NIG International Symposium on "Gene Functions to Cell Differentiation" organized by Junichi Tomizawa, former Director-General. We invited 21 foreign guests and 25 Japanese speakers. We also utilized this occasion to present many contributions from our Institute, and asked for comments, advice and reviews as to our activities. The reviewer's

critical comments are being used to make our new future plan to improve our standard. We sincerely hope that with guidance from people in and outside NIG and further support from governmental and private sources, we will be able to lead the institute into a more successful future.

Junichi Tomizawa who introduced radical innovations retired at the end of September 1997, and Yoshiki Hotta was elected as a new Director-General. I consider that the contribution of Junichi Tomizawa in modernizing the Institute cannot be overstated. He paved a road for us to maintain our prestigious position in the genetics research. Indeed, most of the works reported here are accomplished under the guidance of the former Director-General.

In the past year we also saw a number of changes in the staff of the institute. Makio Tokunaga joined as associate professor of Structural Biology Center. Dr. Kensuke Horiuchi, Division of Microbial Genetics and Dr. Tomoko Ohta, Division of Population Genetics, retired at the end of March. Promoted as research associates are, Masataka Okabe (Div. of Developmental Genetics) and Tetsuichiro Saito (Genetic Strain Research Center). On the other hand, the following members left NIG to take new positions to extend their career. Among them are Kenji Izuhara (Kyusyu Univ.), Atsushi Higashitani (Tohoku Univ.), Yasuo Ina (Biomol. Eng. Res. Inst.), Hideo Goto (Nat. Inst. Animal Health), Kengo Kanamaru (Univ. of Tokyo) and Kenichi Matsumoto (Hokkaido Univ.). In the Department of Administration, Hitoshi Sunada succeeded to the Dept. Head, and Hideo Kuroda moved to Nagasaki Univ.

Yoshiki Hotta

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TATENO, Yoshio; National Institute of Genetics

RESEARCH ACTIVITIES IN 1997

A. DEPARTMENT OF MOLECULAR GENETICS

A-a. Division of Molecular Genetics

(1) Molecular Anatomy of the α Subunit C-terminal Domain of *Escherichia coli* RNA Polymerase: Positioning of Two α Subunit Carboxy-Terminal Domains on Promoters by Two Transcription Factors

Katsuhiko MURAKAMI, Nobuyuki FUJITA, Olga N. OZOLINE, Jeffrey T. OWENS[1], Tamara A. BELYAEVA[2], Claude F. MEARES[1], Stephen J.W. BUSBY[2] and Akira ISHIHAMA ([1]Univ. California, Davis, USA; [2]Univ. Birmingham, Birmingham, UK)

The RNA polymerase holoenzyme of *Escherichia coli* is composed of the core enzyme with subunit structure $\alpha_2\beta\beta'$ and one of multiple species of σ subunit with promoter recognition activity. Promoter selectivity of the holoenzyme is modulated by direct interaction with one or two of about 100 species of transcription factors. The RNA polymerase activity is also enhanced by direct interaction with DNA UP elements. The best characterized target on the RNA polymerase involved in molecular communication with transcription factors is the carboxy-terminal domain (CTD) of α subunit that contains the contact sites for class-I transcription factors and DNA UP elements.

The binding sites of two αCTDs within a single RNA polymerase on the UP element DNA were determined by hydroxyl radical-based DNA cleavage mediated by Fe-BABE [(*p*-bromoacetamidobenzyl)-EDTA-Fe] which was bound at Cys-269 on the UP-recognition surface of one or both α subunits. The results clearly indicated that the two α subunits bind in tandem to two helix turns of the *rrnB* P1 UP element, the β- and β'-associated α subunit being bound to the promoter-proximal and promoter-distal region, respectively. Using the

same DNA cleavage method by α-conjugated Fe-BABE, interactions between cAMP receptor protein (CRP) and αCTD were also analyzed at promoters carrying tandem DNA sites for CRP binding. Each CRP dimer was found to direct the positioning of one of the two CTDs, indicating that two CRP dimers can interact independently with the two αCTDs leading to direct their positioning along the promoter DNA. If other transcription factors interact with the σ, β or β' subunits independently, RNA polymerase could potentially participate in regulatory complexes involving three or more different transcription factors. For details see Refs. 7, 11, 26 and 27.

(2) Molecular Anatomy of the α Subunit C-terminal Domain of *Escherichia coli* RNA Polymerase: Monitoring of RNA Polymerase-DNA UP Element Interaction by a Fluorescent Probe Conjugated to α Subunit

Olga N. OZOLINE, Nobuyuki FUJITA, Katsuhiko MURAKAMI and Akira ISHIHAMA

Single Cys mutant α subunits were used for specific labeling with a monomercury derivative of fluoresceine acetate (FMMA). The FMMA-modified RNA polymerase only at Cys269 of the α subunits was used to investigate RNA polymerase interaction with different promoters either with or without an UP element. Interaction with the *rrnB* P1 UP element led to substantial alterations in the spectral parameters of the reporter label. A pronounced spectral blue shift suggests that the labeled surface of αCTD closely approaches the charged UP DNA helix. The spectral blue shift was also observed when the modified RNA polymerase interacted with *trp* P, supporting the prediction that this promoter carries an *rrnB* P1-type UP element.

A new approach employing a fluorescent reporter label positioned in the multiple sites within the αCTD was then used to compare conformational changes of individual structural segments of the protein upon transcription complex formation. FMMA was conjugated to a single Cys residue placed by the site-directed mutagenesis at 9 different positions of αCTD. Spectral parameters of FMMA were compared between free and promoter-bound forms of the RNA polymerases, each carrying a modified α at a single position. Reliable conformational changes were observed for binary complexes formed

with UP element-dependent promoter *rrnB*P1 and ternary complexes formed with cAMP-CRP at class I promoter *uxuAB*. Our results confirm the previous data indicating that the helix I of αCTD and the loop region between helices III and IV are involved in these complex formation and indicate that similar pathways may be used by the RNA polymerase to realize the activation signal accepted from either the UP-DNA or the activator protein.

Some structural changes were also observed when the FMMA-modified RNA polymerase was mixed with cAMP-CRP in the absence of DNA, confirming the direct protein-protein contact. These changes were different from those observed for ternary complexes formed on the *uxuAB* promoter. For details see Refs. 11, 33, 34 and 35.

(3) Molecular Anatomy of the α Subunit N-terminal Domain of *Escherichia coli* RNA Polymerase: Contact Sites for Dimeric Association Studied by Cleavage with Fe-BABE Conjugated to Single Cysteine Residues

Reiko MIYAKE[1], Katsuhiko MURAKAMI, Jeffrey T.OWENS[1], Nobuyuki FUJITA, Claude F. MEARES[1] and Akira ISHIHAMA ([1]Univ. California, Davis, USA)

The *E. coli* RNA polymerase core enzyme is assembled *in vivo* and *in vitro* in the sequence: $2\ \alpha \rightarrow \alpha_2 \rightarrow \alpha_2\ \beta \rightarrow \alpha_2\ \beta\beta'$ (premature core) → E (active core). Genetic and biochemical studies indicated that the subunit-subunit contact sites on α including the sites for α dimerization and the contact sites with β and β' subunits are all located within the amino-terminal domain (NTD) down to residue 235, whereas the carboxy-terminal domain (CTD) is involved in transcription regulation through direct interaction with class-I transcription factors and DNA UP elements. These two functional domains form independent structural domains, each being connected by a long flexible linker.

Proximity relationships between the two associated monomers of RNA polymerase α subunit were studied using a set of four mutant α subunits, each carrying a single Cys residue at one of the naturally occuring positions. These mutants α subunits were conjugated with the cutting reagent Fe-BABE, and

the peptide backbone was cleaved at locations near the modified Cys. Analysis of the cleavage sites identified segments within approximately 12 Å from the conjugation site. The results show that intra-molecular cutting was observed only within the individual αNTD and αCTD, supporting that the two functional domains form independent structural domains. On the other hand, for inter-molecular cutting, segments of the amino-terminal subunit assembly domain of one α subunit and the linker region between αNTD and αCTD of the other subunit are located close to each other on the three dimensional structure, implying that the two αNTD are aligned in anti-parallel fashion. For details see Refs. 7, 15 and 24.

(4) Molecular Anatomy of the β and β' Subunits of *Escherichia coli* RNA Polymerase: Mapping of Subunit-subunit Contact Sites Studied by Proteolytic Cleavage

Tasuku NOMURA, Akira KATAYAMA, Dipankar CHATTERJI, Nobuyuki FUJITA and Akira ISHIHAMA

The α subunit of *E. coli* RNA polymerase plays a key role in the assembly of core enzyme by providing the contact surface for both β and β' subunits. In the assembled core enzyme, one α subunit contacts with β subunit while the other α makes direct contact with β' subunit. We have been concerned with the mapping of subunit-subunit contact sites on the α subunit. For mapping of the subunit-subunit contact sites on the two large subunits, we employed two approaches: (i) analysis of proteolytic cleavage sites on both β and β' subunits for unassembled free subunits, the intermediate subassembly $\alpha_2\beta$ complex and the core enzyme; and (ii) analysis of complex formation between β fragments and intact α subunit or between β' fragments and the $\alpha_2\beta$ complex. Results so far obtained indicate that two regions of the β subunit is involved in the full activity of α binding, *i.e.*, the primary contact site between residues 737 and 904 and the secondary region with assembly control activity downstream from residue 1138. All the α subunit- β fragment binary complexes were found to bind β' subunit and form pseudo-core complexes, indicating that the regions of β involved in α-subunit contact also participate in interaction with the β' subunit.

Recently we succeded to cross-link ppGpp, the regulatory molecular of stringent control, with the β subunit, indicating that β is the target of ppGpp action. The ppGpp-binding site seems to overlap with the primary contact site of α subunit, while the binding sites for substrates and rifampicin are located upstream from the α contact site.

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

Tryptic cleavage of unassembled free β' subunit indicated that the initial cleavage always takes place at sites between residues 800 and 950, splitting this large polypeptide into two fragments. The N-terminal large fragment is further cleaved around residue 350 into two subfragments. Significant difference in the β' cleavage pattern was not observed for that assembled into the core enzyme. Attempts to isolate β' tryptic fragments which specifically associate with $\alpha_2\beta$ complex, the assembly intermediate, appeares to be not so useful. We then started to express and isolate β' fragments using cloned recombinant genes and test their abilities of subunit assembly. Refs. 4 and 11.

(5) Molecular Anatomy of the σ Subunits of *Escherichia coli* RNA Polymerase: Mapping of Contact Sites with Promoter DNA and Core Enzyme Subunits Studied by Cleavage with Fe-BABE Conjugated to Single Cysteine Residues

Jeffrey T. OWENS[1], Jon BOWN[2], Katsuhiko MURAKAMI, Nobuyuki FUJITA, Claude F. MEARES[1], Stephen J.W. BUSBY[2], Stephen D. MINCHIN[2] and Akira ISHIHAMA ([1]Univ. California, Davis, USA; [2]Univ. Birmingham, Birmingham, UK)

Base-specific interactions between promoter DNA and RNA polymerase are regulated by σ subunits during transcription initiation. To map spacial relations between the primary and major σ subunit (σ^{70}) and each DNA strand along the *lac* UV5 promoter in the transcriptionally active open complex, we used a Cys-tethered cutting reagent FeBABE to cleave DNA strands. This chemical nuclease was conjugated to single-Cys mutants of σ^{70} at sites 132C, 376C, 396C, 422C, 496C, 517C or 581C. After formation of open promoter complexes, we observed DNA cleavage spanning at least 60 bases, between positions -48 and +12. The results show that σ^{70} region 2.1 is proximal to the nontemplate strand between the -10 element and positions as far downstream of the transcription start site as +12. The conserved region 3.2 is proximal to the template strand near +1, and region 3.1 is positioned between the -10 and -35 elements.

This FeBABE-mediated DNA cleavage method was also employed to locate the position of region 2.5 of σ^{70} in transcriptionally competent complexes. Genetic studies indicated that the region 2.5 is involved in recognition of the extended -10 element with TGnTATAAT sequence. FeBABE was then tethered at 454C, 458C, 459C and 461C near or in region 2.5. Results of hydroxy-radical based DNA cleavage indeed indicated that the region 2.5 is in close proximity to promoter at position -14/-15 and this positioning is independent of promoter sequence.

FeBABE covalently tethered at various positions of σ^{70} was also used to locate the σ^{70}-binding sites on the core enzyme. The protein-cutting probe was covalently conjugated in or near the four conserved regions of σ^{70} using the seven single Cys mutants. Each FeBABE-conjugated σ^{70} was bound to the core enzyme, which led to cleavage of nearby sites on the β and β' subunits, but not α subunit. The cut sites from different probe-modified σ^{70} subunits are clustered in distinct regions of the two large core subunits. On the β subunit, cleavage is observed in two regions, one between residues 383 and 354, including the conserved C and Rif regions; and the other between residues 854 and 1022, including the conserved G region, regions of ppGpp sensitivity, and one of the segments forming the catalytic center of RNA polymerase. On the β' subunit, the cleavage was identified within the sequence 228-461, including the β' conserved regions C and D, which comprise part of the catalytic

center. For details see Refs. 7, 31 and 32.

(6) Regulation of the Activity of Sigma Subunits from *Escherichia coli*: Functional Interaction of *Escherichia coli* RNA Polymerase with Inorganic Polyphosphate

Shuichi KUSANO, J. GOWRISHANKAR and Akira ISHIHAMA

Upon entry into the stationary phase, transcription of σ^{70}-dependent genes in *E. coli* is mostly turned off while that of σ^{38} (σ^{S})-dependent genes is switched on, even though the intracellular level of σ^{70} stays at a constant level without being degraded. In order to reveal the control mechanism(s) for preferential utilization of σ^{38} in the stationary phase, we tested possible effects of the increase in trehalose, glycine betaine, glycogen or polyphosphate concentrations and the decrease in DNA superhelicity on σ^{70}- and σ^{38}-dependent transcription *in vitro*. All these changes have been recognized for the stationary-phase *E. coli* cells. The optimum concentrations of glutamate and trehalose giving maximum transcription by Eσ^{38} were higher than those by Eσ^{70}. The activation of Eσ^{38} by trehalose was additive with the transcription enhancement by decreased superhelicity of template DNA prepared from stationary-phase cells. We thus propose that the selective activation of transcription by Eσ^{38} holoenzyme takes place in the presence of specific condition(s) and factor(s) present under stress conditions.

The ubiquitous occurrence of poly P in the stationary phase bacteria is suggestive of some important physiological role(s) for this polymer. Mutants defective in the *ppk* gene encoding polyphosphate kinase (PPK) and thus lacking long-chain poly P are defective in survival in the stationary phase. Previously we observed the association of yet unidentified acidic compound(s) with the RNA polymerase. Several lines of evidence indicated that the stationary-phase RNA polymerase is associated with poly P. Functional assays indicate that at low salt concentrations, poly P inhibits transcription by both σ^{70} and σ^{38} holoenzymes, but at high concentrations of potassium glutamate, the inhibition is relieved. In the presence of high concentrations of potassium glutamate, however, only the Eσ^{38} holoenzyme is allowed to function. These results altogether suggest that poly P plays a role

in the promoter selectivity control of RNA polymerase under stress conditions. For details see Refs. 11, 12, 20, 21, 22 and 36.

(7) A Stationary-Phase Protein in *Escherichia coli* with Binding Activity to the Major Sigma Subunit of RNA Polymerase

Miki JISHAGE, Akira IWATA[1], Nobuyuki FUJITA, Susumu UEDA[1] and Akira ISHIHAMA ([1]Nippon Institute for Biological Science, Ohme, Tokyo)

E. coli contains seven different molecular species of the σ subunit, the promoter recogniztion subunit of RNA polymerase. Using a quantitative Western immnoblot analysis, the intracellular levels of all seven σ subunits were determined. The concentration of σ^{70} (the product of the *rpoD* gene) required for transcription of most of the genes expressed during exponential phase is about 700 molecules per cell at both log and stationary phases, while σ^{38} (σ^{S}, the *rpoS* gene product) is detected only in the stationary phase, reaching to about 200-300 molecules per cell. The levels of σ^{54} (σ^{N}, the *rpoN* gene product) and σ^{28} (σ^{F}, the *rpoF* gene product) are maintained at 10 and 50% the level of σ^{70} at both exponential and stationary phases, while other minor σ subunit, σ^{32} (σ^{H}, the *rpoH* gene product), σ^{24} (σ^{E}, the *rpoE* gene product) and σ^{FecI} (the *fecI* gene product), were hardly detected under normal growth conditions.

In the course of these studies, we realized that heterogeneity exists among the laboratory stocks of *E. coli* strains with respect to two σ subunits, σ^{F} and σ^{S}. In particular, marked heterogeneity was observed in not only the intracellular level but also the structure of σ^{38}.

Upon entry into stationary phase, the level of σ^{S} increases to as much as about 30-35% the level of σ^{70}, while the level of σ^{70} stays constant, even though the frequency of transcription of genes under the control of σ^{70} decreases by more than 10-fold. Known factors which contribute to the preferential utilization of σ^{S} rather than σ^{70} in the stationary phase or under stress conditions include increases in intracellular concentrations of potassium glutamate, trehalose, glycine betaine and polyphosphate and a decrease in DNA superhelicity, all of which tend to reduce the activity of E σ^{70} but increases that of E σ^{S}. In addition, we found that in stationary phase cells,

σ^{70} but not other σ subunits forms a complex with a hitherto unidentified stationary phase-specific protein. This protein begins to be synthesized during the transition from exponential growth to stationary phase. *In vitro* transcription studies indicated that the protein interferes with the engagement of σ^{70} in the transcription cycle. After sequencing, the protein was identified as a product of one URF (unidentified reading frame) revealed by the genome sequence analysis. We propose that this protein, designated Rsd (Regulator of Sigma D), with the σ^{70}-binding activity plays a role in controlling the σ^{70} function in stationary-phase *E. coli*. Possible role of Rsd as an anti- σ^{70} factor is being examined. For details see Refs. 11, 12, 16 and 17.

(8) Sequence Specificity and Affinity of DNA Binding for Twelve Species of *Escherichia coli* DNA-binding Proteins

TALKUDER Ali Azam and Akira ISHIHAMA

The genome of *Escherichia coli* is composed of a single molecule of circular DNA with the length of 4.7 Mb, to which about 10 major DNA-binding proteins are associated, altogether forming the nucleoid. The species and amounts of these genome-associated proteins are considered to affect the template activity of genome DNA in transcription. As an initial attempt to reveal the overall configutation of *E. coli* genome DNA under various growth conditions, we compared the DNA-binding specificities of twelve species of *E. coli* DNA-binding proteins: CbpA, curved DNA-binding protein A; CbpB, curved DNA-binding protein B; DnaA, DNA-binding protein A; Dps, DNA-binding protein from starved cells; Fis, factor for inversion stimulation; Hfq, host factor for phage Q_β; H-NS, histone-like nucleoid structuring protein; HU, heat-unstable nucleoid protein; IciA, inhibitor of chromosome initiation A; IHF, integration host factor; Lrp, leucine-responsive regulatory protein; StpA, suppressor of td- phenotype A. Up to the present time, our knowledge on the specifity of DNA binding by these proteins is limited. In particular, little is known on the specificity of DNA recognition by Dpa, Hfq, H-NS and StpA. We then purified all these DNA-binding proteins using the respective cloned genes, and examined the sequence specificity and the affinity of DNA binding *in vitro* using gel-mobility shift assays of various synthetic and natu-

ral DNA probes. Five (CpbB, DnaA, Fis, IHF and Lrp) out of the twelve DNA-binding proteins showed sequence-specific DNA-binding, while the remaining seven proteins (CbpA, Dps, Hfq, H-NS, HU, IciA and StpA) showed sequence-nonspecific DNA-binding. From the apparent dissociation constant (K_d, app) determined using the DNA probes used, the order of DNA-binding affinity was determined to be: CbpB>Fis>Lrp>IciA>IHF>StpA>Dps>HU>H-NS>CbpA>Hfq.

(9) Gene Organization and Protein Sequence of the Subunits of the Fission Yeast *Schizosaccharomyces pombe* RNA polymerase II

Hitomi SAKURAI and Akira ISHIHAMA

RNA polymerase II purified from the fission yeast *Schizosaccharomyces pombe* contains more than ten different species of polypeptides, tentatively designated as subunits. Previously, we cloned and sequenced both cDNA and the genes encoding the four large subunits, Rpb1, Rpb2, Rpb3 and Rpb5. Later, other groups isolated the genes for Rpb6 and Rpb12 and cDNA for Rpb10. For cloning of the genes for other small-sized subunits, purified RNA polymerase II was separated into individual subunits by SDS-PAGE and each gel band was treated for partial proteolytic digestion and proteolytic fragments were subjected to micro-sequencing. Using primers designated from the partial amino acid sequences or the published DNA sequences, we cloned both cDNA and the genes encoding four small subunits, Rpb7, Rpb8, Rpb10 and Rpb11. These genes were found to encode Rpb7, Rpb8, Rpb10 and Rpb11 consisting of 172 (Mr 19,103), 125 (Mr 14,300), 71 (Mr 8,276) and 123 (Mr 14,127) amino acid residues, respectively. All these four subunits are homologous to the corresponding subunits of *Saccharomyces cerevisiae* RNA polymerase II. The *rpb7* gene contains one intron while the *rpb8*, *rpb10* and *rpb11* genes contain two introns.

In addition, using degenarate primers from the known sequences of subunit 9 from other organisms, we also cloned both the *rpb9* gene and its cDNA encoding the subunit 9 of RNA polymerase II. From the DNA sequences, Rpb9 was predicted to consist of 113 amino acid residues with the molecular mass of 13,175. *S. pombe* Rpb9 is 47, 40 and 36% identical in amino acid sequence to

the corresponding subunits from *Saccharomyces cerevisiae*, human and *Drosophilla melanogaster*, respectively. Previously we failed to detect Rpb9 in the purified RNA polymerase II by amino-terminal micro-sequencing of proteolytic fragments of subunits separated by SDS-gel electrophporesis. After Western blot analysis using antibodies raised against the protein product of the newly isolated *rpb9* gene, we found that the purified RNA polymerase II contains Rpb9. Taken all our previous observations together the gene organization and the predicated protein sequence have been determined for eleven subunits of *S. pombe* RNA polymerase II. However, it is not established yet whether all these eleven putative subunits are necessary for RNA polymerase functions or not. For details see Refs. 13 and 37.

(10) Two Large Subunits of the Fission Yeast RNA Polymerase II Provide Platforms for the Assembly of Small Subunits

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

The two large subunits, Rpb1 and Rpb2, of *S. pombe* RNA polymerase II share high levels of sequence similarity with the β' and β subunits, respectively, of prokaryotic RNA polymerases. The sequence of amino-terminal domain (NTD) of α subunit, which plays a key role in the subunit assembly by providing the contact surface for both β' and β subunitsis, is conserved in both subunit 3 (Rpb3) and 11 (Rpb11) of eukaryotic RNA polymerase II. Since no prokaryotic counterparts have been identified for other putative subunits of RNA polymerase II, little is known about the functions and the assembly mechanism of other subunits.

The subunit-subunit contact network was then analyzed for the *Schizosaccharomyces pombe* RNA polymerase II consisting of more than ten putative subunits. Previously we carried out far-Western blot analysis of bimolecular interaction with radio-labeled subunit 3 and 5 probes. This year we extended the analysis using other six small-sized subunits, Rpb6, Rpb7, Rpb8, Rpb10, Rpb11 and Rpb12, as probes. Taken the results together the subunit-subunit interaction was observed for a total 18 (or 19) combinations.

All eight small-sized subunits exhibited binding activities to the two large subunits, Rpb1 and Rpb2. In addition, bimolecular interaction was observed for the combinations of Rpb3-Rpb5, Rpb3-Rpb11 (and Rpb5-Rpb8/11). The subunit-subunit contact within the assembled RNA polymerase was then analyzed by protein-protein cross-linking using five species of bifunctional cross-linkers with different length and specificity, *i.e*. dimethylsuberimidate (DMS), 2-iminothiolane hydrochloride (ITL), *N,N'*-o-phenylene dimaleimide (PDM), dimethyl 3,3'-dithio-bis propionimidate (DTBP) and diepoxybutane (DEB). Cross-linking was observed for a total of 19 combinations, including five combinations between small subunits, Rpb3-Rpb10, Rpb3-Rpb11, Rpb5-Rpb6, Rpb6-Rpb7 and Rpb6-Rpb8. The results altogether indicate that two large subunits Rpb1 and Rpb2 provide the platform for the assembly of small subunits and besides the small subunits interact each other for limited combinations. Direct contact of the two large subunits, Rpb1 and Rpb2, was also demonstrated by cross-linking. Taken the results altogether we propose a model of the subunit topology within the *S. pombe* RNA polymerase II. For details see Refs. 10 and 13.

(11) Molecular Composition of a Core Subassembly of *S. pombe* RNA Polymerase II

Makoto KIMURA, Akira ISHIGURO and Akira ISHIHAMA

RNA polymerase II purified from the fission yeast *Schizosaccharomyces pombe* consists of more than ten putative subunits. We introduced a histidine cluster tag sequence into the chromosomal *rpb1* and *rpb3* genes which encode subunit 1 (Rpb1) and subunit 3 (Rpb3), respectively. Using these fission yeast strains, we could easily purified RNA polymerase II by Ni^{2+}-affinity chromatography. Furthermore, the RNA polymerase can be obtained as a regin-bound form. After step-wise dissociation of the Rpb1-tagged and the Rpb3-tagged RNA polymerases fixed on Ni^{2+}-resin by increasing concentrations of urea or guanidium hydrochloride, Rpb2-Rpb3-Rpb11 or Rpb2-Rpb3-Rpb11-Rpb10 complexes were obtained. Since the complex consisting of Rpb2, Rpb3 and Rpb11 cannot be dissociated even after treatment with 6 M urea buffer, we propose that this complex represents a core subassembly of the

RNA polymerase II, analogous to the $\alpha_2\beta$ complex in the assembly of *Escherichia coli* RNA polymerase.

Using the isolated subunits and subassemblies, we detected direct binding of the isolated Rpb1 protein and of the Rpb2-Rpb3-Rpb11 subassembly to DNA in aqueous solution. The affinity for DNA was, however, weaker for both the Rpb1 subunit and the Rpb2-Rpb3-Rpb11 subassembly than the intact pol II, suggesting that both the Rpb1 and Rpb2 subunits in the intact pol II participate in the high affinity binding. For details see Refs. 10 and 19.

(12) Mapping of the Subunit-Subunit Contact Sites on Rpb1, Rpb2 and Rpb3 of the Fission Yeast RNA Polymerase II

Kiyoshi YASUI, Takenori MIYAO, Akira ISHIGURO, Ayae HONDA, Zhangyi QU, Makoto KIMURA, Hiroshi MITSUZAWA and Akira ISHIHAMA

Far-Western, GST pull-down and chemical cross-linking experiments indicated that the two large subunits of the fission yeast *S. pombe* RNA polymerase II provide platforms for the assembly of small subunits. Mapping of the contact sites on two large subunits, Rpb1 and Rpb2, with two small subunits, Rpb3 and Rpb5, was carried out using the two-hybrid screening system in the budding yeast *Saccharomyces cerevisiae*. The Rpb5 was found to interact with any fragment of Rpb1 containing the region H which is conserved among the subunit 1 homologues of all RNA polymerases, including the β' subunit of prokaryotic RNA polymerases. In agreement with the fact that Rpb5 is shared among all three forms of eukaryotic RNA polymerases, the region H of RNA polymerase I subunit 1 (Rpa190) was also found to interact with Rpb5.

On the other hand, the two-hybrid screening of Rpb2 fragments from the RNA polymerase II indicated the presence of Rpb3 contact site on the region H which is conserved among the subunit 2 homologues of all RNA polymerases, including the β subunit of prokaryotic RNA polymerases.

Previously we indicated that Rpb3 plays an essential role in subunit assembly because it interacts with at least five subunits, two large subunits (Rpb1 and Rpb2) and three small subunits (Rpb3, Rpb5 and Rpb11), and it constitutes a core subassembly consisting of Rpb2, Rpb3 and Rpb11. By making a

set of Rpb3 deletion derivatives, we carried out mapping of the Rpb5- and Rpb11-contact sites on Rpb3. By far-Western blot and GST pull-down assays, we found that the amino acid sequence between residues 105-263 of Rpb3 is involved in binding Rpb5, and the sequence between residues 105-297 is required for binding Rpb11. Although the Rpb5- and Rpb11-contact sites on Rpb3 overlap each other, both subunits are able to associate with Rpb3 simultaneously. The binding of Rpb5 stabilizes the Rpb3-Rpb11 heterodimer. For details see Refs. 11, 25 and 41.

(13) Mapping of the Functional Sites on Two Large Subunits of the RNA Polymerase II: Photo-affinity Cross-linking of 3'-Termini of Nascent RNA

Wjatschesslaw A. WLASSOFF, Makoto KIMURA and Akira ISHIHAMA

To locate the catalytic site for RNA polymerization of *S. pombe* RNA polymerase II, the catalytically competent transcription complex was photo-affinity labeled with photo-reactive nucleotide analogues, and the proteins were separated by SDS-PAGE for identification of the labeled subunits. Proteolytic cleavage analysis of ^{32}P-labeled subunits allowed us to identify the exact location of the sites of labeling on the subunits of RNA polymerase II. Using this approach, we tried to cross-link transcriptionally competent elongation complexes with photo-reactive nucleotides located at 3'-terminal ends of growing RNA chains.

Results altogether indicate that: (i) the RNA 3'-end binding site locates between amino acids 934 and 994 of Rpb2; and (ii) regions 306-542 of Rpb2 and 509-917 of Rpb1 are situated in the vicinity of active center, probably forming the catalytic site. All these regions include conserved motifs of RNA polymerases, and the active site belongs to the N-terminal part of conservative motif H. Proteolytic analysis of subunits Rpb1 and Rpb2 has revealed their complex multidomain organization, with the most available site of proteolysis being located in the junctions between the conservative motifs.

(14) Genetic Mapping of the Functional Sites on Rpb3 and Rpb11, Homologues of Prokaryotic Alpha Subunits

Jiro MITOBE, Miwa KOMOTO, Kiyoshi YASUI, Hiroshi MITSUZAWA and Akira ISHIHAMA

RNA polymerase II of *S. pombe* consists of more than ten putative subunits. To understand how these subunits interact with one another and with other transcription factors, we have isolated temperature-sensitive *S. pombe* mutants with mutations in one of the subunit genes. As a first step, we chose *rpb3* and *rpb11*, whose products show homology to the *E. coli* RNA polymerase α subunit and form a core subassembly together with Rpb2 (β homologue). The *rpb3* or *rpb11* genes were randomly mutagenized by PCR and the amplified fragments were used to replace the chromosomal copy of *rpb3* or *rpb11*. Transformants were then screened for temperature-sensitive growth by replica-plating. Nine mutations for the *rpb3* gene and seven mutations for the *rpb11* gene, all of which result in single amino acid change, were identified so far.

For *rpb3* mutations, RNA polymerase II was affinity-purified from two *rpb3*^{ts} strains as well as a wild-type strain. Weaker interactions of the mutant Rpb3 subunits with Rpb2, Rpb5 and Rpb7 were detected by treating the resin-bound enzymes with increasing concentrations of urea. The isolation of extragenic suppressor mutations of *rpb3*^{ts} mutations is in progress. For *rpb11* mutations, recombinant mutant proteins were produced and the interaction with Rbp3 protein was tested by GST pull-down assays. Some mutant Rpb11 protein showed weaker interactions with Rpb3 protein. The isolation of multicopy suppressor genes of *rpb11* mutations is in progress.

(15) The Molecular Anatomy of Influenza Virus RNA Polymerase: Identification of the RNA Cap-binding Site on PB2 Subunit

Ayae HONDA, Kiyohisa MIZUMOTO[1] and Akira ISHIHAMA ([1]Div. Nucleic Acid Chem. and Kitazato Univ.)

Influenza virus genome is composed of eight RNA segments of negative polarity, which is transcribed into plus-strand translatable RNA by the virus-

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

On the other hand, capped RNA with ^{32}P label only at its 5' cap-1 structure was photo-affinity cross-linked to the PB2 subunit. Isolated radio-labeled PB2 was digested, after mixing with unlabeled PB2, with V8 protease and cleavage fragments were separated by SDS-polyacrylamide gel electrophoresis. Fragments with cross-linked cap-1 were eluted from gels and subjected to amino acid sequencing. Results indicated that two regions of PB2 are involved in the binding of 5' cap-1 structure of capped RNA, one at the N-terminal proximal region downstream from the PB1-contact site and the other around 500 amino acid residue region overlapping the motif commonly found in cellular cap-binding proteins. For details see Refs. 1 and 8.

(16) The Molecular Anatomy of Influenza Virus RNA Polymerase: Identification of Host Factor(s) for Interconversion between Transcriptase into Replicase

Ayae HONDA, Takuto OKAMOTO and Akira ISHIHAMA

Transcription of the genome RNA by the viral RNA polymerase is initiated by using host cell capped RNAs as primers, while the mechanism of replication initiation remained unsolved. Analysis of the 5'-terminal structure of genome RNAs indicated that RNA synthesis for replication is initiated *de novo* without using primers. Both purified and reconstuted 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. We have searched for the putative host factor(s) using yeast two hybrid screening system. So far more than 10 cellular proteins have been identified, which interact with either PB1 or PB2 protein. Some of these putative host factors were found to form complexes *in vitro* by mixing with the PB1 or PB2 proteins. Functional conversion of the RNA polymerase after interaction with each of these proteins is being analyzed. For details see Refs. 8 and 9.

(17) Isolation of 130K/180K Heterodimer with RNA-dependent RNA Polymerase Activity from TMV-Infected Tobacco

Takato WATANABE, Ayae HONDA, Akira IWATA[1], Susumu UEDA[1], Tadaaki HIBI[2] and Akira ISHIHAMA ([1]Nippon Institute for Biological Science, Ohme, Tokyo; and [2]University of Tokyo, Department of Agricultural and Environmental Biology)

The genome of tobacco mosaic virus (TMV) consists of a single-stranded RNA molecule of about 6,400 nucleotides in length with positive polarity, which encodes at least four polypeptides, 126 and 183 kDa proteins required for transcription and replication (referred to as 130K and 180K proteins, respectively), 30 kDa (30K) protein for cell-to-cell virus movement in infected plants, and 18 kDa protein for virus coat formation. The complete nucleotide sequence was determined for the putative RNA polymerase (180K protein) gene of tobacco mosaic virus (TMV) OM strain, which differed from the related strain, *Vulgare*, by 51 positions in nucleotide sequence and 5 residues in amino acid sequence. Three segments of this 180K protein, each containing the sequence motifs of methyltransferase (M), helicase (H) or RNA-dependent RNA polymerase (P), were expressed in *Escherichia coli* as fusion proteins with hexa-histidine tag, and domain-specific antibodies were raised against purified His-tagged M and P proteins. By immunoaffinity column chromatography, a heterodimer(s) consisting of one molecule each of the 130K (amino terminal-proximal portion of 180K protein) and full-length 180K viral proteins was isolated, which exhibited the activity of model viral RNA template-dependent RNA synthesis *in vitro*. We propose that the TMV RNA poly-

merase protomer is composed of one molecule each of the 130K and 180K proteins.

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A-b. Division of Mutagenesis

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

Hiroaki SEINO and Fumiaki YAMAO

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

ter chromatid holding protein(s)" at the onset of anaphase and possibly of mitotic cyclin at the exit of mitosis (2). These phenotypes were confirmed by using temperature-sensitive mutant of *ubcP4*, which indicated that progression of each cell cycle step mentioned above was blocked by dysfunction of the UbcP4 pathway.

Cig2, another B-type cyclin in fission yeast, has the same type of destruction signal as Cdc13. We examined the possibility of the degradation of Cig2 via UbcP4 pathway, providing the genetic interaction between genes encoding the two proteins. In order to identify genes that could be functioning in or around the UbcP4/APC pathway, two approaches were adopted, screening of the multicopy suppressors of *ubcP4* mutations, and search of conditional mutants suppressible by UbcP4 overproduction. Characterization of the candidates from these screening is now undergoing.

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

Tsutomu KISHI and Fumiaki YAMAO

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

We have isolated extragenic suppressors of a *cdc34-1 sic1* mutant. One group of suppressors was recessive, and was found to be allelic to *GRR1*, a gene shown to be involved in degradation of G1 cyclins (Cln1 and Cln2) as well as glucose repression. Conversely, overproduction of Grr1 in *cdc34 sic1* cells impaired colony formation at 30℃. We isolated *MGO1* (Multicopy suppressor of Grr1 Overproduction defect) which suppressed this growth defect when introduced on a multicopy vector. Sequence analysis revealed that *MGO1* is identical to *SKP1*, which has been shown to be required for the ubiquitin-meadiated proteolysis of Cln2 and Sic1 (4).

We found that Grr1 binds Skp1 in vitro and in vivo. The interaction was

dependent on the F-box in Grr1. We also found that Grr1 binds phosphorylated Cln2 in vivo. The interaction was dependent on the leucine-rich repeats (LRR). Furthermore, we demonstrated that (1) Cln2 is stabilized in cells with mutant Grr1 (Grr1-dF), which is defective in Grr1-Skp1 association, (2) Cln2 is stabilized in cells with mutant Grr1 (Grr1-dL), which is defective in Grr1-Cln2 association, and that (3) phosphorylated Cln2 associated with Skp1 dependently on Grr1 in vivo. These results indicate that Grr1 links phosphorylated Cln2 with Skp1 (submitted).

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A-c. Division of Nucleic Acid Chemistry

(1) Enzyme mechanism of the 5'-terminal capping of eukaryotic mRNA

Kiyohisa MIZUMOTO (School of Pharmaceutical Sciences, Kitasato University)

Previous studies demonstrated that the mammalian mRNA capping enzyme is a bifunctional enzyme containing RNA 5'-triphosphatase and RNA

guanylyltransferase activities in a single polypeptide. In yeast, these two activities are separated into two different subunits, α(RNA guanylyltransferase) and β (RNA 5'-triphosphatase), the genes for which we have cloned recently. It is thus interesting to compare the structural and functional relationships between the mammalian and yeast capping enzymes. We isolated two human cDNAs encoding mRNA capping enzymes termed *hCAP1a* and *hCAP1b* which code for 597 and 541 amino acids, respectively. They are different only at the region coding for the C-terminal portion of the enzyme. Comparison of the deduced amino acid sequences with other cellular and viral capping enzymes showed that all the regions conserved among RNA guanylyltransferases including the yeast α subunit are observed in our clones except one conserved C-terminal region which was absent in the hCAP1b protein. The purified recombinant *hCAP1a* gene product, hCAP1a, exhibited both RNA 5'-triphosphatase and RNA guanylyltransferase activities. Deletion mutant analysis of hCAP1a showed that the N-terminal 213 amino acid fragment containing a tyrosine specific protein phosphatase (PTP) motif catalyzed the RNA 5'-triphosphatase activity, and the C-terminal 369 amino acid fragment had the mRNA guanylyltransferase activity. On the other hand, hCAP1b showed RNA 5'-triphosphatase activity, but exhibited neither the enzyme-GMP covalent complex formation nor the cap structure formation. Interestingly, comparison of the amino acid sequences between the N-terminal RNA triphosphatase domain of hCAP1a and the yeast β subunit showed no similarity, and the β subunit had no PTP motif, although they catalyze the same reaction.

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B. DEPARTMENT OF CELL GENETICS

B-a. Division of Cytogenetics

(1) *S. cerevisiae recA* homologues *RAD51* and *DMC1* have both distinct and overlapping roles in meiotic recombination

Tomoko OGAWA

Rad51 and Dmc1 are *Saccharomyces cerevisae* homologues of the *Escherichia coli* recombination proteins RecA. Mutant analysis has shown that both proteins are required for normal meiotic recombination, for timely and efficient formation of synaptonemal complex and for normal progression out from meiotic prophase.

We have further characterized *rad51* and *dmc1* single mutants. A *dmc1* mutation confers more severe defects in double strand break (DSB) resolution, crossover recombination and meiotic progression than does a *rad51* mutant; in contrast, during return to growth, a *rad51* mutation confers more severe defects in viability and intrachromosomal recombination than does a *dmc1* mutation. Analysis of a *rad51 dmc1* double mutant, in parallel with single mutants, shows that the double mutant is more defective with respect to the formation of crossovers during meiosis and, especially strikingly, with respect to interhomologue and intrachromosomal recombination during return to growth. Consistent with the observation of *DMC1* -dependent recombination in a *rad51* mutant, subnuclear complexes of Dmc1 protein were detected for the first time in this mutant. In contrast to the effects on recombination, the effect of double mutant on meiotic progression was similar to that of the *rad51* single mutant.

Rad51 and Dmc1 each make unique contributions to meiotic recombination. However, the two proteins are capable of substituting for one another under some circumstances, implying that they most likely share at least one recombination function. Recombination and cell cycle phenotypes are all consistent with the possibility that a *dmc1* mutation causes an arrest of the post-

DSB recombination complexes at a later, more stable stage than does a *rad51* mutation. see Ref. 1

(2) Complete Nucleotide Sequence of the Chloroplast Genome from the Green Alga Chlorella *vulgaris* ; The Existence of Genes Possibly Involved in Chloroplast Division

Tsutomu OHTA

The complete nucleotide sequence of the chloroplast genome (150,613bp) from the unicellular green alga *Chlorella vulgaris* C-27 has been determined. The genome contains no large inverted repeat and has one copy of rRNA gene cluster consisting of 16S, 23S, 5S rRNA genes. It contains 31 tRNA genes, of which the tRNA Leu (GAG) gene has not been found in land plant chloroplast DNAs analyzed so far. Sixty-nine protein genes and eight ORFs conserved with those found in land plant chloroplasts have also been found. The most striking is the existence of two adjacent genes homologous to bacterial genes involved in cell division, *minD* and *minE*, which are arranged in the same order in *Escherichia coli*. This findings suggests that the mechanism of chloroplast division is similar to bacterial division. see Ref. 2

(3) A Recombinational Defect in the C-terminal Domain of *Escherichia coli* RecA2278-5 Protein is Compensated by Protein Binding to ATP

Andrei ALEXEEVE and Tomoko OGAWA

RecA2278-5 is a mutant RecA protein (RecA mut) bearing two amino acid substitutions, Gly-278 to Thr and Val-275 to Phe, in the alpha-helix H of the C-terminal subdomain of the protein. RecA2278-5 mutant cells are unusual in that they are thermosensitive for recombination but almost normal for DNA repair of UV damage and the SOS response. Biochemical analysis of purified RecA mutant protein revealed that its temperature sensitivity is suppressed by prior binding of this protein to its ligand. In fact, the preheating of RecA mutant protein for several minutes at a restrictive temperature (42℃) in the absence of ATP resulted in inhibition at 42℃ of many activities

related to homologous recombination including ss- and dsDNA binding, high-affinity binding for ATP, ss- or dsDNA-dependent ATPase, RecA-RecA interaction, and strand transfer capability. The binary complex RecA mutant::ATP under the same conditions showed a decrease in only two activities, i.e. dsDNA binding and high-affinity binding for ATP. Besides ATP, sodium acetate (1.5 M) was shown to be another factor that can stabilize the RecA mutant protein at 42℃, judging by restoration of its DNA-free ATPase activity. The similarity of influence of high salt (with its non-specific binding) and ATP (binding specifically) on the apparent protein folding stability suggests that the structural stability of the RecA C-terminal domain is one of the conditions for correct interaction between RecA protein and ATP in the RecA::ATP::ssDNA presynaptic complex formation. The decrease in affinity for ATP was suggested to be the factor that determined a particular recombinational (but not repair) thermosensitivity of the RecA-mutant protein. Finally, we show that the stability of C-terminal domain appeared to be necessary for the dsDNA-binding activity of the protein. see Ref 3.

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gene expression in the cells with DNA damage and those in meiosis. Third European Conference on "Meiosis" The Netherland, Wageningen, April.

3. SHINOHARA, A. and OGAWA, T.: Stimulation of Rad51-mediated Recombination by Rad52 in Saccharomyces cerevisiae. FASEB Meeting on "Genetic Recombination and gene rearrangement", USA, Aug.
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B-b. Division of Microbial Genetics

(1) Characterization of the *envC* mutation that affects septation and leads to chain formation in *E. coli*

Hiroshi HARA, Setsuko NARITA[1,2], Yoshihiro YAMAMOTO[3], and Yukinobu NISHIMURA[1] ([1]Faculty of Sciences, Toho University, [2]Department of Clinical Laboratory, Sanritsu Co. Ltd., [3]Department of Genetics, Hyogo College of Medicine)

PM61 is a non-conditional morphological mutant of *E. coli* that forms chains of cells of irregular lengths. It carries the only known mutant allele of *envC* that appears to disturb septum formation and cell separation. The mutation also causes hypersensitivity to crystal violet, leakage of periplasmic proteins, and thermosensitivity on salt-free L agar. We cloned the *envC* gene that complemented all these defects and identified the mutation site of the *envC61* allele ($His^{366} \rightarrow Tyr$). The gene was named *yibP* in the DNA databases and the map position was 81.5 min. There was a report of the cloning of a gene at 73.5 min as *envC*, but that gene was probably a multicopy suppressor of the crystal violet sensitivity and was not the true *envC*.

The *envC* gene product was identified as a protein of about 45 kDa found mainly in the periplasm after the cleavage of the signal peptide. The sequence of the N-terminal half of the mature form has features characteristic of the coiled coil structure. The C-terminal third of EnvC shows homology to the C-terminal regions of *E. coli* NlpD and YebA, and the mature form of lysostaphin, an extracellular peptidoglycan-degrading enzyme of *Staphylococcus*. As was suggested for NlpD, EnvC may also be involved in the remodeling of the peptidoglycan sacculus during cell growth and division. Overproduction of NlpD was reported to cause bulge formation and eventually cell lysis.

Disruption of the chromosomal *envC* gene by insertion of an antibiotic resistance gene resulted in the same defects that the *envC61* mutant showed. The *envC* gene is dispensable for growth, but important for the normal septation/separation and the maintenance of integrity of the cell surface structure. The *envC::cat* allele truncates the product to about one-third. It seems likely that the *envC* mutation (H366Y) also leads to the loss of function.

When the wild-type *envC* was overexpressed, the growth was inhibited and morphological aberration was observed. Overexpression of the *envC61* mutant allele showed an even stronger inhibitory effect, and caused similar morphological aberration and cell lysis. The inhibition was possibly due to the interaction of the overproduced wild-type/mutant EnvC proteins with a second protein.

(2) Contribution of the P_{mra} promoter to expression of genes in the *Escherichia coli mra* cluster of cell envelope biosynthesis and cell division genes

Dominique MENGIN-LECREULX[1], Juan AYALA[2], Ahmed BOUHSS[1], Jean VAN HEIJENOORT[1], Claudine PARQUET[1], and Hiroshi HARA ([1]Laboratoire des enveloppes bacteiennes, Centre National de la Recherche Scientifique, Universite Paris-sud, France, [2]Centro de Biologia Molecular Severo Ochoa, Consejo Superior de Investigationes Cientificas, Universidad Autonoma de Madrid, Spain)

Recently, a promoter for the essential gene *ftsI*, which encodes penicillin-

binding protein 3 of *Escherichia coli,* was precisely localized 1.9 bp upstream from this gene, at the beginning of the *mra* cluster of cell division and cell envelope biosynthesis genes. Disruption of this promoter (P_{mra}) on the chromosome and its replacement by the *lac* promoter (P_{mra}::P_{lac}) led to isopropyl-β-D-thiogalacto pyranoside (IPTG)-dependent cells that lysed in the absence of inducer, a defect which was complemented only when the whole region from P_{mra} to *ftsW*, the fifth gene downstream from *ftsI*, was provided *in trans* on a plasmid.

In the present work, the levels of various proteins involved in peptidoglycan synthesis and cell division were precisely determined in cells in which P_{mra}::P_{lac} promoter expression was repressed or fully induced. It was confirmed that the P_{mra} promoter is required for expression of the first nine genes of the *mra* cluster: *mraZ* (*orfC*), *mraW* (*orfB*), *ftsL* (*mraR*), *ftsI*, *murE*, *murF*, *murD*, and *ftsW*. Interestingly, three- to sixfold- decreased levels of MurG and MurC enzymes were observed in uninduced P_{mra}::P_{lac} cells. This was correlated with accumulation of the nucleotide precursors UDP-N-acetylglucosamine and UDP-N-acetylmuramic acid, substrates of these enzymes, and with a depletion of the pool of UDP-N-acetylmuramyl-pentapeptide, resulting in decreased cell wall peptidoglycan synthesis. Moreover, the expression of *ftsZ*, the penultimate gene from this cluster, was significantly reduced when P_{mra} expression was repressed. It was concluded that the transcription of the genes located downstream from *ftsW* in the *mra* cluster, from *murG* to *ftsZ*, is also mainly (but not exclusively) dependent on the P_{mra} promoter.

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B-c. Division of Cytoplasmic Genetics

(1) Targeted integration of DNA using mutant lox sites in embryonic stem cells

Kimi ARAKI, Masatake ARAKI and Ken-ichi YAMAMURA

Site-directed integration has been achieved by using a pair of mutant lox sites [Albert, H., et al. (1995) Plant J. 7, 649-659.] in embryonic stem (ES) cells. We established ES cell lines carrying single copy of loxP or mutated lox site as a target, and examined the frequency of site-specific integration by Cre transient expression. Since the targeting vector that we constructed contains a complete neo gene, random integrants can also form colonies like a simple gene targetting. In this system , the frequency of site-specific integration via the mutant lox sites reached to maximum 17%. On the contrary, the wild type loxP sites yielded site-specific integration events at quite low frequencies (under 0.5%). This mutated lox system will be useful for "knock-in" using ES cells. For the details, see Ref. 1.

(2) Efficiency of recombination by Cre transient expression in embryonic stem cells: Comparison of various promoters

Kimi ARAKI, Takashi IMAIZUMI, Keiji OKUYAMA, Yuichi OIKE and Ken-ichi YAMAMURA

The Cre-loxP recombination system of bacteriophage P1 is now frequently utilized in genetic manipulation in embryonic stem (ES) cells. The level of Cre expression is critical to induce loxP site-specific recombination in ES cells. In order to compare the efficiency of recombination, we have constructed four cre expression vectors driven by different promoters, that are cytomegarovirus / chicken b-actin (CAG) promoter, human polypeptide chain elongation factor 1a (hEF-1a) promoter, mouse phosphoglycerate kinase-1 (mPGK) promoter and polyoma enhancer / herpes simplex virus thymidine kinase (MC1) promoter. We introduced these cre expression vectors by electroporation into three ES cell lines carrying a single copy of CAG-loxP-chloramphenicol acetyltransferase (CAT) gene-loxP-b-galactosidase (b-gal)

gene construct. Since the Cre-mediated recombination leads to the excision of CAT gene, the efficiency of recombination can be monitored by b-gal expression. No selection system was used in the experiments. The maximum recombination frequency was obtained when the CAG promoter was used, followed by the hEF-1a promoter, the mPGK promoter and the MC1 promoter in order. These results indicate that the efficiency of recombination in transient expression system corelates with the promoter activity of Cre expression vector. Thus, it is important to choose the promoter for effective recombination by Cre. For the details, see Ref. 2.

(3) Nuclear organization in fission yeast meiosis

Yasushi HIRAOKA (Kansai Advanced Research Center, Communications Research Laboratory)

We found that in fission yeast meiotic prophase, telomeres are clustered near the spindle pole body (SPB; a centrosome-equivalent structure in fungi) and take the leading position in chromosome movement while centromeres are separated from the SPB (Chikashige et al., Science 264, 270-273, 1994). The meiotic telomere position contrasts with mitotic nuclear organization in which centromeres remain clustered near the SPB and lead chromosome movement. Thus, nuclear reorganization switching the position of centromeres and telomeres must take place upon entering meiosis. We analyzed the nuclear location of centromeres and telomeres in genetically well-characterized meiotic mutant strains. An intermediate structure for the telomere-centromere switching was observed in haploid cells induced to meiosis by synthetic mating pheromone; fluorescence in situ hybridization revealed that in these cells, both telomeres and centromeres were clustered near the SPB. Further analyses in a series of mutants showed that the telomere-centromere switching takes place in two steps; first, association of telomeres with the SPB and second, dissociation of centromeres from the SPB. The first step can take place in the haploid state in response to mating pheromone, but the second step does not take place in haploid cells and probably depends on conjugation-related events. In addition, a linear minichromosome was also colocalized with authentic telomeres instead of centromeres, suggesting

that telomere clustering plays a role in organizing chromosomes within a meiotic prophase nucleus. (Chikashige et al., 1997)

(4) Chromosome dynamics in living human cells

Yasushi HIRAOKA (Kansai Advanced Research Center, Communications Research Laboratory)

To examine mitotic events, such as nuclear membrane assembly and disassembly, in living mammalian cells on a microscope stage, we designed a microscope system with a precise temperature control (Hiraoka and Haraguchi, Chromosome Research 4, 173-176, 1996). Using the computerized fluorescence microscope system with a precise temperature control, we observed dynamics of chromosomes and microtubules during mitosis in living HeLa cells. Chromosomes were stained for double staining of chromosomes and microtubules, tubulin conjugated with rhodamine was microinjected into cells that had been stained with a DNA-specific fluorescent dye, Hoechst 33342. Microinjected rhodamine-tubulin was incorporated into microtubules and visualized microtubules in living cells. We examined their dynamics in the presence of mitotic inhibitors such as an inhibitor of type II DNA topoisomerase and inhibitors of microtubule polymerization. Continuous observation in individual living cells has also made it possible to visualize effects of mitotic inhibitors on dynamics of chromosomes and microtubules. These inhibitors discoordinated cytoplasmic events and chromosomal events.
(Haraguchi et al., 1997)

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C. DEPARTMENT OF DEVELOPMENTAL GENETICS

C-a. Division of Developmental Genetics

(1) The Inhibition of Ras Signaling Pathway by EDL is Required for the Neural Inducing Ability of Founder Cells

Takuma YAMADA, Masataka OKABE, Hiroyuki KOSE and Yasushi HIROMI

In the developing nervous system, the determination of neurons occurs in a stepwise fashion. For example, two classes of adult photoreceptor cells (R1-R8) and of embryonic chordotonal organ precursors (COPs: C1-C8) can be discerned in Drosophila. Class I (founder cells) comprises the R8 cells in the eye and five *rhomboid* (*rho*)-expressing COPs (C1-C5), determined by a proneural gene *atonal*. Class II cells (R1-R7 and C6-C8) are induced by the founder cells through activation of the Ras signaling and its nuclear target Pointed, an ETS transcription factor. Although requirement of Ras signal activation in Class II cells has been well documented, its role in the founder cells is largely unknown.

To test the function of Ras signaling in the founder cells, we overexpressed Pointed in the region including the founder cells. In both the retina and the chordotonal system, this caused a reduction in the number of neuronal cells surrounding the founder cells rather than production of extra neurons. This unexpected finding suggests that the expression of Pointed in the founder cells resulted in their decreased ability to induce Class II cells. As all the molecular components of the Ras signaling pathway are also expressed in the founder cells, there must be a mechanism to inhibit Ras signaling in these cells. Here we describe a novel gene, *edl*, a candidate inhibitor of the Ras signaling in the founder cells. *edl* product is present in the founder cells, can inhibit the transcriptional activity of Pointed P2 in S2 cells, and directly binds Pointed P2 protein *in vitro*. Loss of *edl* function results in the down regulation of *rho* expression in founder cells and causes reduction in the number of photoreceptor cells and COPs, resembling the effect of overexpression of Pointed. These

results suggest that the inhibition of Ras signaling by EDL is required in the founder cells for their neural inducing ability.

(2) Sprouty, an Extracellular Factor that Antagonizes both FGF and EGF Signaling Pathways

Susanne KRAMER[1], Masataka OKABE, Nir HAKOHEN[2], Mark KRASNOW[2] and Yasushi HIROMI ([1]Dept. Molecular Biology, Princeton University, [2]Dept. Biochemistry, Stanford University)

Extracellular factors such as FGF and EGF control various aspects of morphogenesis, patterning and cellular proliferation in both invertebrates and vertebrates. In most systems, it is primarily the distribution of the factors, mostly governed by their synthesis, processing and diffusion, that controls the differential behavior of the responding cells. Sprouty is an extracellular protein that has been shown to antagonize FGF signaling during tracheal branching in Drosophila (ref. 3). It is a novel type of protein with a cystein rich region, conserved in human Sprouty homologs. In addition to the embryonic tracheal system, *sprouty* is also expressed in the developing eye imaginal disc, embryonic chordotonal organ precursors, and the midline glia. In all of these systems the EGF receptor (DER) signaling is known to participate in the control of the correct cell number (neurons or glia). We have found that in each of these systems, loss of *sprouty* results in supernumerary neurons or glia. Furthermore, overexpression of *sprouty* in other tissues where EGF signaling is required for patterning, such as wing veins or ovarian follicle cells, results in phenotypes that resemble the loss of function phenotypes of *DER*. These results suggest that Sprouty acts as an antagonist to both the FGF and EGF signaling pathways. These receptor tyrosine kinase-mediated pathways may share not only intracellular signaling components but also factors that modulate their signal magnitude in the extracellular compartment.

(3) Functional Dissection of the Ligand Binding Domain of an Orphan Receptor, Seven-up

Hiroyuki KOSE, Steve WEST[1] and Yasushi HIROMI ([1]Dept. Molecular Biology, Princeton University)

Nuclear receptors, such as the estrogen and the retinoic acid receptors, are ligand-dependent transcription factors that possess conserved DNA binding and ligand binding (LBD) domains. Although many members of the nuclear receptor family have been discovered, due to the wide structural variety of their potential ligands (steroids, retinoids, fatty acids, amino acid derivatives), it has been technically difficult to identify specific ligands for them. Thus the majority of nuclear receptors remain "orphan". Expression studies performed in vertebrates and in invertebrates show that many of the orphan receptors are expressed during development, often under exquisite transcriptional control. Their discrete expression patterns suggest that these "receptors" may convey regulatory information for the function of other molecules, rather than acting in response to their ligands. High structural similarity within the "ligand binding domain" among orphan and the ligand-dependent receptors hints that this domain has an important function, even if it does not serve as a "reception domain" for a ligand. This function is likely to be interaction with other proteins and the modulation of their activity.

We are trying to understand the mechanism of action of the LBD of an orphan receptor Seven-up. Seven-up is highly conserved throughout evolution; the human and the Drosophila homologs share more than 90% amino acids in their LBDs. This suggests that molecules that interact with this domain are also conserved between human and Drosophila. The *seven-up* gene plays an important role in cell fate specification in the developing eye; in its absence photoreceptor neurons R3/R4/R1/R6 are transformed towards another fate, the R7 neuron. The proper function of Seven-up depends, in part, on its tight transcriptional control; misexpression of Seven-up in cells that normally do not express *seven-up* causes a variety of cell fate changes that are specific to each cell type. We found that the expression of the Seven-up LBD, separated from its DNA binding domain, produces various cellular phenotypes similar to those observed upon misexpression of the full length Seven-up

protein. These phenotypes therefore are unlikely to be caused by the misregulation of the target genes of Seven-up, but rather results from the titration of another molecule. We propose that this molecule is a protein partner (protein X) for Seven-up as well as for other cell type-specific transcriptional regulators that, like Seven-up, control fates of individual photoreceptor subtypes. A search for protein X is now under way.

(4) Peptide Groups Which Regulate Nerve Cell Differentiation in Hydra

Toshio TAKAHASHI, Hiroshi SHIMIZU, Masayuki HATTA, Seungshic YUM, Osamu KOIZUMI[1], Tsutomu SUGIYAMA[2], Yojiro MUNEOKA[3] and Toshitaka Fujisawa([1]Fukuoka Women's Univ., Fukuoka, [2]Ishinomaki-Senshu Univ., Ishinomaki, [3]Hiroshima University, Hiroshima)

In the process of systematic isolation of peptide signaling molecules from *Hydra magnipapillata* (Takahashi et al., 1997), we identified two groups of peptides which negatively and positively regulate nerve cell differentiation in *Hydra*. The negative regulators belongs to a PW family which consists of 4 members, Hym-33H, Hym-35, Hym-37 and Hym-310. These peptides (5-8 amino acids long) share a common motif of PW in their C-termini. When hydra were treated for two days with one of these peptides, about 50% of new nerve cell differentiation was inhibited. In contrast, the treatment was prolonged to 7 days, the inhibitory effect disappeared. The results were interpreted that the PW family is a negative feed back signal for nerve cell differentiation.

The positive regulator, Hym-355, is 9 amino acid long and its C-terminus is amidated. The antibody raised against its C-terminus recognized nerve cells mostly present in the tentacles, hypostome and basal disk. Thus, Hym-355 is a neuropeptide. Hym-355, when treated for 2 days, enhanced new nerve cell differentiation by 50 %. The co-treatment with Hym-33H, one of the PW peptides, and Hym-355 at the same concentration of 10^{-6}M canceled out their activities. These results suggest that the two groups of peptides are the feedback regulators of nerve cell differentiation in *Hydra*.

(5) Extracellular matrix and hydra pattern formation

Hiroshi SHIMIZU and Michael P. SARRAS (Univ. Kansas, USA)

The goal of the research project is to examine the mechanism of how extracellular matrix (ECM) affects cell activities in hydra. ECM plays a crucial role in various cell activities such as cell proliferation, cell differentiation, cell migration, morphogenesis etc as has been examined in vertebrate cell cultures. However, it is not at all certain whether these pieces of information obtained in cell culture of vertebrates is true of invertebrate systems. To examine this issue, we examined the effect of ECM on cell activities such as mitotic activity and morphogenetic activity. Hydra ECM termed mesoglea is located between two epithelial mono layers, the ectoderm and the endoderm. Mesoglea does upon incision or amputaion retract away the site of the injury stimulus thus giving rise to an area of tissue free of ECM. We then tried to examine if there could be changes in cell activities provoked by the retraction of ECM. So far we found two things. The first is that the mitotic activity of the ectodermal epithelium is not altered by the retraction of ECM. The second is that head regenerative activity is restricted to the area of tissue where retraction occurred suggesting a relationship between morphogenesis and ECM. The first piece of information is of particular interest. It has been thought that the mitotic activity of epithelium is strongly dependent upon the attachment of the cells to ECM. Those which can divide in the absence of ECM at the base as mentioned above have been classified as tumor cells. The present result suggests that invertebrate cells follow different rules from vertebrates and that ECM dependence of cell activities of intertebrates should be thoroughly reexamined.

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C-b. Division of Gene Expression

(1) MBF1 is an evolutionarily conserved transcriptional coactivator that connects a regulatory factor and TATA element-binding protein

Ken-ichi TAKEMARU, Feng-Qian LI, Hitoshi UEDA, and Susumu HIROSE

*M*ultiprotein *b*ridging *f*actor 1 (MBF1) is a transcriptional cofactor that bridges between the TATA element-binding protein (TBP) and the *Drosophila melanogaster* nuclear hormone receptor FTZ-F1 or its silkworm counterpart BmFTZ-F1. A cDNA clone encoding MBF1 was isolated from the silkworm *Bombyx mori* whose sequence predicts a basic protein consisting of 146 amino acids. Bacterially expressed recombinant MBF1 is functional in interactions with TBP and a positive cofactor MBF2. The recombinant MBF1 also makes a direct contact with FTZ-F1 through the C-terminal region of the FTZ-F1 DNA-binding domain and stimulates the FTZ-F1 binding to its recognition site. The central region of MBF1 (residues 35-113) is essential for the binding of FTZ-F1, MBF2 and TBP. When the recombinant MBF1 was added to a HeLa cell nuclear extract in the presence of MBF2 and FTZ622 bearing the

FTZ-F1 DNA-binding domain, it supported selective transcriptional activation of the *fushi tarazu* gene as natural MBF1 did. Mutations disrupting the binding of FTZ622 to DNA or MBF1, or a MBF2 mutation disrupting the binding to MBF1 all abolished the selective activation of transcription. These results suggest that tethering of the positive cofactor MBF2 to a FTZ-F1-binding site through FTZ-F1 and MBF1 is essential for the binding site-dependent activation of transcription. A homology search in the databases revealed that the deduced amino acid sequence of MBF1 is conserved across species from yeast to human. For details, see Ref. 1.

(2) Transcriptional activation through interaction of MBF2 with TFIIA

Feng-Qian LI, Ken-ichi TAKEMARU, Masahide GOTO[1], Hitoshi UEDA, Hiroshi HANDA[1] and Susumu HIROSE ([1]Faculty of Bioscience and Biotechnology, Tokyo Institute of Technology, Yokohama)

Transcriptional activation of the *Drosophila melanogaster fushi tarazu* gene by FTZ-F1 or its silkworm counterpart BmFTZ-F1 requires two cofactors MBF1 and MBF2 which do not directly bind to DNA. MBF1 is a bridging molecule that connects FTZ-F1 (or BmFTZ- F1), MBF2 and TATA binding protein TBP. MBF2 is a positive cofactor that activates transcription.

To elucidate the mechanism of transcriptional activation by MBF2, we isolated a cDNA coding for the factor. Northern blot analyses showed temporally restricted expression of MBF2 mRNA similar to that of BmFTZ-F1 mRNA. The cDNA sequence predicts a polypeptide of 10kDa whereas natural MBF2 is a glycoprotein of 22kDa. The deduced amino acid sequence of the factor showed no homology with proteins in the databases. Farwestern analyses and glutathione *S*-transferase interaction assays demonstrated that MBF2 makes a direct contact with the β-subunit of TFIIA. In a HeLa cell nuclear extract, bacterially expressed recombinant MBF2 activated transcription from various promoters as natural MBF2 did. This activation requires the MBF2-TFIIA interaction. When recombinant MBF2 was added to the HeLa cell nuclear extract in the presence of MBF1 and FTZ622 bearing the DNA-binding region of FTZ-F1, it selectively activated transcription of the

fushi tarazu gene. This selective activation also requires the MBF2-TFIIA interaction.

In sum, MBF2 activates transcription through its interaction with TFIIA. Selective transcriptional activation occurs when MBF2 is recruited to a promoter carrying the FTZ-F1 binding site by FTZ-F1 and MBF1. For details, see Ref. 2.

(3) Temporal regulation of the mid-prepupal gene FTZ-F1: DHR3 early-late gene product is one of plural positive regulators

Yuji KAGEYAMA, Shoko MASUDA, Susumu HIROSE and Hitoshi UEDA

Various ecdysteroid responsive genes play important roles in insect molting and metamorphosis. Late FTZ-F1, a member of the nuclear receptor superfamily, is a unique transcription factor which is induced by a pulse exposure of 20-hydroxyecdysone. Elucidation of the regulation mechanism of this gene during prepupal period will help our understanding of metamorphosis at a molecular level.

Using transgenic fly lines carrying various transcription regulatory regions of the FTZ-F1 gene fused to the *LacZ* gene, we investigated *cis*-regulatory elements in the late FTZ-F1 transcription unit. The region which governs the stage-specific expression during prepupal period was narrowed down to 1.2 kb, from -0.7 to +0.5 kb relative to the transcription start site. Electrophoresis mobility shift assays using staged extracts and various probes within the stage-specific region allowed us to identify binding sites for DHR3, an early-late gene product, around 170 and 450 bp downstream of the transcription initiation site. Mutations disrupting these binding sites reduced the reporter gene expression without affecting the stage specificity.

In sum, our deletion and mutation studies of the *cis*-regulatory element of the FTZ-F1 gene suggest that the DHR3 binding sites located in the 5' non coding region are involved in the prepupal expression of the gene. These DHR3 binding sites confer high level expression while other elements are also involved in stage-specific expression. For details, see Ref. 3.

(4) Genetic Studies on the 2nd (*p*)-Linkage Group in *Bombyx mori* (L.) Are the *p* and *S*-alleles comprising of either two independent alleles or of a common allele?

Akio MURAKAMI

The *p*-linkage group in the mulberry silkworm [*Bombyx* (*mori*) *mori* L.] is discovered by Tanaka (1913) and that is remarkable in which contains 15 known genetic factors as *p*-alleles at 0.0 unit, 4 as *S*-alleles at 6.1 and 7 as *Gr*-alleles at 6.9. Furthermore, four known recessive genetic factors (*i-lem*, *ml*, *wm-b*, and *oal*) and 3 dominant factors (*Y*, *Ict-A*, and *Rc*) are locating at each locus on *p*-linkage group. In addition to those alleles and genes, a few genes/(*pk Rt*, *sme*, etc.) have reported to be linked with this linkage chromosome, but their loci have yet been worked out. In *Bombyx* silkworms, where cover on eleven linkage groups as to larval body-colors and/or marking patterns: *bd* (9-22.9), *Dus* (10-3.9), *L* (4-15.3), *mln* (18-35.3), *ms* (12-5.5), *msn* (19-26.9), *q* (7-0.0), *so* (26-?) *U* (14-40.5) and *Ze* (3-20.8), besides *p* (1-0.0) and *S* (1-6.1). A phenotype of gene p^M (moricaude), one of the *p*-multiple allelic member, is fairly analogous to that of *Bombyx* (*mori*) manadarina *M*. which is widely accepted as a closely related form as a subspecies of *B. mori*. L. Analyses of the larval body marking pattern and/or coloration among variants of *p*-linkage chromosome seem to be useful clues for approach on an original form or a progenitor of *B. mori*.

Although there have been accumulated a large body of data for every variant on this linkage group, certain unresolved questions are remained. There is no difference in the phenotype between the p^S (striped) and *S* (new striped) mutants, for example. The dominant-recessive relationship between the both mutants is yet established, however. Tanaka (1913, '16 '25) indicated that the gene p^M is epistatic to the p^S, while Tazima (1938) proposed a different view that the former is dominant over the latter. If the latter case is acceptable, the p^S becomes an original phenotype (or genotype) in stead of the gene p^M for the larval body marking pattern in *Bombyx* silkworms. In general, a frequency of forward mutations is higher than that of back mutations for many species. But the latter type mutation showed a high frequency in these *p*- and *S*-allelic series than the former, indicating that the situation is

unusual. Also, recombination values between p-or S-allelic members and the gene & (yellow blood): the values between both alleles are small, but to some extent fluctuated. Accordingly, the gene arrangement on p-linkage group seem to be not an appropriate. There is a mysterious observation that the two mutants, S^2 (second new striped) and p^{Sw} (withish-throx striped), are derived from different multiple allelic series. Furthermore, there is a complicated report that the mutant S^2 (second striped) which was obtained from an X-ray-induced treatment of the p^S line has been explained as an inversion (and/or duplication) of the p^S into S-allelic domain (Takasaki, 1947). On the contrary, the p^{Sw} (whitish-thorax striped) was obtained from a high temperature shock treatment of S line (Tanaka, 1951, 1953). According to the mutant list published by Chikushi (1972), however, he noted that the mutant was obtained from the pupae after treatment of X-rays instead of the temperature shock. Such being the context, it brings forward a question whether both p- and S-alleles are independent each other. In other words, it gives rise to a possibility that p- and S-allelic series are comprising of a common allele. In any case, to reveal the question, it will contribute partly to elucidate a gene arrangement of p-linkage group in this insect as well as an original form in *B. mori* L.

(5) Genetic Studies on the 2nd (p)-Linkage Group in *Bombyx mori* (L.) p-allelic series (2-0.0)

Akio MURAKAMI

In this allelic series, spontaneously-appeared four mutants, p^B, p^M, P^S and p^D are listed at present. All of them are dominant over the standard type p^3 or a conventional wild-type designated as $\pm^p$. A mutant insect having either p^M mutant gene or p^S gene, shows normal viability that is no embryonic lethality, but the gene p^B to some extent shows poor in health condition. Six artificially-induced dominant mutants are registering in the list of mutants as follows: p^L (light crescent), p^{Sa} (sable), $p^{Sa\text{-}2}$ (sable-2), p^G (ventral striped), p^{ST} (pale striped) and p^{Sw} (whitish-thorax striped). No embryonic lethality can be observed for the p^L mutant, which was obtained from p stock line pupae treated with a high temperature shock at 42℃ (Chikushi, 1938). While

the two X-ray-induced sable type mutants, p^{Sa} and $p^{Sa\text{-}2}$, are accompanying with a serious embryonic lethality and indicating that they have chromosomal deficiency (Tazima, 1938, '43). The mutant p^{G} which was obtained from an X-ray-treated pupa, has a translocation between p-linkage chromosome and a certain autosome, T(A:^{2})pS, where A indicates the autosome. This translocation is no indication of the embryonic lethality. The genetic nature of an X-ray induced mutant p^{St} (Takasaki, 1947) could not further refer to beyond the original brief memoranda on this mutant. A mutant p^{Sw} was derived from after treatment of pupae homozygous for S with X-rays (Chikushi, 1972). It is very difficult to understand reasons why the mutant was appeared from a different allelic series or the S strain. This mutant seems to be in a very normal health condition.

All of those artificially-induced mutations are what we called a dominant mutation, but every mutational event is directed from the dominant phenotype or genotype. p^{S} or S, to the recessive one, p^{L} and p^{Sa}. Accordingly, it is very logical to say that those mutations are appeared as some structural (of functional) changes on the chromosome or defects of the original dominant phenotype.

The body-marking pattern of larvae heterozygous for p^{S} is clearly lighter in color tone than that of the homozygous ones. Also, the color tone of a trisomic larva for the gene locus makes deeper than a normal diploid one (Takasaki, 1952). A similar situation would be applicable to other p- and S- allelic mutant members: the mutant p^{B} seems to be comprised of either duplicated or triplicated p^{S} (or p^{M}) genes on this 2nd chromosome accompanied with a chromosomal aberration. Furthermore, the gene p^{S} might the also duplicated the p^{M} with unequal crossing-overs in long domestication (or evolutional) processes. Curiously enough, we cannot picture a wild-type of the S-alleles. In any case, such being the situation, the present author would like to propose and alternative view that the so-called standard type of the p-allele, $\pm^{p}$ or p^{3}, should be replaced with the dominant genotype of either p^{S} or p^{M}.

In this connection, it is of interest to note that a phenotype of the mutant p^{M} (moricaude) is identical to a so-called wild-type mulberry silkworm, *B. (mori) mandarina* (*M.*) and/or a closely related subspecies of *B. (mori) mori*

(L.). The dominant-recessive relationship between p^M and p^S is equistatic each other (Tanaka, 1933), but the latter phenotype is regarded as epistatic to the former (Tazima, 1938). Accordingly, the present author has to be inclined to have a view that the p^S is a fundamental body-marking pattern character among the alleles in larvae of *Bombyx* silkworms. As is well known facts that color tone of the p^s is more deepen that of the p^M. However, we cannot say anything certain without further investigations on this point. Because the body marking in heterozygous for the p^S is less color tone than that of homozygous individuals. Furthermore p^S trisomic silkworms show deeper in the color tone of body marking than the disomics (or diploid) (Takasaki, 1952). A similar explanation may adopt the other p - and S allelic members: and origin of mutant p^B conceive of du- (or tri-) plication of the p^M gene or p^S in the chromosome which is accompanying with some kinds of the chromosome aberration.

The history of the gene p^S is also able to be supported to a multiplication of the p^M gene on account of unequal crossing-overs, but the mutant p^S is free from any chromosomal aberrations. Although the p^M like phenotype in the larval body marking pattern has constantly been recorded in various historical documents of sericulture industry from ancient era to the present. While the p^S like mutant has not been described in old documents, so far, so that such the mutant seems to be recently appeared under our eyes (e.g., Yoshitake and Sato, 1988; Yoshitake, 1988). A similar situation will be adaptable to the mutant p^B as well as U (ursa). It seems likely to consider that these mutants, p^B, p^S, S etc. have been appeared in relatively recent as products of some unusual biological events, mutation, unequal crossing-over and so on. Among other things, domestication procedure might be accelerated this event. The assumption has an advantage for the interpretation of allelic structures in both the p-and S-loci and their genetic connection between them. It is also feasible to suppose that both allelic series may formed by the unequal crossing-over as a large gene family.

From these context, thus, the following hypothesis can be proposed that the p^M is an original phenotype (or genotype) of the larval body-making pattern of *B. mori*. L. However, we can not say anything certain without further investigations on this point.

(6) Genetic Studies on the 2nd(p)-Linkage Group in *Bombyx mori* (L.) S-allelic series (2-6.1)

Akio MURAKAMI

This allele apart to some extent from the p-allelic domain at the starting point of p-linkage group. At present, the allele is made up of 4 members, S, S^d (dilute new striped), S^2 and S^w and S^W. Curiously enough, however, we can not make picture a phenotype of the wild type ($+^S$) and recessive types in this allele. The first two mutant genes, S and S^d, are spontaneous origin, while the others are artificially induced mutants (Tanaka, 1929, 1933). The mutants is dominant over the mutant S^d (Tanaka, 1933). The artificially-induced mutant S^w was detected after treatment of pupae with a high temperature (42℃) shock heterozygous for $p/p^M;S/S$ (Chikushi, 1938). The history of this mutant was described as a recessive type mutational event from a gene S, while another explanation may be also possible as a dominant type from a gene S, while another explanation may be also possible as a dominant type mutation from either the p or p^M as seen in a description of p^L mutant (Chikushi, 1938).

The mutant S^2 is a product of the X-ray-treated of the p^S pupae and that has been regarded as a chromosomal inversion (or duplication) of the p^S into the S-allelic series locus (Takasaki, 1947), indicating that this mutational event was resulted in the shift of the p^S gene from its original p-allele. The S^2 mutant history is a quite reverse case of the mutant p^{Sw} among three mutants, S, S^d and S^2. Takasaki (1948) reports slightly different recombination values between the S^2 and $+p$, 1.4% (Takasaki, 1947) and between the S^2 and p, 1.5% (Takasaki, 1948). However, he (1952) also reported a different recombination value between the genes S^2 and p to be as a 6.1% (Takasaki 1952) and he followed the view of Tanaka (1933) who has reported a recombination value of 6.1% between the genes p and S.

Genetics relations between the p- and S-multiple allelic series: As has been discussed above, the mostly mysterious phenomena in both loci is. It remains an unsolved question whether the gene S might be resulted from a multiplication of the p^M gene through the unequal crossing-overs.

Although *Bombyx* silkworms with a phenotype likes the p^M gene for the

larval body marking pattern have often recorded in various historical documents for the seicultural industry from the ancient era to the present, the insects with a phenotype likes the p^S gene did not document in old age, so far. However, it seems to have a tendency that such the phenotypic larvae was recently detected and/or documented (e.g., Yoshitake, 1988; Yoshitake and Sato, 1986) with the development of sericulture industry. A similar tendency is also thought to be mutant S such as p^B, U etc. It is likely to say that several mutants, p^B, p^S, S, U and *so* forth, have been appeared as products of some unusual biological events including mutations, unequal crossing-overs, etc., during a long evolutional (or domestication) process might be accelerated the event. This assumptive view may have an advantage of the explanation for the multiple allelic structure in the p -and S -alleles and their connection. Among other things, there is no denying a possibility that both alleles are made up of what we called a large gene family. In any case, it can be inferred from those discussion above that the p^M seems to be an original phenotype or genotype for the various gene factors as to the larval body marking pattern located on p -linkage group of the *Bombyx* silkworm. However, we can not say anything without further investigations on this point.

(7) Origin of Holometabolous Metamorphosis in Insects

Kiyoshi MINATO

The evolutional origin of holometabolous metamorphosis in insects, which is one of the reasons of the large success of insects in the animal world, was investigated through the available literatures and the observations of many life styles of insects in the fields. The insects with holometabolous metamorphosis or the endopterygotes are characteristic to their larval form relatively simple and dissimilar to the adult one, and the special instar stage of "pupae" existing between the larval and adult one.

Though the origin and meaning of the pupal stage have been controversial until recently, it is now considered to be reasonable that the phase with the evaginated wing anlage is necessary to making the internal space for the full growth of wing muscle, and that it evolved as the last larval stage equivalent to the last nymphal stage in exopterigotes which was retained with the exter-

nal wing anlage contrary to the progression of more or less internalization of it in the previous larval instar stages (Hinton,1963b). However, considering the hormonal condition controlling the ecdysis into pupae, that is, the one under the depletion of juvenile hormone, the pupal stage seems to be belong to rather the adult one than the larval - even if the last - one, and the external form of the pupae the almost same as that of the adult also make the same impression as above and seems almost not to necessitate any other ecdysis of the pupal-adult one.

Then, the larval-pupal ecdysis in endopterygotes may originated from the necessity for the protection from predators through the excretion of newly cuticle and its hardening during the long periods necessary for the large morphological change in the phase.

The success of endopterygotes seems to be depend upon rather their very simple larval forms themselves with few external structures such as wing anlage, compound eyes, and more developed legs than the merits produced through the holometabolous metamorphosis or the independent habitats each other in the two phases of larvae and adult. They make them possible to utilize more efficiently the three dimensional environments such as the inside of soil and water, the inside of leaves, fruits and stems of various plants and trees, and the underside of stones. Actually, they appeared to have adapted to utilize actively the various circumstances the parts of which appeared newly in evolution, especially in about Permian. Furthermore, these simple larval forms may have derived from the some utilization of embryonic forms, possibly by suppressing the further development of some external morphology, even not by the precocious hatching of embryos as in the fascinating theory stated by Berlese (1913).

Publications

1. TAKEMARU, K., LI, F.-Q., UEDA, H. and HIROSE, S.: MBF1 is an evolutionarily conserved transcriptional coactivator that connects a regulatory factor and TATA element-binding protein. Proc. Natl. Acad. Sci. USA, **94**, 7251-7256, 1997.
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prepupal gene FTZ-F1: DHR3 early-late gene product is one of plural positive regulators. Genes to Cells, **2**, 559-569. 1997.

C-c. Division of Physiological Genetics

(1) Interplay between positive and negative elongation factors of RNA polymerase II transcription: drawing a new view of DRB-mediated inhibition of transcription

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

The purine nucleotide analog 5,6-dichloro-1- β-ribofuranosylbenzimidazole (DRB) is a classic inhibitor of transcription by RNA polymerase II (PolII). We have identified a factor from HeLa cell nuclear extracts that is required to confer DRB sensitivity in a cell-free transcription system. This DRB sensitivity inducing factor (DSIF), in conjunction with DRB, inhibits PolII-mediated transcription at the level of elongation. DSIF has been purified and is composed of 160 kDa and 14 kDa subunits. DSIF works as a negative elongation factor in the absence of DRB. Isolation of a corresponding cDNA reveals that DSIF comprises human homolog of yeast SPT5 and SPT4. The convergence of biochemical studies on DSIF and genetic analysis of SPT5 and SPT4 indicates that DSIF works generally as a negative elongation factor and regulates PolII processivity. Although DRB inhibits PolII elongation, its mode of action has been elusive. Two factors essential for DRB action have been identified. They are DSIF, a negative elongation factor, and pTEFb, a positive elongation factor. pTEFb was originally identified in Drosophila extracts by Marshall and Price (J. Biol. Chem., **270**, 12335-12338, 1995). These factors positively and negatively regulate PolII-mediated transcription elongation.

(2) Species-specific codon usage diversity in eleven prokaryotes and one eukaryote whose genomes have been completely sequenced

Shigehiko KANAYA[1,2], Yoshihiro KUDO[1], Toshimichi IKEMURA[3] ([1]Department of Electric and Information Engineering, Faculty of Engineering, Yamagata University, [2]Division of Physiological Genetics, Department of Ontogenetics, National Institute of Genetics, [3]Division of Evolutionary Genetics, Department of Population Genetics, National Institute of Genetics)

We characterized codon usage diversity of genes for eleven bacteria (*Mycoplasma genitalium*, *Mycoplasma pneumoniae*, *Methanococcus jannaschii*, *Methanobacterium thermoautotrophicum*, *Borrelia burgdorferi*, *Helicobacter pylori*, *Bacillus subtilus*, *Escherichia coli*, *Haemophilus influenzae*, *Archaeoglbus fulgidus*, and *Synechocystis* sp.) and Yeast (*Saccharomyces cerevisiae*), whose genomes have been completely sequenced. Quantification of the cellular tRNA content of *E. coli*, and codons optimized for its translation system (optimal codons) were reported previously by our group. Principal component analysis of *E. coli* showed that the codons positively contributing to the first principal component (PC1) almost always correspond to the optimal codons, suggesting that the codon usage in this bacterium is strictly constrained by translation efficiency. Comparison of the gene distribution in the PC1 projection of the other eleven speices with that in *E. coli* indicates that codon usage in these eleven species is also constrained by translation efficiency. The diversity for *H. influenzae* is similar to that for *E. coli*, although the G+C% (38%) of the *H. influenzae* genome is significantly different from that of *E. coli* (50%). In the case of *Synechocystis sp.*, the genes with homologues in the chloroplast genomes of present-day plants (e.g. genes for the photosynthetic system and ribosomal proteins) were located in restricted zones in the multidimensional space obtained by principal component analysis. Furthermore, in *M. jannaschii* and *M. thermoautotrophicum*, most of genes involved in methanogenesis have postive scores for PC1, suggesting that codon usage for methanogenesis is also highly adapted to the optimal codon structure estimated from tRNA contents in cells. For details, see Ref. 1-5.

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D. DEPARTMENT OF POPULATION GENETICS

D-a. Division of Population Genetics

(1) Rate variation of DNA sequence evolution in the *Drosophila* lineages

Toshiyuki S. TAKANO

Rate constancy of DNA sequence evolution was examined for three species of *Drosophila*, using two samples: the published sequences of eight genes from regions of the normal recombination rates and new data of the four *AS-C* (*ac*, *sc*, *l'sc* and *ase*) and *ci* genes. The *AS-C* and *ci* genes were chosen because these genes are located in the regions of very reduced recombination in *D. melanogaster* and their locations remain unchanged throughout the entire lineages involved, yielding less effect of ancestral polymorphism in the study of rate constancy. The synonymous substitution pattern of the three lineages was found to be erratic in both samples. The dispersion index for replacement substitution was relatively high for the *per*, *G6pd* and *ac* genes. A significant heterogeneity was found in the number of synonymous substitutions in the three lineages between the two samples of genes with different recombination rates. This is partly due to a lack of the lineage effect in the *D. melanogaster* and *D. simulans* lineages in the *AS-C* and *ci* genes in contrast to AKASHI's observation of genes in regions of normal recombination. The higher codon bias in *D. yakuba* as compared with *D. melanogaster* and *D. simulans* was observed in the four *AS-C* genes, which suggests change(s) in action of natural selection involved in codon usage on these genes. Fluctuating selection intensity may also be responsible for the observed locus-lineage interaction effects in synonymous substitution. For the details, see Genetics (1998) 149: 959-970.

(2) Study of species difference as observed as interspecific hybrid anomaly in Drosophila

Toshiyuki S. TAKANO

With the aim of revealing genetic variation accumulated among closely related species during the course of evolution, this study focuses on loss of macrochaetae on the notum as one of the developmental anomalies seen in inter-specific hybrids between *D. melanogaster* and its closely related species. We previously presented evidence that bristle loss in inter-specific hybrids is found between *D. melanogaster* and *D. simulans*, but not between pairs of *D. melanogaster* on one hand, and *D. mauritiana* and *D. sechellia* on the other. Use of cell type markers suggests that the defect does not lie in cell fate decisions during bristle development, but in the maintenance of neural fate and/or differentiation of the descendants of sensory mother cells (Genetics, in press). To isolate genes responsible for the bristle anomaly of the hybrids, deficiency screening and QTL (quantitative trait loci) mapping based on the within-species variation of *D. simulans* were performed. The latter analysis showed that a QTL accounted for more than two-thirds of the parental difference, whereas we found two distinct candidate regions from the deficiency screening. The genetic analysis of the bristle anomaly revealed a sex difference between comparable genotypes, indicating a certain degree of sex-specificity in genotypic effect. The sex-specificity may be one reason for the inconsistent results of the two experiments, because the female hybrids were studied in the deficiency screening, but the males in the QTL mapping analysis. Based on the finding that the male hybrids were more susceptible to bristle loss than the female hybrids were, duplication screening was conducted, where the hybrid males were examined for the bristle. We consequently found one candidate region, which is close to the estimated position of the QTL with the greatest effect.

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D-b. Division of Evolutionary Genetics

(1) *In situ* visualization of sequence-dependent high-affinity sites for non-B structure-forming DNAs in the human interphase nucleus. Affinity sites for triplex-forming DNAs are closely but differentially associated with individual centromeres

Mizuki OHNO, Toyoaki TENZEN, Tetsushi YAMAGATA, Shigehiko KANAYA and Toshimichi IKEMURA

Triplex formation occurs readily in polypurine/polypyrimidine sequences leaving single-strand unpaired DNAs that can hybridize with other single-strand DNAs with significant complementarity. This will enable distantly spaced DNAs in genome sequences to associate with each other in interphase nuclei. Distantly spaced DNAs may also form transmolecular triplexes organizing themselves into an ordered array. To investigate mechanisms governing spatial organization of chromosomal DNAs in human interphase nuclei, we searched *in situ* the sequence-dependent high-affinity sites for DNAs with specific characteristics such as triplex formation, by two methods. The one resembled fluorescence *in situ* hybridization used for detecting single-strand RNAs, thus was able to detect single-strand DNAs in nondenatured nuclei. The other was a lipofection assay. Both methods showed polypurine/polypyrimidine sequences, such as (GA/TC)n, (GAA/TTC)n and those present in the human genome, gave clear foci-type signals with different probe sequences resulting in different signal patterns. By combining seven chromosome-specific centromere probes with polypurine/ polypyrimidine probes, differential binding characteristics of individual centromere territories in non-denatured nuclei were revealed, yielding a model in which supramolecular structures with differential affinities to different polypurine/polypyrimidine sequences exist

within and/or near individual centromere territories. For details, Cytogenet Cell Genet, 81, 101-102, 1998.

(2) Triplet repeat polymorphism within the NOTCH4 gene located near the junction of the HLA class II and class III regions in narcolepsy

Asako ANDO[1], Kimihiko SUGAYA[2], Takeo JUJI[3], Toshimichi IKEMURA and Hidetoshi INOKO[1] ([1]School of Medicine, Tokai University, [2]National Institute of Radiological Sciences, [3]Japanese Red Cross Central Blood Center)

A polymorphic (CTG)n microsatellite repeat was found in the signal peptide domain of the NOTCH4 gene located near the junction of the class II and class III regions of the human major histocompatibility complex. This gene belongs to a multigene family of NOTCH originally identified as a differential factor of neuronal cells. To ascertain whether the NOTCH4 gene is involved in the development of neurogenic disease, narcolepsy, which is known to be tightly associated with HLA-DR15, this microsatellite polymorphism of the (CTG)n repeat was analyzed in Japanese patients with narcolepsy. One allele, 9 repetitions of CTG (Leu) was significantly increased in the patient group. However, the significant increase of this allele in the patient group could be explained by a strong linkage disequilibrium with the HLA class II alleles, DRB1*1501, DQA1*0102 and DQB1*0602, which were more strongly associated with the disease. These results suggest that the (CTG)n repeat polymorphism in NOTCH4 dose not primarily determine the susceptibility to narcolepsy. For details, see Tissue Antigens, 50, 646-649, 1997.

(3) Replication Timing for the GC Content Transition Area

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

Mammalian genomes are composed of long-range G+C% mosaic structures thought to be related to chromosome bands. We previously reported an example of a boundary of megabase (Mb)-sized GC% mosaic domains at the junction area between major histocompatibility complex (MHC) classes II and III, proposing it as a possible chromosome band boundary. DNA replication

timing during S phase is known to be correlated cytogenetically with chromosome band zones, and thus the band boundaries have been predicted to contain a switch point for DNA replication timing. In this study, to identify at the nucleotide sequence level the replication switch point during S phase, we determined the precise DNA replication timing for MHC classes II and III, focusing on the junction area. To do this we used PCR-based quantitation of nascent DNA obtained from synchronized human myeloid leukemia HL60 cells. The replication timing changed precisely in the boundary region with a 2-hour difference between the two sides, supporting the prediction that this region may be a chromosome band boundary. We supposed that replication fork movement terminates (pauses) or significantly slows down in the switch region, which contains dense *Alu* clusters, polypurine / polypyrimidine tracts (e.g., 209-bp tracts), di-, tri- or tetranucleotide repeats, and medium reiteration frequency sequences (MERs). We found also the replication switch point in the XIST locus in the X chromosome inactivation center (XIC). For details, see Mol. Cell. Biol. 17, 4043-4050.

(4) Structural analysis of mouse tenascin-X: evolutionary aspects of reduplication of FNIII repeats in the tenascin gene family

Tomoki IKUTA, Norio SOGAWA, Hiroyoshi ARIGA[1], Toshimichi IKEMURA, Kenichi MATSUMOTO ([1]Graduate School of Pharmaceutical Sciences, Hokkaido University)

Tenascin-X (TNX) is an extracellular matrix glycoprotein involved in both primary structural functions and modulating cellular activities in multicellular organisms. We determined the 67,977 bp nucleotide sequence of the entire mouse tenascin-X (*Tnx*) gene, which also includes the last exon of *Creb-rp* and *Cyp21*. We compared it with the orthologous human locus. Conservation of both position and orientation of the three functionally unrelated genes at this position was found. Comparison also revealed that introns 1,4 and 6 of *Tnx* are highly consereved between species. The sequence showed that mouse *Tnx* contains 43 exons separated by 42 introns. The deduced amino-acid sequence (4114 residues) revealed that mouse *Tnx* has a primary structure characteristic of tenascins, which consists of a signal peptide and four heptad

repeats followed by 18.5 epidermal growth factor-like (EGF) repeats, 31 fibronectin type III-like (FNIII) repeats, and a region homologous to fibrinogen. cDNA clones generated by alternative splicing of eight consecutive FNIII repeats (M15-M22) as well as a proximal FNIII repeat (M3) were also identified. The FNIII motifs that were subject to alternative splicing were assigned to the group of recently reduplicated FNIII repeats because they have a high level of amino-acid sequence similarity. We also analyzed the evolution of FNIII repeats in TNX. For details, see Gene, 217, 1-13, 1998.

(5) Genomic organization of human GABAB receptor identified in MHC class I region; proposal for differentiation of two isoform's expression via alternative usage of promoters with and without cAMP-responsive element

Tetsushi YAMAGATA, Asako ANDO[1], Hidetoshi INOKO[1] and Toshimichi IKEMURA
([1]School of Medicine, Tokai University)

Gamma-aminobutyric acid (GABA) is the most abundant and widely distributed inhibitory neurotransmitter present in the central nervous system (CNS). Receptors activated with GABA are classified into ionotropic and metabotropic receptors (GABAA/GABAC and GABAB, respectively). Two mRNA forms of rat's GABAB receptor were characterized and predicted to be produced by alternative splicing. In the telomeric portion of MHC class I, we identified the human GABAB receptor gene which encodes the two isoforms. The gene organization showed that the two isoforms are generated by alternative usage of promoters rather than by alternative splicing, and a cAMP responsive element (CRE) is present only in the promoter for the shorter isoform. Based on the fact that GABAB receptor negatively regulates CRE binding protein (CREB)-mediated transcriptions in CNS, we propose that the activation of GABAB receptor can regulate the relative expression of its two isoforms through alternative usage of the promoters with and without CRE and thus can differentiate numbers and relative abundance of its isoforms via the CRE- and CREB-mediated regulatory system.

(6) Nucleotide sequence analysis of the HLA class I region spanning the 237-kb segment around the HLA-B and -C genes

Nobuhisa MIZUKI[1], Minoru KIMURA[1], Asako ANDO[1], Toshimichi IKEMURA and Hidetoshi INOKO[1] ([1]School of Medicine, Tokai University)

To elucidate the complete gene structure and tc identify new genes involved in the development of HLA class I antigen-associated diseases in the class I region of the human major histocompatibility complex on chromosome 6, a YAC clone (745D12) covering the 146-kb segment around the IkBL and MICA loci was isolated from a YAC library constructed from the B-cell line, BOLETH. A physical map of this region was constructed by isolation of overlapping cosmid clones derived from 745D12. Of these, five contiguous cosmids were chosen for DNA sequencing by the shotgun strategy to give a single contig of 146,601 bp from 2.8 kb telomeric of the IkBL gene to exon 6 of MICA. This region was confirmed to contain five known genes, IkBL, BAT1,MICB,P5-1, and HLA-X (class I fragment), from centromere to telomere, and their exon-intron organizations were determined. The 3.8-1 homologue gene (3.8-1-hom) showing 99.7% identity with the 3.8-1 cDNA clone, which was originally isolated using the 3.8-kb EcoRI fragment between the HLA-54/H and the HLA-G genes, was detected between MICA and MICB and was suggested to represent the cognate 3.8-1 genomic sequence from which the cDNA clone was derived. No evidence for the presence of expressed new genes could be obtained in this region by homology and EST searches or coding and exon prediction analyses. One TA microsatellite repeat spanning 2545 bases with as many as 913 repetitions was found on the centromeric side of the MICA gene and was indicated to be a potential hot spot for genetic recombination. The two segments of approximately 35 kb upstream of the MICA and MICB genes showed high sequence homology (about 85%) to each other, suggesting that segmental genome duplication including the MICA and MICB genes must have occurred during the evolution of the human MHC. For details, see Genomics, 47, 372-382, 1998.

(7) Evolution of primate ABO blood group genes and their homologous genes

Naruya SAITOU and Fumiichiro YAMAMOTO[1]([1]The Burnham Institute, La Jolla, California, U.S.A.)

There are three common alleles (A, B, and O) at the human ABO blood group locus. We compared nucleotide sequences of these alleles, and relatively large numbers of nucleotide differences were found among them. These differences correspond to the divergence time of at least a few million years, which is unusually large for a human allelic divergence under neutral evolution. We constructed phylogenetic networks of human and non-human primate ABO alleles, and at least three independent appearances of B alleles from the ancestral A form were observed. These results suggest that some kind of balancing selection may have been operating at the ABO locus. We also constructed phylogenetic trees of ABO and their evolutionarily related α-1,3-galactosyltransferase genes, and the divergence time between these two gene families was estimated to be roughly 400 million years ago. For details, see Ref. 1.

(8) The phylogenetic relationship of the genus Oncorhynchus species inferred from nuclear and mitochondrial markers

Takashi KITANO, Norimasa MATSUOKA[1], and Naruya SAITOU([1]Faculty of Science, Hirosaki University)

The phylogenetic relationship among Oncorhynchus species have been analyzed by various kind of markers for a long time. However, there are three major disagreement among these studies. First, phylogenetic position of the Pacific trouts group (O. mykiss and O. clarki). Second, the relationship among three Pacific salmons (O. keta, O. nerka, and O. gorbuscha). Third, phylogenetic position of O. masou. In this study, allozyme electrophoresis was used to analyze the phylogeneticc salmon group and the Pacific trout group. Then, mitochondrial DNA sequences (D-loop and Hind III fragment regions cited from Shedlock et al., 1992 and Thomas and Beckenbach, 1989, respectively) were re-analyzed to clear above three problems. It was clear that all Pacific

salmons construct a cluster and O. masou diverged firstly in this group, then, O. keta and O. gorbuscha are sister species in three Pacific salmons by allozyme electrophoresis and mtDNA re-analyses. However, stochastic significance for branching pattern of phylogenetic tree constructed by D-loop data were not obtained. For that reason, it is considered that D-loop sequences are affected by parallel substitutions. These vestiges were observed by the split decomposition graph. Nevertheless, above results were supported by the graph. For details, see Ref. 2.

(9) Genetic origins of the Japanese: A partial support for the "dual structure hypothesis"

Keiichi OMOTO[1], and Naruya SAITOU([1]International Research Center for Japanese Studies)

Based on the morphological characteristics of the skull and teeth, Hanihara (1991) proposed the "dual structure model" for the formation of modern Japanese populations. We examine this model by dividing it into two independent hypotheses: 1) the Upper Paleo-lithic population of Japan that gave rise to the Neolithic Jomon people was of southeast Asian origin, and 2) modern Ainu and Ryukyuan (Okinawa) populations are direct descendants of the Jomon people, while Hondo (Main Island)-Japanese are mainly derived from the migrants from the northeast Asian continent after the Aeneolithic Yayoi period. Our aim is to examine the extent to which the model is supported by genetic evidence from modern populations, particularly from Japan and other Asian areas. Based on genetic distance analyses using data from up to 25 "classic" genetic markers, we find first that the three Japanese populations including Ainu and Ryukyuan clearly belong to a northeast Asian cluster group. This negates the first hypothesis of the model. Then, we find that Ainu and Ryukyuans share a group contrasting with Hondo-Japanese and Korean, supporting the second hypothesis of the model. Based on these results, we propose a modified version of the dual structure model which may explain the genetic, morphological, and archaeological evidence concerning the formation of modern Japanese populations. For details, see Ref. 3.

(10) Reconstruction of phylogenetic trees using the maximum likelihood method in parallel environment using logic programming

Satoshi OOTA, and Naruya SAITOU

With rapid increase of nucleotide and amino acid sequence data, it is required to develop reliable and flexible application programs to infer molecular phylogenetic trees. The maximum likelihood method is known to be robust among many methods for reconstruction of molecular phylogenetic trees, however, this method requires extremely high computational cost. Although parallel computation is a good solution to realize complicated inference such as the maximum likelihood method, generally speaking, it is not so easy to implement parallel programs. In actual data analyses, furthermore, it is often needed to modify or expand application programs. In other words, there is no perfect application program for all data analyses. Logic programming is a good candidate to implement such data analysis programs in natural science fields, because programs in logic programming are easy to write, easy to modify, and easy to implement in parallel environment. We thus have developed an experimental system for reconstruction of phylogenetic trees in parallel environment in logic programming as a part of molecular evolutionary analysis system {¥it DeepForest}. We propose the core algorithm for parallel execution of the maximum likelihood and show its verification according to simulation using amino acid data. For details, see Ref. 4.

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Takagi T. (eds.), Genome Informatics 1997, Universal Academiy Press, Tokyo, pp. 326-327, 1997.

D-c. Division of Theoretical Genetics

(1) The meaning of near-neutrality at coding and non-coding regions

Tomoko OHTA

The nature of weak selection differs between coding and non-coding regions. Coding regions contain genetic information, whereas most non-coding regions do not have any information. Genetic information may be regarded as interaction systems, and the NK model of Kauffman was analysed. This model assumes that each amino acid makes a fitness contribution that depends on the amino acid and on K other amino acids among the N that make the protein. It is known that the fitness landscape is very rugged for $K \geqq 2$. Population genetic analysis of this model suggests that protein evolution obeys the nearly neutral theory and that random genetic drift is important. In other words, evolution becomes rapid in small populations because the proportion of near-neutrality increases among new mutations, and proteins as interactive systems evolve by shifting through random genetic drift on the multipeaked fitness landscape. The variance of the evolutionary rate is not quite as large as data indicate under the model, and additional factors, such as environmental change or population-size fluctuation, need to be considered. Weak selection at non-coding regions may come from chromosome organization, and may be regional in character, which differs from that at coding regions. The problem of genetic load is thought to disappear in these circumstances. For details, see Gene 205,261-267 and J. Mol. Evol. 44, S9-S14.

(2) Role of gene conversion in generating polymorphisms at major histocompatibility complex loci

Tomoko OHTA

Multigene families are known to be evolving under the continued occurrence of unequal crossing-over and gene conversion, and MHC loci are not

exceptions. Population genetic models of diversifying selection, with intra- and inter-locus gene conversion were studied by computer simulations. Weak selection at individual amino acid sites are shown to be quite effective in enhancing polymorphisms, if gene conversion occurs. This is caused by the appearance of coadapted segments of mutations in which recombination tends to be suppressed. In addition to the global type of diversifying selection, an endemic type of selection was introduced in some sets of the simulations to find out a mechanism of the rapid allelic turnover of South American Indians. Strong selection is assumed such that the most common two alleles become lethal in one generation. If this endemic selection occurs periodically, allelic turnover by intralocus gene conversion is shown to become rapid. Hence, interaction of selection and gene conversion has a profound effect on MHC polymorphisms. See for details, Hereditas 127, 97-103.

Publication

1. Role of random genetic drift in the evolution of interactive systems. Journal of Molecular Evolution **44**(Suppl 1):S9-S14, 1997.
2. The meaning of near-neutrality at coding and non-coding regions. Gene 205:261-267,1997.
3. Role of gene conversion in generating polymorphisms at major histocompatibility complex loci. Hereditas **127**:97-103, 1997.
4 The meaning of near neutrality at coding and noncoding regions. Jan. 9, 1997, Guanacaste, Republic of Costa Rica
5 Role of gene conversion in generating polymorphisms at major histocompatibility complex loci. May 25, 1997, Visby, Sweden
6 Evolution by nearly neutral mutations. June 16, 1997, Boulder, U. S. A.

E. DEPARTMENT OF INTEGRATED GENETICS

E-a. Division of Human Genetics

(1) Human Genome Resources and Their Application for the Analysis of Chromosome21

Asao FUJIYAMA, Inaho DANJO[1] and Hong-Seog PARK ([1]Human Genome Center, Tokyo University)

The goal of human genome analysis is not only sequencing entire genome nor cataloging protein coding regions, but to understand functions retained in the human genome and chromosomes. Since most of human chromosomes can be purified by means of dual-laser cell sorting system, such isolated chromosomes are good resources for the studies to understand biological functions retained in individual chromosome. Using purified chromosomes, we have constructed human mono-chromosomal cosmid libraries (except for CM#9 - 12) and #21 fosmid library, and BAC library. Unlike other libraries, our cosmid and fosmid libraries employed random fragmentation/cloning protocol that enabled us to efficiently construct highly randomized libraries. Using these resources, detailed analyses on human 21qcen and 21qter regions are in progress.

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

Inaho DANJO[1], Yong-Sik BONG and Asao FUJIYAMA ([1]Human Genome Center, Tokyo University)

In fission yeast, *Schizosaccharomyces pombe*, deficiency of *ras*1 gene causes abnormal cell shape and abolishes mating ability. However, the signaling pathway in the cell and its target genes are largely unknown because of the lack of appropriate analysis system. To overcome this problem, we categorized genes based on their expression levels in the presence or absence of the

ras1 gene product under different growth conditions. We utilized arrays of clones covering entire genome of the fission yeast. Here, we demonstrate the detection of low molecular weight heat shock protein gene, *hsp16*, and show that it is regulated by a *ras*-mediated signaling pathway, not by the heat shock response in fission yeast.

(3) Severe Lactic Acidosis and Neonatal Death in Pearson Syndrome

Kae MURAKI[1], Yuichi GOTO[2], Ichizo NISHINO[2], M. HAYASHIDANI[1], S. TAKEUCHI[1], Satoshi HORAI, N. SAKURA[1], K. UEDA[1] ([1]Department of Pediatrics, Hiroshima University School of Medicine, [2]Department of Laboratory Medicine, National Center Hospital for Mental, Nervous and Muscular Disorders)

Pearson marrow-pancreas syndrome, a fatal disease associated with mitochondrial DNA rearrangements, is characterized by refractory sideroblastic anaemia during infancy. Only a few neonates with Pearson syndrome have been reported with metabolic acidosis. A female neonate who exhibited severe metabolic acidosis and anaemia at birth is described here. Her condition progressively worsened, with pancytopenia and uncontrollable metabolic acidosis resulting in death at the age of 14 days. A 4988-base pair deletion of mtDNA was detected in the patient's leukocytes, liver and muscle. When a neonate exhibits severe metabolic acidosis of unknown cause, the possibility of Pearson syndrome should be considered. For details, see Ref. 1.

(4) Myoclonus Epilepsy Associated with Ragged-Red Fibers: A G-to-A Mutation at Nucleotide Pair 8363 in Mitochondrial tRNA(Lys) in Two Families

Matsuko OZAWA[1], Ichizo NISHINO[2], Satoshi HORAI, Ikuya NONAKA[1,2], Yu-ichi GOTO[1,2] ([1]Department of Laboratory Medicine, National Center Hospital for Mental, Nervous and Muscular Disorders, [2]Department of Ultrastructural Research, National Insitute of Neuroscience)

In addition to well-known mutations at nucleotide pair 8344 and 8356 in mitochondrial DNA in patients with myoclonus epilepsy associated with ragged-red fibers (MERRF), we found a new G-to-A point mutation at nucleotide 8363

in two Japanese families. The probands had the typical clinical characteristics of MERRF. Since the 8363 mutation was present in a heteroplasmic state, and seen in none of 92 patients with other mitochondrial diseases or 50 normal individuals, this mutation is thought to be disease-related and probably specific to MERRF. As seen in muscle biopsies with the previous two mutations, focal cytochrome c oxidase (CCO) deficiency was the most characteristic finding. With single fiber analysis, the CCO-negative fibers contained a higher percentage of mutant DNA (88.4 +/- 6.6%) than CCO-positive fibers (65.1 +/- 8.0%). These findings suggest that mutations in tRNA(Lys) coding region are related to the MERRF phenotype and are responsible for the reduced CCO activity. For details, see Ref. 5.

(5) Polymorphism of the HLA-DRB1 Locus in Colombian, Ecuadorian, and Chilean Amerinds

N. BLAGITKO[1], C. O'HUIGIN[1], F. FIGUEROA[1], S. HORAI, S. SONODA[2], K.TAJIMA[3], D. WATKINS[1], J. KLEIN[1] ([1]Max-Planck-Institut fur Biologic, Abteilung Immungenetik, Tubingen, [2]Department of Virology, Kagoshima University, [3]Division of Epidemiology, Aichi Cancer Research Institute)

We have characterized the DRB1 genotypes in a sample of 64 South American Indians drawn from populations in Chile, Colombia, and Ecuador. No novel DRB1 alleles were found in the total of 17 different alleles characterized, indicating that rapid allelic generation does not occur at the DRB1 loci, in contrast to HLA-B. Comparison between Chilean and Colombian/Ecuadorian samples revealed no major differences in their allelic frequencies. In the combined Amerind sample the HLA-DRB1*0407 and HLA-DRB1*1402 alleles occurred in the highest frequencies (38% and 22%, respectively). Genetic distance measurement showed the HLA-DRB1 frequencies reported here to agree with findings in other Amerind groups. The high frequencies of both HLA-DRB1*0407 and HLA-DRB1*1402 alleles, in conjunction with their absence in Siberian samples, suggest that migratory groups other than Siberians may have been involved in the peopling of the Americas. For details, see Ref. 7.

(6) The Geographic Distribution of Human Y Chromosome Variation

M. F. HAMMER[1], A. B. SPURDLE[2], T. KARAFET[1], M.R. BONNER[1], E. T. WOOD[1], A. NOVELLETTO[3], P. MALASPINA[3], R. J. MITCHELL[4], S. HORAI, T. JENKINS[2] and S. L. ZEGURA[5] ([1]Laboratory of Molecular Systematics and Evolution, and [5]Deaprtment of Anthropology, University of Arizona, [2]Department of Human Genetics, South African Institute for Medical Research and the University of Witwatersrand, [3]Departimento di Biologia, Universita degli Studi, [4]Department of Genetics and Human Variation, La Trobe University)

We examined variation on the nonrecombining portion of the human Y chromosome to investigate human evolution during the last 200,000 years. The Y-specific polymorphic sites included the Y Alu insertional polymorphism or "YAP" element (DYS287), the poly(A) tail associated with the YAP element, three point mutations in close association with the YAP insertion site, an A-G polymorphic transition (DYS271), and a tetranucleotide microsatellite (DYS19). Global variation at the five bi-allelic sites (DYS271, DYS287, and the three point mutations) gave rise to five "YAP haplotypes" in 60 populations from Africa, Europe, Asia, Australasia, and the New World (n=1500). Combining the multi-allelic variation at the microsatellite loci (poly(A) tail and DYS19) with the YAP haplotypes resulted in a total of 27 "combination haplotypes". All five of the YAP haplotypes and 21 of the 27 combination haplotypes were found in African populations, which had greater haplotype diversity than did populations from other geographical locations. Only subsets of the five YAP haplotypes were found outside of Africa. Patterns of observed variation were compatible with a variety of hypotheses, including multiple human migrations and range expansions. For details, see Ref. 6.

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E-b. Division of Agricultural Genetics

(1) Mapped Genomic Locations for QTLs in Rice Reflect Gene Clusters

Hong-Wei CAI and Hiroko MORISHIMA

Wild and cultivated rices differ in a set of correlated characters called domestication syndromes. All those characters are quantitative traits which could be analyzed only by statistical genetic methods in the past. Recent advances in genome research enabled us to map quantitative trait loci (QTL) on the genome. As preliminarily reported last year, to look into the genetic basis of preferential character association underlying domestication syndromes, we analyzed 125 recombinant inbred lines (RILs) derived from a cross between wild and cultivated rice strains.

First, 12 linkage maps were constructed from segregation data of 140 RFLP and isozume markers. Then QTL analysis was performed by examining cosegregation between 32 quantitative traits and 140 markers. The interval mapping method revealed one to 24 QTLs for every 25 characters that were detected, respectively. Among those traits, diagnostic characters for wild

and cultivated types, such as seed dormancy, awn length, anther length, as well as some Indica /Japonica diagnostic traits, such as $KClO^3$ resistance, apiculus hair length, and germination speed proved to be controlled by relatively large number of QTLs.

It was found that several QTLs each of which controls different wild / cultivated diagnostic characters tend to cluster on the specific regions of chromosomes. Similar clusters were redundantly found over the genome. This cluster phenomenon was understood as multifactorial linkages resulting in domestication syndromes. Multifactorial linkages refer to partial linkages inevitably caused by more or less random distribution of multiple factors for different characters on a limited number of chromosomes. Further, it was found that near the chromosomal regions harboring those gene clusters cultivar alleles segregated at higher frequency than expected among RILs. Directions of gene effect of each QTL forming clusters were coincidently the same as predicted from parental phenotypes. Most probably, gene clusters were inconsciously selected for their coadapted character association under cultivation pressure during developing RILs. This inference was supported by the fact that at the isozyme loci located near the clusters frequencies of cultivar alleles gradualy increased in RILs during F^3 to F^7, and that loci outside of the clusters did not show such a trend. Thus, clusters of QTLs found in the present study must be the product moulded by multifactorial linkages and natural selection which worked on coadapted gene sets. Such a linkage system does not prevent, but does restrict recombination in the evolutionary process.

(2) Phylogenetic Study of AA Genome Wild Rice Species Viewed from Phenotypic and Genic Levels

Masahiro AKIMOTO, Yoshiya SHIMAMOTO and Hiroko MORISHIMA

Variability of 21 quantitative characters and 29 isozyme loci were studied for five AA genome wild rice species; *Oryza rufipogon*, *O. glumaepatula*, *O. meridionalis*, *O. longistaminata* and *O. barthii*. Polymorphism at phenotype and isozyme levels were respectively examined by principal component analysis.

O. rufipogon and *O. glumaepatula*, showing higher phenotype variability than *O. meridionalis* and *O. barthii*, were divided into two and three clusters in the scatter diagram, respectively; namely *O. rufipogon*-I, II and *O. glumaepatula*-I to III. *O. rufipogon*-I and *O. glumaepatula*-I, which were categorized as perennial types, were plotted overlapping each other. While, *O. rufipogon*-II joined *O. meridionalis* and *O. barthii*, forming an annual type group. On the other hand, isozyme analysis showed that five species formed respective clusters without overlapping, indicating that they are genetically differentiated from each other.

Phenotypes are subjected to natural selection. The plants, even with different phylogenetic backgrounds, are likely to develop similar phenotypes when they grow in similar environments. The previously mentioned perennial wild species prefer stable and less disturbed environments where density-dependent mortality is high. While, annual types tend to inhabit unstable and occasionally disturbed environments where density-independent mortality is high. Judging from the incongruence between inter- and intra-species variation pattern revealed by phenotype and genotype, similarities in phenotypes were most probably homoplasy caused by evolutionary convergence.

Diversities of mitochondria DNA and chloroplast DNA were examined for the same materials by RFLP analysis. Results of both analysis reflected geographical distributions of five species rather than their phenotypic variation pattern. This also indicated that the phenotype similarities were homoplasy. For details, see Ref. 1.

(3) New Storage Protein Genes in Rice Detected by Acidic Formate-PAGE Method

Hong-Wei CAI and Hiroko MORISHIMA

Acidic PAGE was widely used to detect variations of salt-soluble proteins of wheat and barley. We applied this method to analyze variation in seed storage proteins of rice using 98 cultivars and 25 wild rice strains. Protein was extracted from the individual ground seeds using 0.05M NaCl and 20% sucrose as an extraction buffer.

Salt-soluble proteins were separated into more than ten bands showing

variability among strains. At least three loci (tentatively named APAGE 1, 2 and 3) could be identified. Segregation of 1:1 ratio for APAGE 1 and 2, respectively, was confirmed in recombinant inbred lines of several crosses. Through linkage analysis with 140 RFLP and isozyme markers, these two protein loci were mapped on chromosome 5 and 11, respectively.

Four alleles were found at PAGE 1 locus. Allele 1 and 2 were frequently found in Japonica and Indica types of cultivars, respectively. Allele 3 was frequent in the perennial type of wild rice, though annual types harbored exclusively allele 2. So far, many gene markers were found to be diagnostic in classifying Indica and Japonica types. But, few markers are known useful to distinguish both wild and cultivated rice, as well as perennial and annual types of wild rice. A PAGE 1 could be a useful marker for the evolutionary study in rice. For details, see Ref.4.

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E-c. Division of Applied Genetics

(1) Composite and clinal distribution of *Glycine soja* in Japan revealed by RFLP analysis of mitochondrial DNA

Azumi TOZUKA[1], Hirohumi FUKUSHI[1], Toshiyuki HIRATA[1], Masashi OHARA[1], Akira KANAZAWA[1], Tetsuo MIKAMI[1], Jun ABE[1] and Yoshiya SHIMAMOTO[1] ([1]Faculty of Agriculture, Hokkaido University, Sapporo)

Wild soybean (*Glycine soja* Sieb. et Zucc.), regarded as the progenitor of cultivated soybean (*G. max* (L.) Merr.), is widely distributed in East Asia. We have collected 1097 *G. soja* plants from all over Japan and analyzed restriction fragment length polymorphism (RFLPs) of mitochondrial DNA (mtDNA). Based on the RFLPs detected by gel blot analysis using coxII and atp6 as probes, the collected plants were divided into 18 groups. Five mtDNA types accounted for 94% of the plants examined. The geographic distribution of mtDNA types revealed that wild soybeans grown in Japan consisted of a mixture of plants with different types of mtDNA in many regions, occasionally even within sites. Some of the mtDNA types showed marked geographic clines among the regions. Some wild soybeans possessed mtDNA types that were identical to those widely detected in cultivated soybeans. Our results suggest that the analysis of mtDNA could solve the maternal lineage among plants of the genus *Glycine* subgenus *Soja*. For details, see Ref.1.

(2) Genetic diversity, geographical differentiation and evolution in the cytoplasmic genome of the wild soybean, *Glycine soja*, growing in China

Yoshiya SHIMAMOTO[1], Hirohumi FUKUSHI[1], Jun ABE[1], Akira KANAZAWA[1], Junyi GAI[2], Zhong GAO[2] and Donghe XU[2] ([1]Faculty of Agriculture, Hokkaido University, Sapporo, [2]Soybean Research Institute, Nangjing Agriculture University, Nangjing)

RFLPs of chloroplast and mitochondrial DNA have been used to characterize the cytoplasmic genome of wild soybean, *Glycine soja*, growing in China. We have found from the RFLPs of chloroplast (cp) and mitochondria (mt) that

the cytoplasmic genome of most Chinese wild soybeans have a combination of cpIII with mt-a or a combination of cpII with mt-b accompanied by mtIV or mtV. CpII was not observed in combination with mt-a and cpIII was not observed in combination with mt-b. The regional distribution of these two types showed clines with opposite directions. The Yangtze River Valley had the greatest diversity in cp and two mt profiles detected in this study and the cytoplasmic genome combining these three profiles showed the highest degree of polymorphism in this region. The Yangtze River Valley may be a center of cytoplasmic diversity of wild soybean and may contain various genetic resources of soybean. For details, see Ref.2.

(3) Molecular Genetic Studies of Genomic Imprinting

Hiroyuki SASAKI (Institute of Genetic Information, Kyushu University)

Genomic or parental imprinting refers to a biological process that modifies the genome differently in the male and female germline, leading to differential activity of the parental genomes in the offspring. In order to understand the molecular mechanisms involved in imprinting, we study the imprinted gene cluster in the distal portion of mouse chromosome 7, in which the insulin 2 (*Ins2*), insulin-like growth factor 2 (*Igf2*) and *H19* are contained. The results obtained last year were as follows.

(1) We cloned and characterized a biallelically expressed gene, called *L23mrp*, which is located downstream of *H19*. Furthermore, we showed that *L23mrp* is functionally insulated from the *H19* enhancers, suggesting that there may be a domain boundary between *H19* and *L23mrp* (ref. 1). (2) We found a new feature common to many imprinted genes, i.e., the arrest-specific up-regulation in embryonic fibroblast cells in culture, which may give us a clue to understand the evolution of imprinting (ref. 2). (3) We identified and characterized multiple sense and antisense transcripts in the mouse *Igf2* region, which are also subject to genomic imprinting (ref. 3). (4) We showed that *Igf2*, which has three different promoters, is controlled mainly by a locus-wide mechanism (in press). (5) We have sequenced a 30-kb putative boundary region of the imprinted *Ins2/Igf2/H19* domain located between *H19* and *L23mrp* (in press). (6) A convenient method to detect and localize CpG islands in a large

genomic region has been developed (submitted). This technique should be useful in identifying new imprinted genes. (7) We have determined the timing of the erasure and establishment of the methylation imprint, which is present in the 5' flank of *H19*, in the parental germline (submitted). (8) To study the structure and function of the imprinted domain on distal chromosome 7, we constructed YAC, BAC and cosmid contigs covering the region and are now trying to close the final gaps. We also plan to sequence the entire region.

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F. GENETIC STRAINS RESEARCH CENTER

F-a. Mammalian Genetics Laboratory

(1) Analysis of the Genetic Structure of *Pb* Hotspot in the ClassII of MHC

Taku ISOBE[1], Masayasu YOSHINO[2], Kirsten FISHER LINDAHAL[2], Kenichi MIZUNO[3], Tsuyoshi KOIDE, Kazuo MORIWAKI[1], Toshihiko SHIROISHI ([1]Grad.Univ.Advanced.Studies, [2]Univ. Texas, [3]Inst.Phys.Che. Res.)

In mammals, the murine major histcompatibility complex (MHC) is a unique region where breakpoints of meiotic recombination have been systematically studied at molecular level. In this region, meiotic recombinations do not occur at random but are clustered in restricted regions known as hotspot. Thus far, four hotspots have been identified in this region. Among four, *Pb* hotspot has been poorly characterized. In the present study, we constructed a fine restriction map of the 15kbp of DNA fragment which contains the recombinational breakpoints. Molecular mapping revealed that five recombinants were confined to a 2.4 kb DNA segment located proximal to the 3'end of *Pb* gene. Comparing the molecular structure of the four hotspots including *Pb* hotspot in the mouse MHC class II, we found that all hotspots are located in introns or 3' end of genes, but not at 5' end of genes. This contrasts to hotspots identified in budding yeast, in which most of hotspots are located at 5' end of genes overlapping with transcription initiation sites. Furthermore, we could not find the consensus sequenses and motifs common to all four hotspots .

We have started analysis of higher chromatin structure around the hotspots in meiotic prophase, focusing on topological relation of the hotspots to structure of synaptonemal complex. We have established the protocol using the FISH method and immunostaining method together.

(2) Fine Mapping of a Preaxial Polydactyly Mutant, *Rim4*

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

In vertebrate limb development, the anteroposterior axis formation is mediated by a signal molecule, Sonic hedgehog. The localized expression of Sonic hedgehog at the posterior margin of developing limb (limb bud) is disrupted in several preaxial polydactyly mutants in mouse. *Rim4* is a preaxial polydactyly mutation which induces the ectopic Sonic hedgehog expression at the anterior margin of limb bud.

Toward positional cloning of *Rim4* gene, we have carried out the fine mapping of *Rim4* gene in crosses with two laboratory strains, DBA/2J or NZB. Because the penetrance of *Rim4* was incomplete, we used only the backcross progeny which showed polydactyly. As a result, *Rim4* was mapped to Chromosome 6 and between *D6Mit279* and *D6Mit320*. For the assignment of the genetic markers in this region, we conducted a linkage analysis based on the backcross panel using the intersubspecific cross; (C57BL10 X MSM)F1 x C57BL10. We mapped 13 microsatellite markers in the *Rim4* critical region by SSLP analysis using 1500 progeny. Analysis of YAC clones which contains these genetic markers revealed that gene members which belong *IgVk* familiy (*IgVk10*, *33*, *23* and *28*), *CD8* familiy (*CD8a* and *CD8b*), *IgJk* and *IgCk* located in this region.

(3) Genetic Analysis and Physical Mapping of Polysynductylous Mouse Hemimelic extra toes (*Hx*)

Tomoko SAGAI, Hiroshi MASUYA and Toshihiko SHIROISHI

Many limb mutants are known in the mouse. Some of them have abnormal three axes (proximo-distal, antero-posterior and dorso-ventral) formation governed by particular signaling centers. One of the mutants, hemimelic extra toes (*Hx*) shows preaxial polyductyly on all four feet, associated with hemimelia, shortening of the radius, tibia and talus. *Hx* is located on the proximal region of Chr 5 and is very closely linked to hammer-toe (*Hm*) mutation, which shows interdigital webbing regression probably due to an impair-

ment of apoptosis. Because sonic hedgehog (*shh*) gene that mediates antero-posterior signaling has been also mapped to this region and ectopic expression of *shh* has been detected in the limb bud of *Hx*, it has been proposed that *shh* is a candidate gene for *Hx*.

We have mated the several laboratory and wild inbred strains with *Hx* heterozygotes and observed the phenotypes of the progeny. The progeny from different mating crosses exhibited characteristic phenotypes. In particular, hemimelia that characterized the original mutant phenotype reproducibly disappeared in the progeny from some crosses. The results suggested a possibility that genetic background of the strains modified the phenotype of *Hx* mutation. We are trying to fix these phenotypes by continuous backcross to the corresponding strains.

The linkage analysis of *Hx* mutation using 1570 backcross progeny from intersubspecific cross have mapped the candidate gene to a 0.6 cM segment. One of the recombinants could separate the coding region of *shh* from *Hx* gene, and excluded the coding region of *shh* gene as a candidate for *Hx* gene. We assume that there is at least one or two genes in this region in addition to *shh*, which may be responsible for the antero-posterior specification and the apoptotic cell death. In human, a complex bilateral limb deformity (Nicolei-Hamel polysynductyly) has been mapped to the syntenic region of chromosome 7q36. For the positional cloning of *Hx* and *Hm* genes, we have established a YAC contig covering the *Hx* region. The result showed that *Hx* and *shh* genes are colocalized within a 1.5 Mb single YAC clone snd *Hx* gene is included in a 600 kb single YAC clone. The construction of BAC contig covering the 1.5 Mb DNA which includes *Hx* and *shh* genes is now under way.

(4) Mesenchymal Dysplasia (*mes*), which Exhibits Preaxial Polydactyly

Shigeru MAKINO[1], Hiroshi MASUYA, Mizuyo TSUGANE[2] and Toshihiko SHIROISHI ([1]Grad. Univ. Adv. Stud., [2]Sapporo Univ. Medic.)

Numerous preaxial polydactyly mutations in mouse are caused by duplication of zone of polarizing activity (ZPA), which is coupled with mirror-image duplication of digits. A recessive mutation, *mes*, shows preaxial polydactyly.

In addition, *mes* causes multiple skeletal anomalies, including a shortened and wider face with wide-set eyes, a domened head and kinky tail. Excess skin and increased musculature in the shoulders and hips are seen in the mutant.

We examined whether *mes* exhibits symmetrical digit duplication similar to other preaxial polydactyly. Toluidine blue and alizarin red staining indicated that *mes* has thickened feet and bifurcate metacarpal bones and metatarsal bones, but not mirror-image duplications of the skeletal pattern of the digits and truncated zeugopod like *Rim4*, which exhibits ectopic expression of *Shh* and *Fgf4* genes at the anterior margin of the limb. Furthermore, *mes* had abnormally shaped xiphisternum and bifurcate sternum. On the basis of these findings, it appeared that *mes* mutation alters normal growth and differentiation of mesenchyme-derived cells. Preaxial polydactyly may not be due to abnormally axial pattern formation .

To determine the chromosomal location of *mes*, we carried out linkage analysis by cross with MSM strain. As a result of 79 backcross progeny, *mes* was mapped between *D13Mit42* and *D13Mit210*. This region includes six potential candidate genes for *mes*. Three candidates, *Ptc*, *Pitxl* and *mdac*, play a role in positional specification during the pattern formation. As *mes* may be a mutation in a gene involved in early mesenchyme formation or growth, the other three, *Mad3*, *Gasl* and *Fgfr4*, which play a role in cell growth or differentiation, are better candidates.

(5) Effect of *Ts* Mutation on Mouse Development

Junko ISHIJIMA, Akihiko MITA, Kikue UCHIDA, Toshihiko SHIROISHI

Mice with the Tail-short (*Ts*) mutation have a shortened, kinky tail and numerous skeletal abnormalities, including a homeotic anteroposterior patterning problem involving the axial skeleton. The phenotype is variable on different genetic backgrounds as is seen in many other mouse dysmorphogenic mutants. The viability of *Ts* heterozygotes varies dramatically, depending on the mouse strain crossed with the mutant strain. At the extremes, the heterozygotes are viable or lethal prenatally. Detailed linkage analysis indicated that a single chromosomal region, genetically inseparable from the *Ts*

locus, is responsible for the difference. This result suggests that there exists polymorphism at *Ts* locus among inbred strains, and the allelism affects the viability of the *Ts* heterozygotes.

The different manifestations of the phenotype of *Ts* heterozygotes between these two crosses was useful to understand the function of the *Ts* gene in different stages of mouse embryogenesis. We investigated the phenotype of the *Ts* mutation in crosses with the two groups of strains, which give viable and lethal *Ts* embryos respectively. Morphological and histological analyse indicated that *Ts* embryos from the lethal cross exhibit more severe developmental defects. In neural plate stage, the embryonic region was poorly developed compared with the extraembryonic region due to formation of excess mesoderm in the extraembryonic region. The inappropriate allocation of the mesodermal cells at the early gastrulation stage may cause shortage of progenitors of the embryonic mesodermal cell in the primitive streak of *Ts* heterozygotes. In the later developmental stage, several lines of evidence for the disturbance in establishment of the segmental identity were observed in *Ts* heterozygous embryos, which is associated with alternations of expression pattern of marker genes. Among neural tube defects, malformation of the hindbrain was common phenotype of *Ts* heterozygotes. The expression boundary of some genes which thought to be important for maintaining the segmental identity of hindbrain was altered. Additionally, in the viable cross *Ts* heterozygous mice showed several homeotic transformations in the axial skeletal formation with high reproducibility. Most characteristic two phenotypes were posterior transformation of the cervical vertebra to the thoracic vertebra, and an additional vertebra in the upper thoracic region with anterior transformation of all the prevertebrae caudal to the additional vertebra. We hypothesize that *Ts* gene plays a crucial role in establishment of mouse axial body plan from early gastrulation stage through to early fetal stage. Further study to connect the complex mutant phenotypes observed beyond different developmental stages would certainly provide a clue to clarify the functions of *Ts* gene.

(6) Positional Cloning of the Mouse Skeletal Mutation, *Tail short* (*Ts*)

Kunihiko SHIMIZU[1], Tuyoshi KOIDE, Akihiko MITA, Kikue UCHIDA, Shigeharu WAKANA[2], Yoshiaki KIKKAWA[3], Hiromichi YONEKAWA[3], Hiroki SASAKI[4] and Toshihiko SHIROISHI

([1]Department of Pediatric Dentistry, Nihon University School of Dentistry at Matsudo, [2]Central Institute for Experimental Animals, [3]Department of Laboratory Experimental Animal Science, The Tokyo Metropolitan Institute for Medical Science, [4]Genetics Division, National Cancer Reserch Insutitute)

A mouse mutation Tail-short (*Ts*) exhibits shortened kinky tail and numerous skeletal abnormalities including homeotic anteroposterior patterning problem along the axial skeleton. *Ts* gene was previously mapped to the teromeric region of chromosome 11. *Ts* is likely to be a mouse model for a human skeletal dysmorphology known as Meckel syndrome (MES;OMIM2400), since MES has phenotype similar to *Ts* and has been mapped to human syntenic region, 17q21-24.

To elucidate the function of the *Ts* gene in mouse embryogenesis and verify whether it is a model for MES, we are trying to clone the gene by using the method of positional cloning. First, we employed a fine mapping of this gene based on a large scale intersubspecific backcross between the mutant stock TsJ/Le-*Ts*/+ and Japanese wild mouse-derived MSM strains. *Ts* gene was mapped to a 0.16cM region between two microsatellite markers, *D11Mit128* and *D11Mit256*. We screened mouse YAC and BAC libraries with the microsatellite markers tightly linked to the *Ts* locus and have obtained YAC and BAC clones. Further chromosome walking with the isolated clones allowed us to construct a complete BAC contig covering the *Ts* causative gene. This contig consists of 6 BAC clones, which spans a 250-300 kb DNA fragment. We have isolated several cDNA clones from the critical region by directed cDNA selection using the corresponding BAC clones to search for candidate genes for *Ts*. Characterization of these cDNA clones is now underway to identify the *Ts* causative gene.

(7) A susceptibility gene *Idd4* controls onset of IDDM : an allele from non- diabetic MSM strain is associated with early onset of diabetes in mice

Shigeharu WAKANA[1], Toshihiko SHIROISHI[2], Kazuo MORIWAKI[3], Tatsuji NOMURA[1] ([1]Central Institute for Experimental Animals, [2]National Institute of Genetics, [3]The Graduate Univ. for Advanced Studies)

The NOD mouse is a widely used model of human insulin-dependent diabetes mellitus (IDDM). Because genetic loci responsible for IDDM are as many as eightteen, the NOD mouse is also used as a model for studying genetic complex traits. The susceptibility loci have been identified by linkage analysis in crosses between the NOD mouse and other non-diabetic mouse strains. We have studied the inheritance of the susceptibility to IDDM by genetic cross between NOD/Shi and a Japanese wild mouse-derived MSM strain. Three loci *Idd1*, *Idd3* and *Idd10* appeared to control the incidence of insulitis. However, the contributions of other *Idd* loci including *Idd4* were not detected in the above cross. To investigate the effect of MSM allele of *Idd4* without influence of the other susceptibility genes, we have established the congenic strain for *Idd4* by introducing the chromosomal segment containing *Idd4* locus from MSM strain into the NOD genetic background. The incidence of diabetes and insulitis in the NOD/Shi. *Idd4*$^{nod/msm}$ congenic mice was not lower than that in NOD mice. On the other hand, the incidence of diabetes at early onset (~20wks) in the female NOD/Shi. *Idd4*$^{msm/msm}$ mice was rather higher than that in NOD mice or the NOD/Shi. *Idd4*$^{nod/msm}$ congenic mice. This result indicated that an allele from non-diabetic MSM strain is associated with early onset of diabetes in mice. For fine mapping the *Idd4*, we have produced a series of six recombinant strains which carry various segment from MSM chromosome in the congenic region containing *Idd4*. Comparing with the incidence of early onset diabetes among the congenic strains, we mapped I*dd4* within a short segment approximately less than 0.8cM on chromosome 11.

(8) Characterization of Hyperactive Behavior in MSM Derived from Japanese Wild Mouse, and Hypoactive Behavior in JF1 Established from Japanese Fancy Mouse

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

Behavior of mouse has been studied from many aspects using only small numbers of laboratory strains. However, those laboratory mice have been domesticated in the course of establishing them as inbred strains, and hence their characteristic behavior has been lost by the domestication process. On the other hand, wild mice behave differently from the laboratory mice. This implies that laboratory mice that are used in many fields of brain research have accumulated spontaneous mutations in genes which control their behavior. In this point of view, use of inbred strains derived from wild mice will be more important in future brain research and behavioral studies. We have started a new project in that behavioral patterns of inbred strains established from wild mice and laboratory strains is compared in many aspects of mouse behavior, and the difference between the strains is genetically dissected.

An inbred strain JF1 has been established from Japanese fancy mice which was highly domesticated and obedient. On the other hand, another inbred strain MSM which was established from Japanese wild mouse is still very active. To quantify the general activity in these two mouse strains, locomotor activity of each mouse strain was measured for continuous four days in the cycle of 12 hours light and 12 hours dark. Both of JF1 and MSM strains are active in the dark, but the overall activity of MSM is extremely higher than that of JF1. This difference in the locomotor activity is studied by genetical analysis. We have inter-crossed (JF1 and MSM)F1 progeny, and resultant F2 progeny were tested for the locomotor activity. Based on the linkage analysis of the progeny, we intend to map the loci that control the difference in locomotor activity between JF1 and MSM strains.

(9) Construction of Mouse Mutant Bank by CHL Mutagenesis

Toshihiko SHIROISHI, Tatsuya FUKUDA[1], Kikue UCHIDA, Miyoko MASUYA and Akira MITA ([1]Chugai Pharmaceutical Co.)

Chemotherapic agent chlorambucil (CHL) is highly mutagenic in male germ cells of the mouse. Post meiotic germ cells especially early spermatids are the most sensitive to the mutagenic effects of this agent. It is established that CHL induces predominantly chromosomal deletions. The optimum rate of mutation is estimated to be 10^{-3} per locus per gamete, when progeny sired from males treated with CHL is screened. This implies that we can expect to obtain a mutation at a given locus by screening 1, 000 progeny at about 80% probability.

We have started a project to construct mouse mutant bank by CHL mutagenesis. In this project, males of MSM established from Japanese wild mouse, *Mus musculus molossinus*, are treated with CHL and crossed with females of strain C3H/HeJ. The F1 progeny generated from the above cross are expected to have random chromosomal deletions over MSM genome. Since MSM and C3H/HeJ strains are genetically very remote, the polymorphism between these two strains allows to detect deletions of MSM alleles at the most loci. We have confirmed that ordinary nusive agarose gel electrophoresis is useful to distingush alleles of MSM from that of C3H/HeJ by RFLP analysis for PCR-amplified fragments of 3' non-coding region of the most genes. We have also tested whether it is possible to store sperms of F1 hybrids generated from cross of (C3H/HeJ x MSM) in liquid nitrogen and to use for *in vitro* fertilization to produce mice with deletion mutations. It appeared that the frozen sperms of the F1 hybrids were preserved and used for *in vitro* fertilization without loss of capability of fertilization.

Currently, we are expanding the F1 progeny sired from mutagenized males of MSM strain toward construction of the sperm bank with saturated mutations. We also intend to develop the system to detect chromosomal deletions by genome scanning based on two dimensional acrylamide gel electrophoresis.

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F-b. Mammalian Development Laboratory

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

Norio NAKATSUJI, Yasuaki SHIRAYOSHI and Tetsuichiro SAITO

We have been studying proliferation and growth regulation of mouse primordial germ cells (PGCs) during migratory stages (7.5 - 10.5 days post coitum) by using a culture system. They show a temporary proliferation in culture, but their growth is arrested at a time corresponding to 12.5 -13.5 dpc when they differentiate into gonia after arriving at fetal gonads. We tried a few methods for gene transfection of mouse primordial germ cells (PGCs) in culture as a prelude to the investigation of molecular mechanisms of germ cell development. Using the CaPO4 co-precipitation method, 18 % of PGCs transfected with plasmid *pSV-LT* expressed simian virus 40 large tumor antigen (*SV 40 T-Ag*) transiently. However, we did not detect any effects on the proliferation and survival of PGCs obtained from embryonic gonads at 11.5 dpc during 2 days of culture after transfection. PGCs isolated from 11.5 dpc gonads change from spread- to round-shape and exhibit growth arrest during a few days of culture, and these rounded PGCs quickly disappear from the culture. We found that the transfection and expression of *Bcl-Xl* or adenovirus type 2 E1B 19,000-molecular-weight protein (*E1B 19K*) significantly pro-

moted the survival of PGCs and retarded the disappearance of rounded PGCs from the culture system. These results suggest that *Bcl-XL* or *E1B 19K* can prevent the apoptosis of PGCs and inhibit cell death of rounded PGCs in culture. For details, see Ref. 1.

To extend our studies on PGCs, we are carrying out two lines of experiments. Firstly, we are developing a co-culture system of germ cells and gonadal somatic cells to investigate cell interaction during the entry into meiosis or mitotic arrest by germ cells. Secondly, we have been trying to identify novel genes involved in sex-differentiation of germ cells and gonads at these stages. Sex-differentiation of mouse fetal gonads starts by appearance of testis cords in testis at 12.5 dpc, followed by mitotic arrest of germ cells in testis and initiation of meiosis in ovary starting at 13.5 dpc. It has been known that male somatic cells direct germ cells into the spermatogenic differentiation in the testis. Such initial sex-differentiation is proceeded by expression of *Sry* gene in precursors of sertoli cells of male gonads at around 11.5 dpc. Since the identification of *Sry*, a transcription factor, as the testis-determining gene, vigorous attempts have been made to discover the following cascade of gene groups which should be in play to bring about differentiation of the male germ cells and testicular somatic tissues, but with only a limited success. Previous studies have also identified several genes important for sex differentiation in mammals, including *SF-1/Ad4BP*, *WT-1*, *Sox 9* and *Dax1*. However, a number of other unknown genes should be involved in sex differentiation of gonads. We have been trying to identify such genes in mice by using the subtraction and differential hybridization method to obtain genes whose expression is specific for the 13.5 dpc testis after subtraction by the female gonad cDNA.

Thirty-two clones were analyzed, and 20 were found to be known male-gonad-specific genes, including *Mullerian inhibiting substance* and *Desert hedgehog*. Remaining 12 clones represent novel sequences. We chose a novel gene containing typical basic helix-loop-helix domains for further investigation. Its cDNA size was 1,237 bp and it contained a protein coding region of 540 bp that presumably codes 179 amino acids. We named it *nephgonadin* because its strong expression was observed in the kidney and gonad. At 13.5 dpc, expression of *nephgonadin* was stronger in the testis

than ovary. In adults, however, the expression level was decreased in the testis, while it was increased in the ovary. We are now examining functions of *nephgonadin* in gonad development.

(2) Differentiation and Migration of Neural Cells in Mouse Central Nervous System Development

Tetsuichiro SAITO and Norio NAKATSUJI

The mammalian central nervous system comprises an enormous number of cell types that develop from the neuroepithelium. We have been trying several approaches at the both cellular and molecular levels to understand how the different cell types are generated. Members of the family of basic-helix-loop-helix (bHLH) transcription factors, such as *Mash1* and *Neurogenin*, have been established to play important roles in mammalian neurogenesis. We have focused on the regulatory cascades, i.e. upstream and downstream genes, of the neural bHLH genes. *PHD1*, a member of the Paired-like homeodomain (PHD) family is expressed downstream of *Mash1* during the differentiation of both dorsal spinal cord and olfactory sensory neurons. In contrast, other PHD genes, such as *Phox2* and *DRG11*, are expressed downstream of the bHLH genes, in different lineages of neurons, suggesting that the cascade from bHLH to PHD transcription factors may be important for neuronal identity determination. Moreover, *unc-4*, which is most closely-related gene to *PHD1*, has been shown to determine the identity of a motorneuron in C. elegans. These suggest that the cascade during neurogenesis is conserved even among species.

Another homeobox gene, *MBH1* (mammalian *BarH* homologue), is expressed in an exactly complementary pattern to *Mash1* and overlapping with *Neurogenin2* in the developing nervous system. Forced expression of *MBH1* down-regulates *Mash1* expression and up-regulates *Neurogenin2* in differentiating P19 cells, suggesting that *MBH1* is a regulator of the neural bHLH genes. In order to clarify the relation between *MBH1* and *bHLH* genes and between bHLH and PHD genes, we are trying to identify downstream target genes of *MBH1* and the bHLH genes.

We also established immortalized cell lines from early neuroepithelium

through isolation of the forebrain region at E8.5 or E9.5 from mouse embryos harboring transgene tsA58, the temperature sensitive *SV40 T-antigen* gene (*T-Ag*) driven by the *H2-Kb* promoter. NES8 lines obtained from such embryos at E8.5 expressed marker proteins of the neural precursor cells, but they could not be induced to differentiate into neurons or glia in various culture conditions. Half of the NES9 lines established from embryos at E9.5 were similar to the NES8 lines, but the remaining half expressed neuronal or glial differentiation marker proteins dependent on culture conditions. These results suggest that the early neuroepithelium at E8.5 is made of homogeneous cell population of the early stem cells, and there is a beginning of cell determination and differentiation at E9.5 producing various types of multipotent stem cells and neuronal progenitor cells. Also, in collaboration with Dr. I. Nagata at Tokyo Metropolitan Institute for Neuroscience and Dr. K. Ono at Shimane Medical College, we studied migration patterns of granule cells in development of mouse cerebellum. For details, see Ref. 3.

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

Yasuaki SHIRAYOSHI and Norio NAKATSUJI

We cloned one *Notch* related gene "*int-3*" from the 8.5 day cDNA library. The entire structure of the *int-3* gene resembles that of *Notch* or vertebrate *Notch* homologues, and the *int-3* gene showed 50-60% identity to the related *Notch* homologues in its amino acid sequence. We consider *int-3* to be one of the mouse homologues of *Notch*. RT-PCR and in situ hybridization analysis revealed that *int-3* was expressed in blood vessels in 9.5-10.5 day old embryos. The expression pattern of *int-3* coincided with the expression of receptor tyrosine kinase *Flk-1*, which is regarded as a major regulator of vasculogenesis and angiogenesis. These results suggested that *int-3* might be involved in the formation of blood vessels in early mouse embryos. For details, see Ref. 2.

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F-c. Plant Genetics Laboratory

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

Yukihiro ITO, Mitsugu EIGUCHI, Nori KURATA

To elucidate the gene expression network essential for early embryogenesis in rice, we conducted cloning and structural and functional analyses of transcription activating factor genes specifically expressed in very early embryos. By analysing many genes having roles in the gene expression cascade essential for early embryogenesis, we aim to clarify genetic program of embryogenesis in the monocot plant. We constructed cDNA libraries of embryos at 1 and 3 days after pollination (DAP) and selected 34 cDNA clones probed by homeo-domain-containing PCR fragments isolated originally from the 3 DAP embryo. These clones were categorized into 9 kinds of clones. In addition, we cloned another cDNA and genomic sequences carrying homeobox using related genes of other plants. To detect the expression specificity of these genes, RT-PCR and *in situ* hybridization analyses were carried out using fixed-cDNA libraries and embryo sections of 0 to 6 DAP embryos. Four of these HOS (Homeobox gene in *Oryza sativa*) clones out of 9 showed stage and region specific expression patterns in the embryo. The results showed that HOS 24 expresses on the shoot/radicle primordium at 3~4 DAP and HOS 13

expresses in the restricted regions of early embryo at 2-3 DAP. These genes might have important roles in early rice embryogenesis. To know the functional properties of these genes, we constructed constitutive expression plasmid of genes and introduced into rice cells to generate transformed rice plants. Analysis of the resulting transformants are now in progress. For detecting the role and situation of these genes in the genetic program of embryogenesis, we cloned genomic regions of some of these genes and sequenced. To restrict the control regions for gene expression situating upstream of the genes, plasmids connecting a reporter gene under the upstream region of the genes were constructed and transformed rice calli with them. These experiments would reveal the cis-elements and trans-acting nuclear factors expressed just before the genes we examine. We would like to find out a nember of genes essentially included in the gene expression cascade in early embryogenesis of rice, through the detection of such cis- and trans-factors at successive multiple steps.

(2) Molecular Analysis of the Developmental Program during Regeneration Process of Rice Plant from Somatic Embryo

Nori KURATA and Mitsugu EIGUCHI

We have started this project to analyse genetic program in somatic embryogenesis and compare it to that of seed embryogenesis. Are the programs in two embryogenetic process almost same or different in several steps ? To see this we are performing in situ hybridization and RT-PCR analysis also using regenerating rice somatic callus. A part of genes specially expressed in the seed embryogenesis apeared to be transcribed in a simmilar manner in the regenerating callus.

In this project, we are also trying to bring on plantlet regeneration from the callus of various *odm* (organ differentiation mutant) plants for exploring possible alternative pathways used in the regenaration process of somatic embryogenesis.

The rice homologue with the very useful cell marker gene expressed specifically in regenerating cells of carrot has been also isolated and analysed whether it will be useful for cell fate analysis in rice.

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

Yoshiaki HARUSHIMA and Nori KURATA

Site specific reduced gene transmission, which is called segregation distortion, has been detected in several chromosomal regions in F2 progenies of crosses between *japonica* and *indica* rices. Intensive analysis of three different F2 progenies using high-density DNA markers revealed many distorters with different strength and combinational expression. Among those causative genes (or factors) for the segregation distortion, the most seviere distorter designated *ga2* was mapped on chromosome 3. For positional cloning of the *ga2*, fine mapping of the gene was done. Flanking DNA markers very near the locus were detected and physical mapping will be continued in 1998.

(4) Isolation and Characterization of Rice Centromeric DNA Sequences for the Construction of Rice Artificial Chromosome

Kenichi NONOMURA and Nori KURATA

This study focused on unravelling the mechanisms of spacial and functional organization of chromosomes in the nucleus. For this purpose, we decided to construct plant artificial chromosome with rice as an efficient tool for analysing structural and functional components and organization of chromosome/nucleus. The cereal centromeric sequence (CCS1) conserved in some *Gramineae* species contains a 17-bp motif similar to the CENP-B box, which is the binding site for centromere specific protein CENP-B in humans. To isolate as many divergent centromeric units of rice (*Oryza sativa* L.) as possible, we performed the polymerase chain reaction (PCR) using the CENP-B box-like sequence (CBLS) as primers. A 264-bp clone, called RCS1516, containing this fragment appeared to be a member of the CCS1 family, sharing about 60% identity with the nucleotide sequences of other cereals. Several genomic clones carrying multiple copies of CBLS were isolated. A 14-kb long genomic clone λRCB11 was identified by screening with RCS1516 probe. Characterization and nucleotide sequencing of a 14 kb clone was carried out. It was found that the clone contained 3 copies of a long direct repeat RCE1 with 5.1- and 1.7-kb

intervening sequences. The 1.9-kb RCE1 repeat unit consists of three distinct parts: a variable, a semi-variable, and a conserved region. Fluorescent *in situ* hybridization (FISH) analysis revealed that RCE1 repeat units are located in the centromeric heterochromatin of rice chromosomes. Together with further efforts for isolating a longer DNA fragments containing RCE1 repeat suitable for centromere function, several other candidate clones containing other type of centromere repeat units are successively being screened and characterized.

(5) Generation of Enhancer Trap Lines in Rice

Yukihiro ITO, Mitsugu EIGUCHI and Nori KURATA

To establish and to screen a lot of mutants affecting the steps of early embryogenesis and of chromosome organization in rice nuclei, we are generating enhancer trap lines for most rice genes. For efficient making of trap lines, we decided to use an Ac/Ds transposon system of maize, GUS or GFP genes as reporters and the T-DNA mediated gene transfer system of *Agrobacterium*. We chose useful constructs already used in the Arabidopsis gene/enhancer trapping system and replaced some components with fragments suitable for rice transformation system. We reconstructed the Ds vectors fitting for rice transformation and transformed rice callus with either the vector carrying Ac-transposase or that carrying Ds + GUS gene. We have already obtained above 100 transformants of Ds-GUS constructs in this year. Transformants are selected for low copy number of the Ds-GUS sequence and will be located the integrated sites by mapping of each flanking sequence of the integration. We will conduct to cross these Ds-GUS transformed plants and Ac transformants to obtain a lot of Ds-GUS transposed enhancer trapped progenies.

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F-d. Microbial Genetics Laboratory

(1) Diadenosine 5',5'''-P1,P4-tetraphosphate (Ap4A) controls the timing of cell division in *Escherichia coli*

Akiko NISHIMURA

The timing of the cell division in *Escherichia coli* is highly regulated, but the mechanism has not been identified. We found previously that *cfcA1* 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 of cfcA11, a derivative of cfcA1, and another cfc mutant, cfcB1.

The cfc mutants divided prior to the ordinary stage of cell division, and produced many small cells with nucleoid. However, the cells grew exponentially, and the length of a cell cycle and the initiation mass for chromosome replication were not altered by the cfc mutations. These properties of the mutants resulted from reduction of the period between the nucleoid division and the cell division in a cell cycle and compensatory elongation of the period between the cell division and the initiation of the next round of DNA replication. CfcA11 has a mutation in *glySa* which encodes the α-subunit of glycyl-tRNA synthetase, and cfcB1 has an IS2 insertion in *apaH* which encodes Ap4A hydrolase. The cfc properties of both cfc mutants were suppressed by a multicopy plasmid carrying *apaH*$^+$, and the intracellular level of Ap4A in cfcA1 was fifteen times and cfcB1 was one-hundred times 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.

Ap4A is a signal for induction of cell division. High level of Ap4A is responsible for the initiation of cell division. The *glyS* mutation allows efficient synthesis of Ap4A. For details, see Ref. 1 and 3.

(2) *ftsE*^{ts} affects translocation of K^+-pump proteins into the cytoplasmic membrane of *Escherichia coli*

Hideki UKAI, Hiroshi MATSUZAWA[1], Koreaki ITO[2], Mamoru YAMADA[3] and Akiko NISHIMURA ([1]Department of Biotechnology, The University of Tokyo, [2]Institute for Virus Research, Kyoto University, [3]Faculty of Agriculture, Yamaguchi University)

The *ftsE*^{ts} mutation of *Escherichia coli* causes defects in cell division and cell growth. We expressed alkaline phosphatase (PhoA) fusion proteins of KdpA, Kup, and TrkH, all of which proved functional in vivo as K^+ ion pumps, in the mutant cells. During growth at 41℃, these proteins were progressively lost from the membrane fraction. The reduction in the abundance of these proteins inversely correlated with cell growth, but the preformed proteins in the membrane were stable at 41℃, indicating that the molecules synthesized at the permissive temperature were diluted in a growth-dependent manner at a high temperature. Pulse-chase experiments showed that KdpA-PhoA was synthesized, but the synthesized protein did not translocate into the membrane of the ftsE^{ts} cells at 41℃ and degraded very rapidly. The loss of KdpA-PhoA from the membrane fractions of *ftsE*^{ts} cells was suppressed by a multicopy plasmid carrying the *ftsE*$^{+}$ gene. While cell growth stopped when the abundance of these proteins decreased 15-fold, the addition of high concentration of K^+ ions specifically alleviated the growth defect of ftsE^{ts} cells but not cell division, and the cells elongated more than 100-fold. We conclude that one of the causes of growth cessation in the ftsE^{ts} mutants is a defect in the translocation of K^+-pump proteins into the cytoplasmic membrane. For details, see Ref. 2.

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F-e. Invertebrate Genetics Laboratory

(1) Leg pattern formation: specification in the embryo and proximo-distal pattern formation in the larva

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

Two thoracic limbs of *Drosophila* , the leg and the wing, originate from a common cluster of cells including the sources of two secreted signaling molecules Decapentaplegic and Wingless. Wingless induces Distal-less expression which marks the limb primordium. We showed that graded activity of Decapentaplegic expressed in the dorsal side of the limb primordium is responsible for the specification of the proximal leg and wing cell identity. Development of distal leg cells requires Wingless, but does not require detectable contribution of Decapentaplegic, and thus appears to be the default state. These inductive events, together with cell migration appear to be responsible for the formation of the leg disc that is composed of the proximal and the distal domains, and the wing disc. For detail, see ref. 1.

Specification of the leg cells requires an additional signal involving the *Drosophila* EGF receptor DER that is transiently activated within the limb primordium. In the absence of DER activity, proximal leg cells lose their specific marker gene expression and the morphology of the leg disc is disrupted. Thus a combinatorial action of DER and Decapentaplegic is important in the patterning of the leg.

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

During the course of this work, we developed a method to fluorescently detect a protein and a mRNA in whole-mount embryos. We showed that this method provides a single cellular resolution useful to analyze the dynamics of gene expression and cellular movement during induction of the imaginal disc. For detail, see ref. 3.

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

Miho TANAKA-MATAKATSU, Tomoatsu IKEYA and Shigeo HAYASHI

Coordination of cell motility and adhesion is essential for concerted movement of tissues during animal morphogenesis. The *Drosophila* tracheal network is formed by branching, migration and fusion of tubular ectodermal epithelia. The tracheal tip cell is located at the end of each branch that are going to fuse. We have previously described the details of changes in cell shape and cell surface properties in tip cells during branch fusion (Tanaka-Matakatsu et al., Development 122: 3697-3705, 1996). To further investigate the cellular mechanism that drives these processes, we are studying the changes in cytoskeltal structures and its correlation between distribution of cell surface and secreted molecules. The study revealed that reorganization of microtubular array prior to branch fusion appears to play a central role in the dynamic change of apical-basal cell polarity in the tip cell.

Only a single tip cell differentiates in each branch of the trachea. To understand how cell-cell communication is involved in this singling out process, we analyzed the function of the transmembrane receptor Notch in the trachea. We found that Notch is required for selecting the correct number of the tip cell.

(3) Cdc2 dependent checkpoint couples M phase completion to initiation of S phase

Shigeo HAYASHI

Coupling of M phase completion to initiation of S phase is essential for the maintenance of ploidy during cell proliferation. I have previously shown that the essential M phase regulator Cdc2 kinase is also required for inhibition of

S phase prior to the completion of M phase. To further investigate the mechanism of Cdc2 dependent inhibition of S phase, I am using a genetic and biochemical approach to test a requirement for the kinase activity of Cdc2 in this process.

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

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

Veins on the adult wing are a good landmark for the anteroposterior and dorsoventral positional informations. It also provides a stage where the EGF receptor, Decapentaplegic and Notch / Delta signaling pathways interact. The gene *plexus* is required for the supression of extra veins in specific intervein positions (between vein two and three, and between vein four and five). We have cloned the *plexus* gene and determined the sequence of one of its transcript. Further molecular and genetical analysis of the gene is under way.

(5) An enhancer trap screen for genes involved in pattern formation

Satoshi GOTO, Hiroko TAKEUCHI, Misako TANIGUCHI and Shigeo HAYASHI

To identify novel genes and gene functions in the pattern formation of imaginal disc and the trachea, we are conducting an enhancer trap screen using the Gal4-UAS system. About 500 lines were established and were examined for the activity of enhancers flanking the inserts in the embryo, larva and adult. In collaboration with other groups in Japan, we will continue to screen more lines.

(6) Determination of the dorsoventral domains of neurogenesis by the *Drosophila* EGF receptor

Yoshimasa YAGI[1] and Shigeo HAYASHI ([1]Bio-oriented Technology Research Advancement Institution and School of Pharmaceutical Science, University of Tokyo)

Primary neurogenesis in the central nervous system of insects and vertebrates occurs in three dorsoventral domains in each side of the neuroectoderm. We have previously shown that the EGF receptor homologue (DER) plays a cruicial role in the dorsoventral subdivision of neuroectoderm by repressing the expression of the zinc-finger transcription factor Escargot (ref. 2). We further extended this study to show that DER also represses proneural genes *achaete* and *scute*, and is required for the formation of the neuroblasts in the intermediate column. The activity of DER appears to be mediated by MAP kinase.

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G. CENTER FOR GENETIC RESOURCE INFORMATION

G-a. Genetic Informatics Laboratory

(1) Cross-species functional gene database

Yukiko YAMAZAKI

The goal of this project is full cross referencing among different species. Last year an integrated database of wheat and rice genetic resource information was experimentally developed. The construction of a cross-species functional gene database started this year.

The cross-species functional gene database on trial compiled genetic information of four different organisms, that is, rice, wheat, drosophila and mouse. Three different data sources; (1)DNA sequence database, (2) genetic resource database which we have been working on and (3) gene symbol dictionary provided by the genome group, were used for the database construction. The genetic information was directly extracted from the DNA sequence database and from the genetic resource database. Gathering these information indexed with accession number, a Gene-Table was created. The table consists of organism-name, strain-name, DNA-accession-number and gene-name. The gene-name of the current Gene-Table includes gene symbol, gene name and gene product name. Redundancy was eliminated from the Gene-Table manually according to the description of gene symbol dictionary. Although the lack of controlled vocabulary in the database makes the task difficult to accomplish, the database will be useful as the raw material for a wide range of biological sciences including comparative genomics study in the future.

(2) Genetic Resources Database

2-1. Wheat Genetic Resources Database -KOMUGI

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

The KOMUGI database working group has started preparation of the second version of the database. While reducing the redundancy of current database, 1400 image files have been added to the database and the DNA repository data have also joined KOMUGI in collaboration with Dr. Ogiwara (Kihara Institute for Biological Research). The current DNA repository database comprises 240 accessions including DNA and cDNA clones which will be commonly usable for Triticeae groups.

2-2. Rice Genetic Resources in Japan

Yukiko YAMAZAKI, Mari SAITO, Toshiro KINOSHITA[1] and Keiko MORISHIMA
([1]Hokkaido University)

A hardcopy catalogue, Rice Genetic Resources in Japan-1997, 703 pages, has been published and distributed to over 220 sites in the world. The catalogue includes not only genetic resource information but also basic biological knowledge such as anatomical data, gene designations, a morphological linkage map and the citations. The information is also available through the internet.

Dr. Nori Kurata (National Institute of Genetics) has started establishing the networking group of rice researchers in Japan for the new approach of rice bioinformatics including rice genomic information.

2-3. Barley Germplasm Database

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

A barley germplasm database was constructed in cooperation with Okayama University, a major stock center for barley strains in Japan. The database lists about 5000 strains and consists of 32 items including agro-morphologi-

cal, physiological, pathological and genetic traits. The database is still for internal use only but it soon will be available through the internet.

2-4. Mouse Microsatellite Database

Toshihiko SHIROISHI, Rie TSUCHIYA and Yukiko YAMAZAKI

The mouse microsatellite database in Japan (MMDBJ) has been constructed and is now on-line accessible. The database provides SSLP (simple sequence length polymorphism) information among different strains with PCR condition. The current database has ca.1000 entries and an on-line direct data submission system has been developed so that researchers can deposit their own data through the network.

2-5. Experimental Animal Database

Rie TSUCHIYA, Yukiko YAMAZAKI and Tetsuo ICHIKAWA[1]
([1]Japan Experimental Animal Cooperative)

The database comprises about 1500 experimental animal strains maintained by 51 Japanese breeders and 1 institution. The current database only provides the strain name and address to obtain the strain. The next plan is to collect as much information relevant to the strain as possible.

2-6. Cloning Vector Database

Seiichi YASUDA, Mari SAITO and Yukiko YAMAZAKI

An on-line data management system ,through which the clone maintainer can update his own data by connecting to the remote computer running the databases, has been developed. The system is composed of three parts, (1) data transfer mechanism, (2) data checking mechanism, and (3) tools for creating the Web pages.

Publications

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H. STRUCTURAL BIOLOGY CENTER

H-a. Biological Macromolecules Laboratory

(1) Single Molecule Imaging of Enzymatic Reactions Using Objective-type Evanescent Illumination

Makio TOKUNAGA, Kiwamu SAITO[1], Kazuo KITAMURA[2], Atsuko IWANE[2] and Toshio YANAGIDA[2] ([1]Department of Physics, Kanazawa University, [2]Department of Physiology I, Osaka University Medical School)

Imaging of single fluorescent molecules has been achieved in a relatively simple manner using objective-type total internal reflection fluorescence microscopy (TIRFM). This method allowed visualization of single molecules under scanning probe microscopes. Taking advantage of the technique of single molecule imaging, individual ATP turnovers have been visualized with a fluorescent ATP analogue, Cy3-ATP, using a simple experimental strategy. Clear on/off signals were obtained that correspond to the association and dissociation of single Cy3-ATP/ADP molecules with a single myosin head molecule. This method will allow a variety of single-molecular assays of biomolecular functions to be performed using fluorescently labeled substrates, ligands, messengers and so on. For details, see Ref. 4.

(2) Intermolecular Interactions measured by "Subpiconewton Intermolecular Force Microscopy"

Takaaki AOKI[1], Michio HIROSHIMA[2], Toshio YANAGIDA[1] and Makio TOKUNAGA ([1]Department of Physiology I, Osaka University Medical School, [2]Department of Biophysical Engineering, Osaka University)

Force sensitivity of scanning probe force microscopy was increased by incorporating a cantilever with very low stiffness, 0.1pN/nm, which is over 1000-fold more flexible than is typically used in conventional atomic force microscopy. Thermal bending motions of the cantilever were reduced to less

than 1 nm by exerting feed-back positioning with laser radiation pressure. The system was tested by measuring electrostatic repulsive forces or hydrophobic attractive forces in aqueous solutions. Subpiconewton intermolecular forces were resolved at controlled gaps in the nanometer range between the probe and a material surface. Interaction forces between hydrophobic surfaces of a probe and a coverslip were measured, and a long-range attractive force was observed out to 100 nm in pure water. This result suggests that very long range hydrophobic forces also operate between objects of the size of a protein molecule. For details, see Ref. 2 and 5.

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

Kazuo KITAMURA[1], Atsuko IWANE[1], Toshio YANAGIDA[1] and Makio TOKUNAGA
([1]Department of Physiology I, Osaka University Medical School)

A single myosin head molecule (myosin subfragment-1, S1) was captured onto the tip of a scanning microprobe, using a flexibleglass microneedle, and manipulated with subnanometer resolution. Single molecules were confirmed by examining it in the fluorescence glass microneedle at the same time using objective-type TIRFM. Movements and forces resulting from the interaction of a captured single S1 molecule with actin filaments were measured. Mean displacement amplitudes of individual S1 molecules ranged from 4 to 30 nm, certainly depending upon conditions for interactions of individual molecules. Moreover, it was found that a myosin head processively moves along the actin filament(s) with 5.3 nm steps, and undergoes ~5 steps to produce a maximum displacement of ~30 nm for each ATP hydrolysis. The results proves the loose coupling mechanism.

(4) A New Model of Molecular Motors

Makio TOKUNAGA

Our single-molecule researches have provided direct evidence of the loose coupling mechanism on protein motors. I have shown that a new simple model named "asymmetric interaction model" can explain the results of the single-

molecule experiments. Because this model is based only on a simple assumption that the interaction potential between motor proteins has a asymmetric shape, the theoretical formulation is very simple. Therefore, this model leads to a spread use of its application.

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H-b. Molecular Biomechanism Laboratory

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

Ranjan SEN[1], Tomoko KUBORI[1], Hiroki NAGAI[1], V. Jim HERNANDEZ[2] and Nobuo SHIMAMOTO[1] ([1]Structural Biology Center, National Institute of Genetics, [2]Microbiology, SUNY/Buffalo)

Transcription initiation is conventionally supposed to be a series of steps composed of promoter binding, isomerization, RNA synthesis, and promoter clearance. KUBORI and SHIMAMOTO have presented that kinetics of abortive and productive transcription at λPR promoter violates this sequential mechanism and rather indicates a branched pathway mechanism (*Nuc. Acids Res.* **24**, 1380-1381 (1996): *J. Mol. Biol.* 256, 449-457 (1996)). In the new mechanism one of a branch leads to dead-end inactivated complexes via complexes which only produce abortive transcripts, moribund complex. These studies have opened up a possibility of revision of general initiation mechanism and re-definition of promoter clearance.

To prove the existence of branched mechanism, the yields of full-length transcripts on the same amounts of templates harboring T7A1, λPR, and *lac* UV5 promoters were compared in a single-round transcription condition. The results showed that at least 60% and 97% of holoenzyme bound to the λPR, and *lac* UV5 promoters, respectively, remained inactive in terms of the synthesis of full-length transcripts. This kinetically demonstrates the existence of dead-end pathway (ref. 1).

Next we searched a switch which turns reaction pathway from productive to promoter-arrested branched pathway. We first found that misincorporation occurs at the 5 th and 6 th positions of the λPRAL transcription unit and the non-templated transcripts are aborted. The misincorporation and other abortive synthesis are enhanced by the presence of holoenzyme only in excess to the template DNA, and even more by a stall of RNA polymerase at +32 position. A hydroxide radical footprinting showed that the stall at +32 can accommodate one more enzyme molecule at the promoter. The enhancement is not intro-

duced by a similar stall at +73. These result suggested that an interaction, probably physical contact, between two enzyme molecules tend to switch the trailing molecule into promoter-arrested state (ref. 1).

Thirdly, we have investigated the mechanism of a switch which mitigate the arrest at the promoter. The S506F mutation in σ^{70}, isolated by V. J. Hernandez , is known to reduce abortive synthesis at all the promoters tested. It also enhances full-length synthesis from weak promoters, but not from strong ones. By kinetic and footprinting analyses we showed that the mutant is not an up mutant, but rather shifts the equilibrium of isomerization towards closed complexes at the λPRAL promoter. The most distinctive change is the 10-100 fold stimulation of rates of over-all dissociation of both productive and arrested complexes with no change of binding affinity. Moribund complexes are rapidly dissociated (backward reaction) and inactivated (forward reaction), reducing the level of its accumulation. This reduces abortive synthesis and decreases the yield of full-length transcripts from this strong promoter. These results proposes the significance of introduction of reversibility into otherwise irreversible separation into the two branched pathways as a switch (SEN et al., J. Biol. Chem. (1998)).

(2) Detection and characterization of conformation changes of proteins by a high-resolution protein footprinting

Hiroki NAGAI and Nobuo SHIMAMOTO (Structural Biology Center, National Institute of Genetics)

The term "conformation change" of proteins has been used mostly in speculative sense rather than in description of their distinctive structural change. The recently developed footprinting technique, hydroxide radical protein footprinting for example, enables to identify which part of protein changes its exposure to solvent at a level of amino-acid residues. Therefore, genetic information can be interpreted into concrete structural changes in parts of a protein.

We have chosen σ^{70} as a test protein for establishing the correlation of genetics and structural biology because of abundance of its genetic information and the presence of conformational change which has been speculated

from several biochemical studies. The protein has 4 regions (region 1 to 4) which are conserved among other sigma factors and other bacteria, and the regions are further divided into subdomains.

We put a oligopeptide tag which can be phosphorylated by heart muscle kinase (HMK tag) at C-terminus of σ^{70}, enabling direct visualization of footprinting. Holoenzyme containing the phosphorylate σ^{70} and binary complexes with the λPRAL promoter are prepared and challenged to hydroxide radical generated with Fenton Reaction under a single-cutting condition. The cutting positions are referred by a series of markers which had been generated by several chemical and enzymatic cleavage of σ^{70} (*Gene. Cells* **2**, 725-734 (1998)).

The major cleavage sites well corresponded the borders of functional domains. Region 1.1 is dispensable for basic activity, but a tryptic fragment containing 1.2 and 2 can form a complex with core enzyme which binds to the non-template strand of a promoter DNA. The most strong cleavage was found to the border of these distinct functional domains. The second preferential cleavage site was found in region 3.2, which agrees with the observation that C-terminal truncated sigma factors can promote the initiation of transcription from the extended -10 promoter but not from -35-dependent promoters.

Some genetic models was refined by this study. A N-terminal portion of region 2 was thought to be involved in binding to core enzyme, being supposed to be core-interacting site. Consistently, a moderate protection was observed in this domain, but regions 1.1, and most parts of regions 2, 3, and 4 were also protected, suggesting the presence of more extensive contact sites between σ^{70} and core enzyme than have been proposed by studies using deletion mutants. Two regions, 2.4 and 4.2, have been genetically proved to interact with promoter DNA. Consistently small segments in these regions become protected on binding to DNA. However most parts of these regions are already protected on the stage of holoenzyme before binding to DNA. This indicates that the major structural change occurs upon formation of holoenzyme rather than upon binary complex formation. The acidic region between 1.1 and 1.2 has been suggested to inhibit DNA binding activity of free σ^{70}, but the footprinting clearly showed this region affected only in the presence of DNA, denying this model. Another model claims that region 1.1 masks a

DNA binding activity region 4.2 by intramolecular interaction, preventing free σ^{70} from binding to promoter DNA but not holoenzyme. However, our results do not provide the evidence for interaction between regions 1.1 and 3/4. Further study is required to prove (or disprove) the possible interaction between regions 1.1 and 3/4.

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

Hiroyuki KABATA[1,2], Minoru TAKEUCHI[1], Takashi KINEBUCHI[1], Nobuo SHIMAMOTO[1], Osamu KUROSAWA[2,3], Hironori ARAMAKI[4], 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.

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

ously obtained in kinetic assays of sliding.

We have been developing manipulating techniques for single molecules (*in Structural tools for the analysis of protein-nucleic acid complexes* D(Birkhäuser Verlag AG, Basel, 1992) pp. 241-253). As a fluorescent technique to detect single molecules, we have been improving the *A. victoria* Green Fluorescent Protein.

(4) Functional domain of *Escherichia coli* Single-stranded DNA binding protein

Takashi KINEBUCHI[1,2], Heizaburo SHINDO[2], Hiroki NAGAI[1], Nobuo SHIMAMOTO[1], Mitsuhiro SHIMIZU[2] ([1]Structural Biology Center, National Institute of Genetics, [2]Department of Pharmacology, Tokyo College of Pharmaceutical Science)

E. coli Single-stranded DNA binding protein (SSB) is an essential protein in replication and recombination. It exists as a homotetramer throughout its physiological environment. It cooperatively binds to single-stranded DNA, and makes clusters which is recognized by DnaG primase and RecA recombinase. The role of tetramer formation for DNA binding is studied by a deletion analysis.

A series of mutants with N-terminus or C-terminus deletion was constructed and proteins were purified. The oligomeric state of mutant proteins were determined by gel permeation chromatography with Superdex-75, and DNA binding was determined by gel mobility assay.

The results showed that all the N-terminus deletions hampers DNA binding activity and induced oligomerization more than octamer. The C-terminus deletion mutants up to 115 aa also hampers DNA binding activity and tetramer formation. This correlation of DNA binding activity with tetramer formation indicates that the DNA binding site is created only on homotetramer molecule.

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H-c. Multicellular Organization Laboratory

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

Masaya TAKEUCHI, Akane MOMI, Takeshi ISHIHARA and Isao KATSURA

Fluoride-resistant mutations of *C. elegans* are all recessive and grouped into two categories: class 1 mutations (in *flr-1*, *flr-3* and *flr-4*) that show strong fluoride-resistance and slow growth, and class 2 mutations (in *flr-2* and *flr-5*) that show weak fluoride-resistance, normal growth and suppression of the slow growth of class 1 mutations (Katsura, I. *et al*. (1994) Genetics 136, 145-154).

flr-1 encodes an ion channel belonging to the DEG/ENaC superfamily, which contains mechano-sensory ion channels of *C. elegans* and amiloride-sensitive epithelial ion channels of mammals. *flr-4* encodes a novel Ser/Thr protein kinase having a hydrophobic domain on the carboxyl-terminal side. A genomic DNA fragment that rescues *flr-3* mutations produces a polycistronic mRNA encoding a kinase-like protein and a novel protein.

A *flr-1*::GFP fusion gene is expressed only in the intestinal cells, beginning from the comma stage of embryos to the adult stage. A *flr-4*-GFP fusion gene is expressed in the intestinal cells from the 1.5-fold stage, in the isthmus of the pharynx from the 3-fold stage and in a pair of head neurons called AUA from L1 larvae stage to adults. In those fusion genes, GFP cDNA is connected

in frame to the 3' end of the coding region of the *flr* genes. Since they can rescue the corresponding mutant phenotypes, the expression at the site and the time mentioned above should be enough for the animal to show the wild-type phenotypes.

All the six *flr-1* mutations were missense mutations at or near the amino acid residues that are functionally important in degenerins and ENaCs, showing functional similarity between FLR-1 and those ion channels. Four *flr-4* mutations consisted of two missense mutations at conserved residues in the kinase domain, a mutation at a splice-acceptor sequence, and a missense mutation in the C-terminal hydrophobic region.

Class 1 mutants show pleiotropic phenotypes, including slow growth, short defecation cycle periods, constitutive dauer larva formation in a certain mutant background (See (3)), hypersensitivity to high osmotic pressure, and abnormal gonad shape. Some of these phenotypes are suppressed by class 2 mutations, while others are not. We think that FLR-1, FLR-3 and FLR-4 probably constitute a regulatory system in the intestine. We plan to study (a) what kind of proteins are encoded by class 2 genes, (b) How the regulatory system in the intestine can control putative neuronal functions such as the regulation of defecation cycle periods and dauer larva formation, and (c) how the *flr* gene products act each other in the regulatory system.

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

Ryuichi HISHIDA, Takeshi ISHIHARA and Isao KATSURA

Mutants in *hch-1* gene cannot digest protein components of the eggshell and have abnormality in the migration of a post-embryonic neuroblast called QL and its descendants (Hedgecock, E.M. *et al*. (1987) Development 100, 365-382). *hch-1* encodes a protein that has a signal peptide, a Zn protease domain, an EGF domain and a CUB domain, and that resembles the *Drosophila* TOLLOID and the mammalian BMP-1. Whole-mount *in situ* hybridization of embryos showed that the mRNA is expressed in the mid-embryonic stage, first in the dorsal and lateral sides of the middle and posterior part of embryos and then only on the lateral sides. Experiments using *hch-1*::GFP fu-

sion genes showed essentially the same results except that weak expression continues until just after hatching (Hishida, R. *et al*., 1996, EMBO J., 15: 4111-4122).

We measured protease activity in the hatching fluid *in vitro*, using the soft eggshells of *hch-1* embryos as the substrate. Activity in the hatching fluid of a *hch-1* null mutant was not detectable, whereas a transformant having many copies of the wild-type *hch-1* gene exhibited about 10 times greater protease activity than the wild-type animal. Thus, *hch-1* gene seems to encode the hatching enzyme itself or another factor that determines the level of hatching enzyme activity. The protease activity was inhibited by metalloprotease inhibitors but not by inhibitors of other proteases such as Ser-, Cys-, and Asp-proteases.

A nonsense mutation in the N-terminal protease domain, a putative null mutation, was recessive and had low penetrance in the cell migration abnormality. In contrast, a missense mutation in the C-terminal Cys-rich region was semi-dominant and had high penetrance in the cell migration abnormality. Furthermore, a transposon-insertion mutation had low penetrance in the cell migration abnormality, but showed a stronger abnormality in hatching than the former two mutations. The poor correlation between the two phenotypes indicates that delayed hatching may not be the only cause of the cell migration abnormality.

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

Isao KATSURA, Norio SUZUKI and Takeshi ISHIHARA

If newly hatched larvae of *C. elegans* are in a crowded state and given a limited food supply, they deviate from the normal life cycle and develop to enduring, non-feeding larvae called dauer larvae. The developmental decision to become dauer larvae is regulated by the neural circuit in a pair of sensory organs called amphids, with the dauer pheromone and food as the input. Since the assay of dauer formation is much less time-consuming than behavioral assays such as chemotaxis assays, we are analyzing the head neural circuit by detecting dauer formation as the output. We found that muta-

tions 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 two different neurons are often positioned in parallel so that two mutations may be required to block signals in both neurons.

We found that the pattern of combinations for the Sdf-c phenotype is specific and can be explained by a model assuming three parallel pathways. We also found suppressor mutations of the Sdf-c phenotype of various double-mutants and estimated the positions where the genes of the suppressor mutations act in the parallel pathways.

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 becomes dauer-constitutive by the killing of ASI neurons. Eight of the mutants were alleles of 4 known genes (*tax-2, osm-6, che-11,* and *aex-3*), but most of the remaining 36 mutations, which map in at least 13 genes, seem to be alleles of novel genes. We continue to map them, and plan to characterize them further to elucidate their roles in sensory reception, transduction, or processing.

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

Takeshi ISHIHARA, Manabi FUJIWARA and Isao KATSURA

To study the formation and function of the neural circuit in *C. elegans*, we identified about 20 promoters giving expression in specific sets of neurons by analyzing their GFP fusion gene. Three of the promoters were selected by the promoter trapping method, and others by looking at the genome DNA sequence. Most of the cells that express GFP were identified for each promoter. Of the promoters obtained by promoter trapping, H20 gave expression in all neurons, H13 (promoter for a Zn finger transcriptional factor) only in AFD neurons, and I85 (promoter for a collagen gene) in the post-embryonic blast cell G2 and its descendants, including RMF neurons.

Of the genes selected by the DNA sequence, we investigated three glycine/$GABA_A$ receptor homologs and two metabotropic glutamate receptor homologs in more detail. We determined their cDNA sequence by RT-PCR, 5'-RACE, and 3'-RACE and isolated their deletion mutants, whose behavioral phenotypes are now under study (See (5)). We also isolated a deletion mutant of the gene encoding a Zn finger protein expressed in AFD neurons (H13). Although AFD neurons are known to play a role in thermotaxis, the mutant showed normal thermotaxis.

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

Takeshi ISHIHARA and Isao KATSURA

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

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

While the measurements were performed usually with well-fed animals, we found that animals starved for 5 hours had a preference for odorants, because starved animals had weaker response to copper ion. This behavioral change seems to make sense for starved worms, because they can look for food over a wide area.

The genes for metabotropic glutamate receptors (*mgl*) are expressed in part

of the interneurons that are considered to act in this interaction. We isolated deletion mutants in these genes and analyzed them. A mutant in *mgl-1* (Gi-coupled type) had preference for chemotaxis to odorants over avoidance to copper ion, although it showed no abnormality in each of the single behaviors. On the other hand, a mutant in *mgl-2* (Gq-coupled type) lacked the effect of starvation. Furthermore, *mgl-1; mgl-2* double-mutant worms hardly cross the copper ion barrier even when they are starved. We are now testing if those phenotypes are caused by the mutations in the *mgl* genes or by side mutations.

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

(6) Functional Analysis of Neural Adhesion Molecules by Introduction of Clones Producing Antisense RNA

Manabi FUJIWARA, Takeshi ISHIHARA and Isao KATSURA

In *C. elegans*, gene disruption requires a lot of work, and the mutants obtained in this way lack the function of the gene completely in all the cells that express it. Hence, if we can reduce, with much less labor, the function of a gene specifically in certain cells, it would be useful for many studies. As the expression of maternal genes can be repressed by the introduction of antisense RNA into the gonad, we tried to establish a method in which the gene function is reduced by the expression of the antisense RNA in somatic cells.

Looking at the DNA sequence of the *C. elegans* genome project, we selected 10 genes encoding a protein that has the FNIII domain and/or Ig domain and that is expected to be a neural adhesion molecule. We made DNA constructs which, under the H20 promoter (See (4)), produce antisense RNA corresponding to part of the mRNA of those genes. Worms were transformed by these DNA constructs and tested for their effect. Abnormality in locomotion was detected for three of the genes, which encode homologs of L1, TAG1 and NCAM, respectively. We studied the gene for the L1 (NgCAM, Neuroglian) homolog

in more detail. Antisense RNA corresponding to different parts of the gene caused abnormality in locomotion, while sense RNA had little effect. The animals in which the antisense RNA was expressed under a neuronal promoter showed misdirection of the motor neuron processes that normally extend from the ventral to the dorsal side. The mechanism of the action of this antisense RNA remains to be elucidated, since we could not detect the reduction of gene expression by the antisense RNA, as monitored by a GFP fusion gene.

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H-d. Biomolecular Structure Laboratory

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

Yasuo SHIRAKIHARA

F1-ATPase, with a subunit composition of $\alpha_3 \beta_3 \gamma \delta \varepsilon$, is a catalytic sector of the membrane bound ATP synthase which plays a central role in energy conversion in mitochondria, chloroplasts and bacteria, generating ATP from ADP and inorganic phosphate using energy derived from a trans-membrane electro-chemical potential. Both the $\alpha_3 \beta_3$ sub-assembly and F1-ATPase and have been challenging targets for crystallographic study because of their large sizes e.g. 320kDa for the $\alpha_3 \beta_3$ sub-assembly and 380 kDa for F1. We have solved the structure of the $\alpha_3 \beta_3$ sub-assembly of F1-ATPase from a thermophilic bacterium Bacillus PS3. We have extended the structural study to another crystal form of the $\alpha_3 \beta_3$ sub-assembly, mutant $\alpha_3 \beta_3$ sub-assembly and $\alpha_3 \beta_3$g sub-assembly.

The $\alpha_3 \beta_3$ sub-assembly is an active complex with 20-25% of the ATPase activity of intact F1, and has similar catalytic properties to those of F1. The refined structure of the nucleotide-free form of $\alpha_3 \beta_3$ sub-assembly from Ba-

cillus PS3 F1has been published this year (Ref. 1), and deposited in the Protein Data Bank with accession code 1SKY. The final model had R factor of 22.2% (free R factor 29.9%) and had a reasonable stereochemistry. In the absence of nucleotides and the single copy subunits, the $\alpha_3\beta_3$ sub-assembly adopts a strictly symmetrical structure: the three α and three β subunits have identical conformations. The β subunits adopt a conformation essentially identical to that of the nucleotide-free β subunit in asymmetrical mitochondrial F1 structure. The α subunits have a conformation similar to three mutually similar conformations of α subunits in mitochondrial F1. When compared to the mitochondrial enzyme, the major differences in structure are localised in membrane-proximal half of the enzyme, while the remainder of the structure is highly conserved.

As an extension of the structural study of the α_3 β_3 sub-assembly, we have examined structure of the nucleotide-soaked crystal of the $\alpha_3\beta_3$ sub-assembly, hopefully assuming the structure in it to represent an nucleotide-bound form. Diffraction data from the crystals were taken before they cracked eventually, as the α_3 β_3 sub-assembly is destabilized by presence of nucleotides. From a preliminary analysis of the data sets, ATP and MgADP soaked crystals, among 12 crystals soaked in different conditions, are found to be candidates in which large structural difference from the nucleotide-free form may be observed.

As another extension, we also examined structure of a mutant $\alpha_3\beta_3$ sub-assembly where three copies of a Glu190Gln mutant β subunit join instead of the wild type β subunit. Mutational studies indicate that Glu190 is a catalytic residue and that an α_3 β_3 γ sub-assembly containing Glu190Gln mutant β subunit is inactive. The structure of the a mutant $\alpha_3\beta_3$ sub-assembly is nearly identical to that of the wild-type $\alpha_3\beta_3$ sub-assembly, except the Gln side chain pointing to the same direction as that of the Glu side chain. The structure suggests that loss of the activity is entirely due to the change of the side chain from carboxyl to amide. The analysis opens up a way to examine structure of mutant molecules of the α and β subunits that are of interest, because subunits alone can not be crystallized.

The $\alpha_3\beta_3\gamma$ sub-assembly exhibits kinetic properties very similar to those of F1, and a number of interesting mutant sub-assemblies have been

engineered. We have searched crystallization conditions for the sub-assembly starting from those for nucleotide-free α_3 β_3 sub-assembly. Although small crystals were obtained in the initial stages, it was extremely difficult to get large well-ordered crystals in spite of extensive search for the crystallization conditions and of efforts to improve purity of the sub-assembly preparation and to eliminate in homogeneous binding of endogenous nucleotides. Further efforts are being made to lead to successful crystallization.

The structure solution of the α_3 β_3 sub-assembly was done in collaboration with Andrew Leslie, Jan Pieter Abrahams and John Walker at MRC Laboratory of Molecular Biology, Cambridge,UK. Rest of the works described here were done in collaboration with Masasuke Yoshida, Toyoki Amano, Eiro Muneyuki, Yasuyuki Kato and Satoshi Tsunoda at Research Laboratory of Resource Utilization, Tokyo Institute of technology.

(2) Crystallographic Study of the Transcription Activator, PhoB

Toshihiko AKIBA and Yasuo SHIRAKIHARA

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

The DNA-binding C-terminal domain, spanning from a residue 125 to the C-terminus(220), of the protein forms good crystals allowing to collect diffraction data to 2.1 A resolution. We have attempted to solve its structure by the molecular replacement method using a structural model of C terminal fragment of OmpR with 32 % sequence identity. We found that this molecular replacement calculation was problematic due to 1) a low signal to noise ratio generally encountered on dealing with small-sized molecule, 2) two molecules in an asymmetric unit and 3) a low sequence identity. In spite of many efforts, current solutions are not yet convincing enough. We are trying to com-

bine the information from a heavy atom derivative to derive a correct molecular replacement solution.

From particular interest to interactive regulatory mechanisms between the N- and the C- domains, crystallization of an intact form of the protein has been attempted. While the protein was overproduced in the cells containing a T7 expression vector, the appreciable expression was achieved rather in the absence of the inducer IPTG. This can be ascribed to the toxicity of the expressed protein. By heparin-affinity chromatography and following cation-exchange chromatography used in a pH gradient mode, the protein was purified to a homogeneity level thought to be sufficient for crystallization.

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

(3) Crystallographic Study of the Transcription Repressor, CamR

Koji FUKUSHI and Yasuo SHIRAKIHARA

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

Two crystal forms have been obtained, one from polyethyleneglycol solution in the presence or absence of D-camphor and the other from Na-K-phosphate solution in the presence of D-camphor. Even after establishing these conditions, we had some time when no good crystals were obtained. Therefore we switched the E.coli over-expression system from a P_L promoter system to a more efficient T7 (pET system) promoter one. Combined with minor modifications in purification procedure, this resulted in more reproducible crystal formation.

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

(4) Crystallographic Study of *Escherichia coli* RNA Polymerase α Subunit

Katsuhiko MURAKAMI, Akira ISHIHAMA and Yasuo SHIRAKIHARA

The *Escherichia coli* RNA polymerase (RNAP) α subunit, consisting of 329 amino acid residues, is composed of the two independent structural domains and is included as a dimer into RNAP. The amino (N)-terminal domain from residue 20 to 235 is responsible for the enzyme assembly through the interactions with β and β' subunits and the carboxyl (C)-terminal domain of 94 residues in length is important for transcription regulation. The solution structure of the C-terminal domain is already resolved by NMR study.

For the purpose of analysis the RNAP assembly mechanism, we have tried to crystallize α subunit. Firstly, we examined the purification method of the α subunit from over-expressing *E.coli* cells and found the conditions for preparing highly purified proteins reproducibly. Screening of crystallization conditions of the α subunit was performed by several methods, including microbatch and vapor-diffusion techniques, using numerous kinds of crystallization solutions containing different precipitants, metal ions and different buffers with different pH. Crystallization of α subunit was also carried out using Crystal Screening Kit solutions (Hampton Research). We found that PEG400/CaCl2 and PEG8000/NaAcetate gave thin-plate crystals reproducibly. We are searching for better crystallization conditions producing thicker and larger crystals.

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H-e. Gene Network Laboratory

(1) Expression pattern map of the *C. elegans* genome

Yuji KOHARA, Tadasu SHIN-I, Tomoko MOTOHASHI, Tokie OHBA, Ikuko SUGIURA, Masumi OBARA, Akiko MIYATA, Masako SANO, Hiroko UESUGI, Hisako WATANABE, Yuko MITANI and Tamami NAGAOKA

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

More than 100,000 cDNA clones were picked up randomly from different cDNA libraries, stored and gridded. cDNA clones derived from already analyzed abundant cDNA species were identified by probing the high density clone grids and removed to avoid unnecessary redundancy of analysis. The remaining clones were subjected to analyses of (1) tag-sequencing from both 5'-ends (with vector primers) and 3'-ends (with anchored oligo-dT primers), (2) mapping onto the genome, and (3) analysis of expression patterns. Thus far, 50,000 clones have been processed, and 36,500 clean 3'-tag sequences were obtained, which were classified into 7,680 unique cDNA species (nearly half of the total genes) by comparing the 3'-tags. About 70% of them were located on the genomic sequence determined by the Sanger Ctr/WASH-U sequencing consortium.

chromosome	I	II	III	IV	V	X	unassigned	total
cDNA clones								
5' tags	7,439	7,242	6,765	6,836	6,119	6,103	7,318	47,822
3' tags	5,734	5,535	5,132	5,144	4,747	4,765	5,408	36,465
both tags*	5,230	5,029	4,686	4,651	4,292	4,399	3,773	32,060
clusters	914	1,062	897	896	923	969	2,020	7,681

*: cDNA clones whose 5' and 3'-tags are available.

We are analyzing the expression patterns during development of the classi-

fied cDNA species using the newly developed method of in situ hybridization on whole mount specimens of embryos, larvae and adults in the standard multi-well format. Thus far, more than 2,000 cDNA groups mostly from chromosomes 3 and X have been analyzed. The cDNA sequence and mapping information has been made available at NEXTDB (Nematode EXpression paTtern DataBase) http://watson.genes.nig.ac.jp:8080/db/index.html

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

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

In *C. elegans*, germ cells arise during early embryogenesis from an invariant sequence of asymmetric divisions that separate germ cell precursors from somatic precursors. We show that maternal-effect lethal mutations in the gene *pos-1* cause germ cell precursors to inappropriately adopt somatic cell fates. During early embryogenesis, *pos-1* mRNA and POS-1 protein are present predominantly in the germ precursors. POS-1 is a novel protein with two copies of a CCCH finger motif previously described in the germline proteins PIE-1 and MEX-1 in *C. elegans*, and in the mammalian TIS11/Nup475/TTP protein. Mutations in *pos-1* cause several defects in the development of the germline blastomeres that are distinct from those caused by mutations in *pie-1* or *mex-1*. An early defect in *pos-1* mutants is a failure to express APX-1 protein from maternally-provided *apx-1* mRNA, suggesting that POS-1 may have an important role in regulating the expression of maternal mRNAs in germline blastomeres.

(3) Development of a method to mesure the size of poly(A) tails in *Caenorhabditis elegans* embryos and its application to some maternal genes

Shuichi ONAMI[1] and Yuji KOHARA ([1]Department of Genetics, The Graduate University for Advanced Studies)

Translational regulation is expected to play an important role in early embryogenesis of the nematode *C.elegans*, which are mostly dependent on the maternally supplied proteins and mRNAs. Although the mechanisms of translational regulation are largely unknown, the size of poly(A) is a possible factor in the regulation, considering the findings in other organisms such as Xenopus lavies. Thus, we developed a PCR-mediated method to measure the poly(A) tail length of given mRNA in *C.elegnas* embryos at a desired stage. Nucleic acid is extracted from 5-15 embryos and an RNA-oligonucleotide-tag is added to its 3'-end. The 3'-end region of target mRNA is examined by RT-PCR using gene-specific and tag-specific primers. Using this method, we measured the poly(A) tail length of *fem-3* mRNA as a positive control to verify the method. We also measured the poly(A) lenth of *glp-1* mRNA, whose translation is controlled temporally and blastomere-specifically during early *C. elegans* embryogenesis. GLP-1 protein is not detected in oocyte, while 2cell-stage embryos express the protein only in the anterior blastomere. We found a correlation between the poly(A) tail length and its protein expression.

(4) Functional analysis of a *Caenorhabditis elegans* T-box gene *tbx-9*

Yoshiki ANDACHI

tbx-9 is one of the genes identified by the cDNA project and encodes a protein with the DNA binding motif T-box. The T-box gene family consists of more than 30 genes in various multicellular organisms, including the mouse transcription factor *Brachyury*. Many of the genes are known to be expressed in embryogenesis and to be involved in morphogenesis. As it is conceivable that the T-box genes play important roles in the development of this organism, I

have been studying one of the T-box genes, *tbx-9*.

Sequence analyses of the cDNA clone yk97a6 and 5'RACE PCR products indicated that the *tbx-9* gene is transcribed to a product of 1.0 kb. The transcript that was detected in embryos by developmental northern blot analysis, and in situ hybridization analysis on whole mount embryos revealed that *tbx-9* is expressed in a few cells of early-stage embryos.

To know the phenotype of worms with mutation in the *tbx-9* gene, I performed a gene disruption method using the transposon Tc1, and obtained a null allele losing more than half of the gene. Worms homozygous for the allele show a phenotype of aberration in shape in the posterior half of their bodies. The aberration occurs during embryogenesis. Immunostaining of mutant embryos with an antibody against body-wall muscle myosin revealed abnormality in body-wall muscle cells, indicating that *tbx-9* is required for proper formation of body-wall muscle in embryogenesis.

To show sequence-specific DNA binding activity of *tbx-9*, I determined the DNA binding sequence of *tbx-9* by a strategy for the enrichment of target DNA fragments. The binding sequence of *tbx-9* turned out to be nearly identical to that of *Brachyury*.

Publication

None

I. CENTER FOR INFORMATION BIOLOGY

I-a. Laboratory for DNA Data Analysis

(1) Genome plasticity as a paradigm of eubacteria evolution

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

To test the hypotheses that eubacterial genomes leave evolutionarily stable structures and that the variety of genome size is brought about through genome doubling during evolution, the genome structures of *Haemophilus influenzae, Mycoplasma genitalium, Escherichia coli*, and *Bacillus subtilis* were compared using the DNA sequences of the entire genome or substantial portions of genome. In these comparisons, the locations of orthologous genes were examined among different genomes. Using orthologous genes for the comparisons guaranteed that differences revealed in physical location would reflect changes in genome structure after speciation. We found that dynamic rearrangements have so frequently occurred in eubacterial genomes as to break operon structures during evolution, even after the relatively recent divergence between *E. coli* and *H. influenzae*. Interestingly, in such eubacterial genomes of high plasticity, we could find several highly conservative regions with the longest conserved region comprising the S10, *spc*, and a operons. This suggest that such exceptional conservative regions have undergone strong structural constraints during evolution. See Ref. 6 for details.

(2) Constrained evolution of hepatitis B virus with overlapping genes

Masashi MIZOKAMI[1], Etsuro ORITO[1], Ken-ichi OHBA[1], Kazuho IKEO, Johnson Y.N. LAU[2], Takashi GOJOBORI ([1]Nagoya City University Medical School, [2]University of Florida)

With the aim of elucidating the evolution of a hepadnavirus family, we constructed molecular phylogenetic trees for 27 strains of hepatitis B virus (HBV)

using both the unweighted pair-grouping and neighbor-joining methods. All five gene regions, P, C, S, X, and preS, were used to construct the phylogenetic trees. Using the phylogenetic trees obtained, we classified these strains into five major groups in which the strains were closely related to each other. Our classification reinforced our previous view that genetic classification is not always compatible with conventional classification determined by serological subtypes. Moreover, constraints on the evolutionary process of HBV were analyzed for amino-acid-altering (nonsynonymous) and silent (synonymous) substitutions, because two-thirds of the open reading frame (ORF), P, contains alternating overlapping ORFs. In our unique analysis of this interesting gene structure of HBV, the most frequent synonymous substitutions were observed in the nonoverlapped parts of the P and C genes. On the other hand, the number of synonymous substitutions per nucleotide site for the S gene was quite low and appeared a strongly constrained evolution. Because the P gene overlaps the S gene in a different frame, the low rate of synonymous substitution for the S gene can be explained by the evolutionary constraints which are imposed on the overlapping gene region. In other words, synonymous substitutions in the S gene can cause amino acid changes in its overlapping region in a different frame. Thus, the evolution of HBV is constrained evolutionarily by the overlapping genes. We propose calling this mode of viral evolution "constrained evolution". The evolution of HBV represents a typical constrained evolution. See Ref. 7 for details.

(3) Evolutionary mechanisms and population dynamics of the third variable envelope region of HIV within single hosts

Yumi YAMAGUCHI and Takashi GOJOBORI

Clonal diversifications of HIV virus were monitored by periodic samplings on each of the six patients with regard to 183- to 335-bp segments of the *env* gene, which invariably included the functionally critical V3 region. Subsequently, six individual phylogenetic trees of viral variants were constructed. It was found that at one time or another during the course of disease progression, viral variants were inexplicably released from a strong negative selection against nonsynonymous base substitutions, possibly indicating positive

selection. This resulted in concentrated amino acid substitutions at five specific sites within the V3 region. It was noted that these sites were often involved as antigenic determinants that provoked the host immune response and that these sites were also involved in the determination of viral phenotypes as to their cell tropism, syncytium formation capability, and replication rates. See Ref. 8 for details.

(4) Ascidian tyrosinase gene: Its unique structure and expression in the developing brain

Shigeru SATO[1], Hiroshi MASUYA, Takaharu NUMAKUNAI[2], Noriyuki SATOH[3], Kazuho IKEO, Takashi GOJOBORI, Koji TAMURA[1], Hiroyuki IDE[1], Takuji TAKEUCHI[4], Hiroaki YAMAMOTO ([1]Biological Institute, Tohoku Univ., [2]The Marine Biological Station of Asamitsu, Tohoku Univ., [3]Dept. of Zoology, Kyoto Univ., [4]Dept. of Biotechnology, Ishinomaki Senshu Univ.)

Tadpole larvae of ascidians have two sensory pigment cells in the brain. One is the otolith cell that functions as a gravity receptor, the other pigment cell is part of a primitive photosensory structure termed the ocellus. These sensory cells, like vertebrate pigment cells, contain membrane-bounded melanin granules and are considered to reflect a crucial position in the evolutionary process of this cell type. To investigate the molecular changes accompanying the evolution of pigment cells, we have isolated from *Halocynthia roretzi* a gene encoding tyrosinase, a key enzyme in melanin biosynthesis. The cDNA has an open reading frame (ORF) of 596 amino acids, which is 36-39% identical in amino acid sequence to vertebrate tyrosinases. In addition, the sequence analysis of both cDNA and genomic clones reveals an unusual organization of the tyrosinase gene, an extraordinary 3' untranslated region of the transcripts with significant homology to the coding sequence, and a single short intron in the sequence encoding a cytoplasmic domain. Expression of the gene is detected first in two pigment precursor cells positioned in the neural plate of early neurulae, and later in two melanin-containing pigment cells within the brain of late tailbud embryos. Its expression pattern correlates well with the appearance of tyrosinase enzyme activity in the developing brain. These results provide the first descrition of pigment cell differentia-

tion at the molecular level in the ascidian embryo, and also will contribute to a better understanding of the evolution of chordate pigment cells. See Ref. 9 for details.

(5) Inference of molecular phylogenetic tree based on minimum model-based complexity method

Hiroshi TANAKA[1], Fengrong REN[1], Toshitsugu OKAYAMA, Takashi GOJOBORI ([1]Medical Research Institute, Tokyo Medical and Dental University)

In this study, starting with a newly introduced concept of data complexity ("empirical data complexity"), we specify the concept of complexity more concretely in relation to mathematical modeling and introduce "model-based complexity (MBC)". Inductive inference based on the minimum model-based complexity method is then applied to the reconstruction of molecular evolutionary tree from DNA sequences. We find that minimum MBC method has good asymptotic property when DNA sequence lengths approach to infinite and compensates the bias of maximum likelihood method due to the difference of tree topology complexity. The efficiency of minimum MBC method for reconstruction of molecular tree is studied by computer simulation, and results suggest that this method is superior to the traditional maximum likelihood method or its modification by Akaike's AIC. See Ref. 10 for details.

(6) Molecular evolution of myelin proteolipid protein. Biochemical and Biophysical Research Communications

Tadashi KURIHARA[1], Mitsuhiro SAKUMA[1], Takashi GOJOBORI ([1]Institute of Life Science, Soka University)

We show that the major membrane protein of central nervous system myelin, proteolipid protein, evolved much more rapidly than it does now more than 300 million years ago. We reason that myelin proteolipid protein evolved rapidly just after its appearance in vertebrates and that its evolutionary rate then gradually decreased. Comparison of the rates between the synonymous and nonsynonymous nucleotide substitutions for the cDNA suggests the possi-

bility that positive selection operated on myelin proteolipid protein at least when it appeared in vertebrates. See Ref. 13 for details.

(7) Father-to-mother-to-infant transmission of HIV-1: clonally transmitted isolate of infant mutates more rapidly than that of the mother and rapidly loses reactivity with neutralizing antibody

Yukari OKAMOTO[1], Koichi SHIOSAKI[2], Yasuyuki EDA[2], Sachio TOKIYOSHI[2], Yumi YAMAGUCHI, Takashi GOJOBORI, Takashi HACHIMORI[3], Shudo YAMAZAKI[1], Mitsuo HONDA[1] ([1]AIDS Research Center, National Institute of Health, [2]Chemo-Sero-Therapeutic Research Institute, [3]Clinical Dept. of Infectious Diseases, Komagome Tokyo Metropolitan Hospital)

The sequences of the V3 loop and surrounding regions of human immunodeficiency virus type-1 from a father-to-mother-to-infant trimmer were studied and the horizontal and vertical transmissions compared. The father's virus was variable for reactivity with neutralizing antibody and sequences of the V3 loop central core sequence. In contrast, the mother's viral sequences were much less diverse than those of the father, and N-glycosylation sites were conserved. By phylogenetic analysis, the major clone, of which V3-peptide reacted with the neutralizing antibody, was found to be transmitted from the mother to her infant; however, the mutated minor clones did not bind to the antibody. These finding suggest that both horizontal and vertical virus transmission were selective, and that the clonally transmitted virus in infants mutates more rapidly than viruses in the mother, to whom the virus was horizontally transmitted. See Ref. 16 for details.

(8) Bacterial features in the genome of methanococcus jannaschii in terms of gene composition and biased base composition in ORFs and their surrounding regions

Hidemi WATANABE, Takashi GOJOBORI, Kin-ichiro MIURA[1] ([1]Institute for Biomelocular Science, Gakushuin University)

As a result of genome projects, the complete nucleotide sequence of the

entire genome of an archaeon, *Methanococcus jannaschii*, was recently determined as well as other complete sequences of bacterial and eucaryal genomes. When all the 1680 predicted protein-cloning genes of *M. jannaschii* were classified on the basis of sequence similarity, it was found that this archaeon had a chimeric set of 1016 bacterial-type, 471 eucaryal-type and 193 species- or archaebacteria-specific genes. However, most of the genes predicted to be involved in translation and transcription pathways including RNA genes were of the eucaryal-type with only a few exceptions such as 16S ribosomal RNA and some translation factor-like genes. This appeared curious since previous studies indicated that methanogens have bacterial features in gene organization and expression. To understand the apparent inconsistency between physiological observations and the result of the classification of genes for transcription and translation, we examined the structural relatedness of the genome of *M. jannaschii* to those of other species. In practice, we compared based compositional patterns in ORFs and their surrounding regions. This made it possible to reveal the relationships among the translation- and transcription-related structures in genomes. In this study, we conducted a statistical test called '*G*-test' to evaluate the base biases around the boundaries of ORFs. We then found that *M. jannaschii* possesses more bacterial features in base biases than eucaryal ones, e.g. strong G biases at the positions corresponding to the Shine-Dalgarno site. This indicates that the few exceptional bacterial genes for translation, such as 16S ribosomal RNA and translation factor-like genes, play crucial roles in the translation pathway in *M. jannaschii*. The possibility that the genome structure in the last common ancestor of all present species was bacterial is discussed. See Ref. 17 for details.

(9) Evolutionary significance of intra-genome duplications on human chromosomes

Toshinori ENDO, Tadashi IMANISHI, Takashi GOJOBORI, Hidetoshi INOKO[1] ([1]Tokai University School of Medicine)

Phylogenetic analyses indicated that a series of paralogous gene pairs, found in two extensive regions on human chromosomal bands 6p21.3 and 9q33-34, were created by at least two independent duplications. The duplicated

genes on chromosomal band 6p21.3 include the genes for type 11 collagen a2 subunit (*COL11A2*), *NOTCH4* (mouse *int*-3 homologue), 70 kDa heat shock protein (*HSPA1A*, *HSPA1B*, and *HSPA1L*), valyl-tRNA synthetase 2 (*VARS2*), complement components (*C2* and *C4*), pre-B cell leukemia transcription factor 2 (*PBX2*), retinoid X receptor *b* (*RXRB*), *NAT/RING3*, and four other proteins. Their paralogous genes on chromosomal band 9q33-34 are genes for type 5 collagen a1 subunit (*COL5A1*), *NOTCH1*, 78 kDa glucose-regulated protein (*HSPA5*), valyl-tRNA synthetase 1 (*VARS1*), complement component C (*C5*), *PBX3*, retinoid X receptor a (*RXRA*), *ORFX/RING3L*, and others. Among these, the genes for collagem complement components, *NAT/RING3*, *PBX*, and *RXR* appear to have been duplicated around the time of vertebrate emergence, supporting the idea that they were duplicated simultaneously at that time. Another group of genes that includes *NOTCH* and *HSP* appear to have diverged long before that time. A comparison of the physical maps of these two regions revealed that the genes which duplicated in the same period were arranged in almost the same order in the two regions, with the assumption of a few chromosomal rearrangements. We propose a possible model for the evolution of these regions, taking into account the molecular mechanisms of regional duplication, gene duplication, translocation, and inversion. We also propose that a comparative mapping of paralogous genes within the human genome would be useful for identifying new genes. See Ref. 18 for details.

(10) Evolution of Nicotinic Acetylcholine Receptor Subunits

Kazuhisa TSUNOYAMA and Takashi GOJOBORI

Acetylcholine (ACh) has long been recognized as a neurotransmitter active in nervous and muscle systems of Bilateria. The nicotinic acetylcholine receptors (nAChR) belong to the superfamily of receptors involved in ligand-gated ion channels in both nervous and muscle systems. Because the nAChR in the muscle system was the first receptor to be isolated, the nAChR of striated muscles is the best-characterized member of the super family. It is known that the nAChR in the muscle system is a hetero-oligomer composed of five subunits ($\alpha 1$, $\beta 1$, γ, δ, and ε). As for the nAChR in the nervous

system, there are eight α-type (named $\alpha 2$ - $\alpha 9$) and three β-type (classified as $\beta 2$ - $\beta 4$) subunits.

It is of particular interest to understand how all 16 subunits are evolutionarily related to each other. In spite of the intensive previous works, the controversy over the evolutionary history of these subunits has not been settled yet. In order to resolve the controversy and to elucidate the evolutionary mechanisms of these subunits, we constructed a phylogenetic tree using 84 nucleotide sequences of receptor subunits from 18 different species. The tree constructed showed that the common ancestor of all subunits may have appeared first in the nervous system. Moreover, we suggested that the $\alpha 1$ subunits in the muscle system originated from the common ancestor of $\alpha 2$, $\alpha 3$, $\alpha 4$, $\alpha 5$, $\alpha 6$, and $\beta 3$ in the nervous system, whereas the $\beta 1$, γ, δ, and ε subunits in the muscle system shared the common ancestor with the $\beta 2$ and $\beta 4$ subunits in the nervous system.

Using the ratio (f) of the number of nonsynonymous substitutions to that of synonymous substitutions, we predicted the functional importance of subunits. We found that the $\alpha 1$ and $\alpha 7$ subunits had the lowest f values in the muscle and nervous system, respectively, indicating that very strong functional constraints work on these subunits. It is consistent with the fact that the $\alpha 1$ subunit has binding sites to the ligand and the $\alpha 7$-containing receptor regulates the release of the transmitter. Moreover, the window analysis of the f values showed that strong functional constraints work on the so-called M2 region in all 5 types of the muscle subunits.

For more details, see Ref.

Reference

TSUNOYAMA, K., GOJOBORI, T.,: Evolution of nicotinic acetylcholine receptor subunits. Mol. Biol. Evol., **15**, 518-527, 1998.

(11) Tie Trees Generated by Distance Methods of Phylogenetic Reconstruction

Naoko TAKEZAKI (National Institute of Genetics, The Pennsylvania State University)

Examining genetic data in recent publications, Backeljau et al. (1996) showed cases in which two or more different trees (tie trees) were constructed from a single data set for the neighbor-joining (NJ) method and the UPGMA. However, it is still unclear how often and in what conditions tie trees are generated. Therefore, I examined these problems by computer simulation. Examination of cases in which tie trees occur shows that tie trees can appear when no substitutions occur along some interior branch(es) on a tree. However, even when some substitutions occur along interior branches, tie trees can appear by chance if parallel or backward substitutions occur at some sites. The simulation results showed that tie trees occur relatively frequently for sequences with a low divergence level or with a small number of sites. For such data, UPGMA sometimes produced tie trees quite frequently, whereas tie trees for the NJ method were generally rare. In the simulation, bootstrap values for clusters (tie clusters) that differed among tie trees were mostly low (<60%). With a small probability, relatively high bootstrap values (at most 70% - 80%) appeared for tie clusters. The bias of the bootstrap values caused by an input order of sequences can be avoided if one of different paths in cycles of making an NJ or UPGMA tree is chosen at random in each bootstrap replication.

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I-b. Laboratory for Gene-Product Informatics

(1) Structural Requirement of Highly-Conserved Residues in Globins

Motonori OTA, Yasuhiro ISOGAI[1] and Ken NISHIKAWA ([1]The Institute of Physical and Chemical Research (RIKEN), Hirosawa, Wako, Saitama)

Glogins have remarkable sequence diversity, and yet maintain a common fold. In spite of the diversity, there are highly-conserved residues at several sites. The conserved residues were examined in terms of the structural stability, by employing the pseudo-energy functions of the structure/sequence compatibility method. The fitness of each residue type to the structural environment was evaluated at seven highly-conserved sites: the Leu (at the B10 site), Phe (CD1), and Leu (F4) residues were found to fit their respective sites due to hydrophobic interactions; and Pro (C2) stabilizes the N-terminal edge of an α-helical structure; and Phe (CD4) is stabilized by backbone hydrogen-bonding to Phe (CD1). On the other hand, the other two residues, His (E7) and His (F8), are poorly suited to the sites from a structural viewpoint, suggesting that their conservation clearly results from a heme-related functional requirement. The invariant Phe residue (CD1) has been suggested to be important for supporting the heme. The present analysis revealed that this residue is also well suited to the site in terms of energy. See Ref. 3 for details.

(2) Prediction of Protein Secondary Structure Using the 3D-1D Compatibility Algorithm

Masahiro ITO[1], Yo MATSUO[2] and Ken NISHIKAWA ([1]Gene Library Laboratory, [2]National Center for Biotechnology Information, National Library of Medicine, NIH, Bethesda, MD 20894, USA)

A new method for the prediction of protein secondary structure is proposed, which relies totally on the global aspect of a protein. The prediction scheme is as follows. A structural library is first scanned with a query sequence by the 3D-1D compatibility method developed before. All the structures examined are sorted with the compatibility score and the top 50 in the list are picked out. Then, all the known secondary structures of the 50 proteins are globally aligned against the query sequence, according to the 3D-1D alignments. Prediction of either α helix, β strand or coil is made by taking the majority among the observations at each residue site. Besides 325 proteins in the structural library, 77 proteins were selected from the latest release of the Brookhaven Protein Data Bank, and they were divided into three data sets. Data set 1 was used as a training set for which several adjustable parameters in the method were optimized. Then, the final form of the method was applied to a testing set (data set 2) which contained proteins of chain length <400 residues. The average prediction accuracy was as high as 69% in the three-state assessment of α, β and coil. On the other hand, data set 3 contains only those proteins of length >400 residues, for which the present method would not work properly because of the size effect inherent in the 3D-1D compatibility method. The proteins in data set 3 were, therefore, subdivided into constituent domains (data set 4) before being fed into the prediction program. The prediction accuracy for data set 4 was 66% on average, a few percent lower than that for data set 2. Possible causes for this discrepancy are discussed. See Ref. 4 for details.

(3) Differences in Dinucleotide Frequencies of Human, Yeast, and *E. coli* Genes

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

Nucleotide sequences coding proteins in human, yeast and *E. coli* genes were analyzed in terms of dinucleotide occurrences. Every gene is plotted as a point in the dinucleotide space, which is spanned by 16 axes corresponding to the 16 components of the dinucleotide. The metric unit in the space is defined using the log-odds ratio of dinucleotide occurrences in a gene. The distribution of points showed that genes from the same organism are clustered in the space. The clusters of human and *E. coli* are completely separated, and the yeast cluster sits in-between, implying that individual genes are classified into the three sources from their location. In fact, they could be identified with accuracy of 90%, using the DNA data alone. Even genes encoding homologous proteins belonging to the same protein superfamily were discriminated by the DNA data, and were correctly identified into their sources with the same accuracy as above. DNA sequences of non-coding regions, including human introns, as well as human genes of GC-rich and GC-poor types, were also analyzed in the same manner. The most significant finding is that human genomic DNA sequences, including genes and introns together, exhibit the largest deviation of dinucleotide occurrence from the random expectation. Possible origins for this phenomenon are discussed. See Ref. 5 for details.

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I-c. Laboratory of Gene Function

(1) Evolutionary Motif and Its Biological and Structural Significance

Yoshio TATENO, Kazuho IKEO, Hidemi WATANABE, Toshinori ENDO, Yumi YAMAGUCHI, Yoshiyuki SUZUKI, Kazunari TAKAHASHI, Kazuhisa TSUNOYAMA, Masato KAWAI[1], Yuichi KAWANISHI[1], Kimitoshi NAITOU[1] and Takashi GOJOBORI ([1] Fujitsu Limited, Makuhari System Laboratory, Mihama-ku, Chiba)

We developed a method for multiple alignment of protein sequences. The main feature of this method is that it takes the evolutionary relationships of the proteins in question into account repeatedly for execution, until the relationships and alignment results are in agreement. We then applied this method to the data of the international DNA sequence databases, which are the most comprehensive and updated DNA databases in the world, in order to estimate the " evolutionary motif " by extensive use of a supercomputer. Through a few problems needed to be solved, we could estimate the length of the motifs in the range of 20 to 200 amino acids, with about 60 the most frequent length. We then discussed their biological and structural significance. We believe that we are now in a position to analyze DNA and protein not only in vivo and in vitro but also in silico. For details, see Ref. 1.

(2) DNA Data Bank of Japan in the Age of Information Biology

Yoshio TATENO and Takashi GOJOBORI

DNA Data Bank of Japan (DDBJ) began its activities in 1986 in collaboration with EMBL in Europe and GenBank in the United States. DDBJ developed a data submission tool called Sakura, by which researchers can submit their newly sequenced data on WWW from every corner of the world. The data bank also built a database management system (Yamato II), incorporating the techniques and functions of the object-oriented database, in order to efficiently process the data it has collected. A number of research activities in information biology are also going on at DDBJ. For details, see Ref. 2.

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I-d. Laboratory for Molecular Classification

(1) Development of the data processing system for DNA Data Bank of Japan (DDBJ)

Hideaki SUGAWARA, Takashi GOJOBORI, Hirotada MORI[1], Takurou TAMURA, Toshitsugu OKAYAMA, Hikaru YAMAMOTO and Kousuke GOTO ([1] NARA Institute of Science and Technology)

The sequence data stored in DDBJ has increased to be more than 1G base-pairs in 1997. To cope with this data explosion, we improved systems for capturing, evaluation, storage and dissemination of sequence data and their annotation. The submission system of SAKURA is now equipped with visualization of annotations along sequences and resulted in supporting the sub-

mission of long sequences. The Genome Information Broker was newly developed for the comprehensive retrieval of microbial genome data. For details, see Ref.5 and 8, 9, 10, 14.

(2) Development of information systems for the study of biodiversity

Hideaki SUGAWARA, Satoru MIYAZAKI

2-1. WFCC-MIRCEN World Data Centre for Microorganisms (WDCM)

The WDCM is a data center of World Federation for Culture Collections (WFCC) and Microbial Resources Centers Network(MIRCEN). The Center has provided WWW server since April 1, 1997. This server was highly evaluated in the field of microbiology by the American Society of Microbiology and accessed 30,000 times a month on average. The WDCM is and will be an important facility for the study of microbial diversity which is introduced in Ref. 3 and 15.

2-2. Utilization of a broad-band network

It is prerequisite for the study of biodiversity that a number of centers distributed on the globe are able to share information. We did real-time experiments of virtual laboratory and distant learning system between Japan and US by use of a dedicated line of 45Mbps. For the details, see Ref. 1 and 9. We also proposed a concept of APBioNet for the promotion of bioinformartics in Asia Pacific region as introduced in Ref. 13. We especially studied an information environment composed of high-performance computers, software and networks. For details, see Ref. 7.

2-3. Classification and identification of microbes

The systematics is the base of the study of biodiversity and requires polyphasic analysis of data. It is at the stage of integration of conventional taxonomy based on phenotypic data and phylogenetic analysis based on molecular data. We designed an information system and also evaluated several methods of phylogenetic analysis for a large scale data. For details, see Ref. 6 and 11.

6 and 11.

(3) Homology modeling of the 3D structure of immunoglobulin

Satoru MIYAZAKI and Hideaki SUGAWARA

The one of the important aims of bioinformatics is to elucidate the relationships of sequences, 3D structure and function of biological macromolecules. We investigated the effects of somatic mutations in immunoglobulin variable region genes on the affinity maturation of antibodies using single precursor B cell-derived anti-DNA monoclonal antibodies generated from an autoimmune desease-prone NZB x NZW F1 mouse. Based on analyses of DNA sequences, homology modeling on a graphic workstation and molecular dynamics simulation of antigen-binding sites, we proposed a theory that the flexibility of antigen-binding Fv loops is associated with affinity-maturation ant-DNA antibodies. For details, see Ref. 2.

Publications

1. AATAGO, T., KIKUCHI, S., TATEYAMA, J., FUKITA, M., HARADA, H., YOKOKAWA, T. SUGAWARA, H. and MIYAZAKI, S.: An, experiment of research collaboration between Japan and U.S.A using broadband research network, **40(5)**, 404-413, 1997 04-413, 1997.
2. MIYAZAKI, S., SHIMURA, J., HIROSE, S., SANOKAWA, R., TSURUI, H., WAKIYA, M., SUGAWARA, H. and SHIRAI, T.: Is structural flexibility of antigen-binding loops involved in the affinity maturation of anti-DNA antibodies ?. International Immunology, Vol. **9**, No. 5, 771-777, 1997.
3. SUGAWARA, H. and MIYAZAKI, S.: Introduction to Biodiversity. Japan Society for Information and Knowledge Newsletter (in Japanese), No. 43, 3-12, 1997.
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6. FUTATSUKI, T., KAWANISHI, Y., NAITO, K., MIYAZAKI, S. and SUGAWARA, H.: Efficient Computation of Sequence Analysis in a Vector-Parallel Computer for the Study of Molecular Evolution. Genome Informatics 1997, Universal Academy Press (Tokyo), 264-265, 1997.

7. SUGAWARA, H. and MIYAZAKI, S.: Information environment for microbiology in the new age. The 5th JST International Symposium New Frontier in Microbiology, Tokyo, January.
8. SUGAWARA, H., GOTO, K., TAMAMOTO, H., KOIKE, T., OKAYAMA, T., ISHII, J., MIZUNUMA, T., TAMURA, T., MIYAZAKI, S., OTA, M., FUKAMI-KOBAYASHI, K., IMANISHI, T., IKEO, T., SAITOH, N., NISHIKAWA, K., TATENO, Y. and GOJOBORI, T.: DNA Data Bank of Japan (DDBJ) in the age of mass submission of sequences, HGM'97, Toronto, March.
9. SUGAWARA, H.: Utilization of broad-band networks for bioinformatics, Lecture Series on Network and Evolution of Molecular Information, Mishima, May.
10. TAMURA, T., OKAYAMA, T., GOTO, K., KOIKE, T., MIYAZAKI, S., GOJOBORI, T. and SUGAWARA, H.: Genome Information Broker: The JAVA Applet for the Processing and Presentation of Genome Data and the CGI Program for the Retrieval of the Data from the DDBJ Database, Objects in Bioinformatics, Hinxton Cambridge UK, June.
11. MIYAZAKI, S., FUTASUKI, T., KAWANISHI, Y. and SUGAWARA, H.: Phylogenetic Analysis of Large Scale Datasets. V-th Annual Meeting of Japan Society for Culture Collections (Wako), June, 1997.
12. SATOU, K., MIYAZAKI, S. and OHYA, M.: Analysis of HIV by Entropy evolution rate. 5th International congress on amino acids, Chalkidiki, Greece, August.
13. SUGAWARA, H. and MIYAZAKI, S.: A proposal of Asia-Pacific Bioinformatics Network (APBioNet). The 8th CODATA/DSAO - New Perspectives on S&T Information Processing, Taejon Korea, November.
14. SUGAWARA, H.: Genome Information Broker for Microbes: International Workshop on Genome Project 1997 The Frontier of Genome Research on Microorganisms. ganisms, Tokyo, December.
15. SUGAWARA, H.: Information on Biological Research Resources as an Infrastructure of the study of Biodiversity. CBI meeting (Tokyo), December, 1997.

J. RADIOISOTOPE CENTER

(1) Feedback loops controlling the initiation of sporulation in *Bacillus subtilis*

Masaya FUJITA and Yoshito SADAIE

The early gene expression required for the development of spores in *Bacillus subtilis* is regulated by the Spo0A transcription factor. Spo0A activity is dependent on phosphorylation. The immediate source for the phosphorylation of Spo0A is the phosphorelay signal transduction system, a more complicated variation of two-component signal transduction systems. The first step is the activation of protein kinases, predominantly the products of the *kinA* and *kinB* genes, leading to ATP-dependent autophosphorylation. A third kinase, the product of the *kinC* gene, is required under some growth conditions. The phosphate group is then transferred to the Spo0F protein. Spo0F-P in turn becomes a phosphodonor for Spo0B, a phosphoprotein-phosphotransferase catalysing the transfer of phosphate to the Spo0A protein. RNA polymerase containing σ^A(E σ^A) or σ^H(E σ^H) transcribes the genes of the phosphorelay components. Phosphorylated Spo0A is also involved in their expression and is required for the induction of σ^H by repressing its repressor gene *abrB*. We have examined the effects of phosphorylated Spo0A (Spo0A-P) and AbrB on *in vitro* transcription of the genes involved in the Spo0A phosphorylation and initiation of sporulation. Spo0A-P repressed E σ^A-dependent transcription of the *kinC* and E σ^H-dependent transcription of *spo0A* and *kinA*. E σ^H-dependent transcription of *spo0F* was stimulated by Spo0A-P at low concentrations but was repressed by higher amounts of Spo0A-P. On the other hand, AbrB repressed E σ^A-dependent transcription of *spo0H*(σ^H gene), *kinC* and *abrB*, although its effect was not strong. The results obtained support the notion of control circuits in which the Spo0A and AbrB proteins activate or repress the initial sporulation pathway. (to be published in J.Biochem.)

(2) Promoter Selectivity of the *Bacillus subtilis* RNA Polymerase σ^A and σ^H Holoenzymes

Masaya FUJITA and Yoshito SADAIE

The $\sigma^H$of *Bacillus subtilis* directs transcription of a large number of early sporulation genes, whereas the principal σ factor, σ^A, is essential for the transcription of the genes for vegetative growth and early sporulation. We have purified $\sigma^A$and σ^H proteins, and characterized their properties. The genes encoding σ^Aor σ^H were separately cloned into an expression vector under the control of T7 promoter. Both proteins were overproduced in *Escherichia coli* BL21(DE3) and purified from inclusion bodies after solubilization with guanidine hydrochloride. Antigenicities and N-terminal amino acid sequences of the overproduced proteins were used to identify both proteins. Unlike σ^A protein, σ^Hprotein showed a DNA-binding ability. To compare the promoter selectivity of the σ^Aprotein with that of the σ^Hprotein, transcription *in vitro* of 16 promoters was performed using RNA polymerase holoenzymes reconstituted from a purified core enzyme with either σ^Hor σ^A. These holoenzymes correctly recognized each of the cognate promoters; σ^H-RNA polymerase recognized σ^Hpromoters but not σ^Apromoters, and *vice versa*. A competition experiment for core RNA polymerase using σ^A and σ^H revealed that σ^A had a stronger affinity. We propose that the predicted replacement of a σ subunit in a holoenzyme from σ^A to σ^H *in vivo* at late logarithmic growth phase may require an additional factor, or the modification of a core enzyme or σ factor.
(to be published in J.Biochem.)

(3) Functional analysis of the *phoB/cotA* region of the *Bacillus subtilis* chromosome containing the konjac glucomannan utilization operon

Yoshito SADAIE and Katsunori YATA

The *phoB-rrnE-groESL-gutR-cotA* region (70kb) of the *Bacillus subtilis* genome contains several operons of unknown or not well known function. They include mannan utilization operon, glycoprotein endopeptidase operon, glucitol utilization operon, and an operon for inherent restriction/modification system

genes. A large operon involved in degradation and incorpolation of mannan consists of eight ORFs whose predicted products includs PTS system proteins as well as mannan digesting enzyme. The expression of this operon was induced by konjac glucomannan and repressed by glucose or mannose. Yeast mannan did not induce the expression of the operon. Disruption of an ORF having a HTH domain resulted in a high expression of the operon suggesting that this ORF is an internal repressor gene of the operon. *Bacillus subtilis* chromosome harbors an operon for utilizing glucomannan, a plant-derived carbon source.

(4) Restricted Transcription from Sigma H or Phosphorylated Spo0A Dependent Promoters in the Temperature-sensitive *secA341* Mutant of *Bacillus subtilis*

Kei ASAI[1], Masaya FUJITA[2], Fujio KAWAMURA[3], Hideo TAKAHASHI[4], Yasuo KOBAYASHI[5] and Yoshito SADAIE[2] ([1,3,4] Institute of Molecular and Cellular Biosciences, The University of Tokyo, [2] Radioisotope Center, National Institute of Genetics, [5] Laboratory of Molecular Biology and Microbial Chemistry, Tokyo University of Agriculture and Technology, [1] Present address : Department of Cell Biology, The graduate School of Biological Sciences, Nara Institute of Science and Thechnology, [3] Present address : Laboratory of Molecular Biology, College of Science, Rikkyo University.)

The temperature-sensitive *secA341* mutation of *Bacillus subtilis* affects sporulation and sporulation-associated events as well as protein secretion and cell septation. With *lacZ* or *bgaB* fusion genes, we examined the expression of the early sporulation genes in the mutant strain. Transcriptional expression of σH dependent *kinA*, *spo0A* (Ps), *phrC*, *spoVG* and *citG* (p2) genes was blocked by the *secA341* mutation at 37C. On the other hand, neither repression of the *abrB* gene nor induction of *spo0H* (σH) gene was affected. Active RNA polymerase containing σH was, however, found to be produced in the mutant cell. Expression of the phosphorylated Spo0A dependent *spoIIG* operon was also blocked. Thus the *secA341* mutation blocks some step(s) or factor(s) required for σH-dependent transcription *in vivo*. (To be published in Biosci.Biotechol.Biochem.)

(5) The *secA341* Mutation Inhibits Expression of the *Bacillus subtilis* Protease Gene, *aprE* by Blocking DegS/DegU depending activation step

Yoshito SADAIE

The production of extracellular protease(s) of *Bacillus subtilis* is induced in the post-exponential growth phase, and is severely reduced in a strain carrying a temperature sensitive mutation (*secA341ts*) in the *secA* gene, the product of which is required for protein secretion. The expression of the extracellular serine protease gene, *aprE*, as monitored by the β-galactosidase activity of an *aprE-lacZ* translational fusion, was inhibited in the *secA341* mutant cell and restored by the introduction of the *degU32*(Hy) mutation which makes the phosphorylated DegU response regulator of a two-component system refractory to dephosphorylation and renders the cell to become a strong producer of AprE. The *secA341* mutation appears to block some activation step required for *aprE* expression. In fact, in the *secA341* mutant at 37C, the expression of the *degR* gene, whose product is a stabilizer of phosphorylated DegU, is completely repressed. This repression was reliefed by the deletion mutation in the anti-sigma D gene, *flgM*. As *degR* gene is transcribed by RNA polymerase containing sigma D, repressed expression of the *degR* in the *secA* mutant cell is due to the undiluted anti-sigma D and results in destabilization of DegU-P leading to repressed expression of *aprE*. (To be published in Biosci.Biotechol.Biochem.)

Publication

1. ASAI, K., KAWAMURA, F., SADAIE, Y. and TAKAHASHI, H.: Isolation and characterization of a sporulation initiation mutation in the *Bacillus subtilis secA* gene. J.Bacteriol. **179**, 544-547.
2. SADAIE, Y., YATA, K., FUJITA, M., SAGAI, H., ITAYA, M., KASAHARA, Y. and OGASAWARA, N.: Nucleotide sequence and analysis of the *phoB-rrnE-groESL* region of the *Bacillus subtilis* chromosome. Microbiol. **143**,1861-1866.
3. KASAHARA, Y., NAKAI, S., OGASAWARA, N., YATA, K. and SADAIE, Y.: Sequence analysis of the *groESL-cotA* region of the *Bacillus subtilis* genome, containing the restriction/ modification system genes. DNA Res. **4**,335-339.
4. KUNST, F.: et al. The complete genome sequence of the Gram-positive bacterium *Bacillus subtilis*. Nature. **390**:249-256.

K. EXPERIMENTAL FARM

(1) Development, Evaluation and Distribution of the Genetic Stocks of Rice

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

We have conducted the reproduction, evaluation and distribution of genetic stocks of wild and cultivated rice. Especially, plenty of the collection of wild species is useful for practical breeding and evolutional studies. It contains *Oryza sativa* complex, which is classfied into AA genome species, *O. officinalis* complex of BB, BBCC, CC, CCDD, EE and FF genome species, and so on. These are also interesting sources for basic science, because rice has the smallest genome size in all cereal crops, corn, barley, wheat, rye, millet and sorghum. These stocks have been distributed as the requests for basic researches. Information about these stocks were collected and made up in the rice genetic stock database (by Drs. Morishima, Kinoshita and Yamazaki) and is accessible at the site of http://www.shigen.nig.ac.jp/NIG_rice/rice.html.

Another trial to develop genetically tagged strains by enhancer trapping are now in progress in cooperation with the plant genetics laboratory. Care of seeding, crossing, screening and distribution of these newly developing genetic materials will be taken by the experimental farm in a few years.

Publication

None

ABSTRACTS OF DIARY FOR 1997

Biological Symposium

488th-Jan. 29	Anaerobic and Nirate Control of Respitory Pathway Gene Expression in Escherichia coli (Robert P.Gunsalus)
489th-Feb. 17	Correct kinetic measurement by surface plasmon resonance:principle and limitation of Biacore (Peter Schuck)
490th-Feb. 21	"Dissecting Hydra Development Using Molecular Biology" (Robert E.Steele)
491st-Feb. 24	Molecular Changes in the Eye Lens during Aging and in Cataract (D.Balasbramanian)
492nd-Mar. 18	"Organization and function of MHC class Ib genes" (Kirsten Fischer Lindahl)
493rd-Mar. 24	"Molecular mechanisms of axon guidance and cell migrations inan invertebrate spinal cord- the nematode C.elegans" (Joseph G.Culotti)
494th-Apr. 4	"Drosophila Wing Morphogenesis" (Antonio Garcia-Bellido)
495th-Apr. 9	"Cell/Extracellular Matrix Interactions in Hydra vulgaris"
496th-Apr. 10	Genetic Control of Morphogenesis and Evolution of the Eye (Walter J.Gehring)
497th-Mar. 21	The structure and function of bovine mitochondrial F1-ATPase;An example of rotational catalysis (Andrew G.W.Leslie)
498th-June 2	Role of General and Gene-Specific Co-activators in Transcription Regulation (Robert G.Regulation)
499th-June 27	"Molecular Insight into Origin and Composition of Microbial Communities" (James M.Tiedje)
500th-July 9	The Role of Apoptosis in the Pathogenesis of Sacral Agenesis (Alisa S W Shum)
501st-June 22	Chlorophyll and Bacteriochlorophyll:Theory and Experi-

	ment in Unison (Ian Gould)
502nd-Aug. 11	"Mammalian Molecular Phylogeny:Everything you know is wrong" (Dan Graur)
503rd-Sep. 12	Coupling of Local DNA Topolpgy to tyrR Promoter Activity (Andrew Travers)
504th-Oct. 8	Molecular mechanisms involved in specific recongnition of pathogens in plants (Fumiaki Katagiri)
505th-Oct. 15	Molecular mechanisms of synaptic initiation:how postsynaptic cells talk back to presynaptic cells (Akira Chiba)
506th-Nov. 4	"Maternal Factors That Guide Early Development Of The Germline In C.elegans." (Susan Strome, Ichiro Kawasaki)
507th-Nov. 5	"Translation termination in higher eukaryotes:news and views" (Lev Kisselev)
508th-Nov. 12	"Germ Line Establishiment in Drosophila melanogaster" (Paul Lasko)
509th-Nov. 19	Beyond Genomes:Emerging Role of Mass Spectrometry in Proteome Studies (Hiroyuki Matsumoto)
510th-Nov. 21	"Role of Bone Morphogenetic Proteins (BMPs) in Forebrain and Eye Development" (Yasuhide Furuta)
511st-Nov. 27	Neurohormone Cascades:A Mechanism for Controlling Behavioral Sequences in Insects (James W.Truman)
512nd-Nov. 27	Orchestration of Insect Molting and Metamorphosis: Hormonal Regulation and Molecular Switches (Lynn M. Riddiford)
513rd-Dec. 3	"The Myosin VIIA Deafness Gene-Genetic and Functional Analysis in Mouse and Human" (Steve D.M.Brown)
514th-Dec. 8	"Electrostatic Properties of Biological Macromolecules" (Barry Honig)
515th-Dec. 9	"Structure-based design" (Tom Blundell)
516th-Dec. 10	"Biological Database Searching and Statistical Significance" (Michael S.Waterman)
517th-Dec. 15	Transcriptional Behavior of Neutrophil White BloodCells (Sherman M.Weissman)

Mishima Geneticists' Club

501st-Jan. 8	Characterization of the ADA transcriptional co-activator complex (Junjiro Horiuchi)
502nd-Jan. 16	New methods for molecular manipulation of DNA (Masao Washizu)
503rd-Feb. 12	Genetic variability and Cell Tropism of HIV (Nobuaki Shimizu)
505th-Apr. 16	Characterization of RuvB like mammalian TIP49 protein whichi has a DNA dependent ATPase/helicase activity (Takaaki Tamura)
506th-May 14	Cascades of transeviptional control factors producing variabilities of mammalian neuroral cells (Tetsuichiro Saito)
507th-May 21	Molecular Evolution of Rotaviruses -Toward New Paradigms- (Osamu Nakagome)
508th-June 6	Small teleost species as valuable genetic resource (Hiroshi Hori)
509th-June 10	Bioinformatics : analyses of *Escherichia coli* and Sccharomyces cerevisiae complete genomes (Katsumi Isono)
510th-June 11	DNA replication machinery and mechanism of Sphase checkpoint (Hiroyuki Araki)
511st-June 12	Cellular function of AAA protease FtsH in Escherichia coli (Hikaru Ogura)
512nd-June 13	Coupling of gene expression and morphogenesis-Bacterial flagella serve as a transcriptional regulator (Kazuhiko Kutsukake)
513rd-June 17	Transcriptional regulation of the yeast rRNA gene-Molecular genetic approach (Yasuhiro Nogi)
514th-June 18	Molecular genetics of clock genes in cyanobacteria (Masahiro Ishimura)
515th-June 23	Role of MAT1 in phosphorylation of Oct-1 transcription factor by CDK activating kinase (Susumu Inamoto)
516th-July 4	Centromere-specific nucleosome structure in human

(Kinya Yota)

517th-Aug. 18 Interspecific chromosome transfer as a useful too for the study of speciation in Drosophila (Kyoichi Sawamura)

518th-Oct. 27 Role of SUMO/UBC9 modifier system in nuclear transport and cell cycle control (Sumihito Saito)

519th-Nov. 13 Cloning of the genes essential for morphogenesis of Zebrafish using the retro virus-mediated insertional mutagenesis (Koichi Kawakami)

520th-Nov. 19 Promoter-transcription activation protein interaction *in vivo* : Roles of cooperative DNA binding and activation-domain function (Masashi Tanaka)

521st-Nov. 13 Hepatitis virus and liver cancer

522nd-Nov. 27 Postembryonic Development of the Drosophila Brain Circuit (Kei Ito)

523rd-Dec. 22 Induction gene-trapping : Identification of genes active down stream of BDNF (Mika Karasawa)

524th-Dec. 22 *Glial cells missing*, gene for the regulation of differenciation between neuron and glia in *Drosophila* (Toshihiko Hosoya)

FOREIGN VISITORS IN 1997

Jan. 26-Feb. 13	J.Gowrishankar,Center for Cellular Molecular Biology, India
Feb. 14-22	Robert Steele,University of California Irvine,U.S.A.
Jan. 27-29	Robert P.Gunsalus,University of California,Los-Angeles,U.S.A.
Jan. 28-29	A.M.Chakrabarty,University of Illinois, Chicago,Chicago Illinois,U.S.A.
Feb. 12-Mar. 7	Dipankar Chatterji,Centre for cellular and Molecular Biology,India
Feb. 12-Mar. 31	Jeffrey T.Owens,University of California, Davis, U.S.A.
Feb. 23-25	D.Balasubramanian,Centre for Cellular and Molecular Biology,India
Mar. 10-Apr. 27	Talat Malik,University of Nottingham, Nottingham, U.S.A.
Mar. 18-19	Kirsten Fischer Lindahl,Howard Hughes Medical Institute U.T.Southwestern Medicine Center,U.S.A.
Mar. 19-June. 1	Masatoshi Nei,Pennsylvania State University,U.S.A
Mar. 24	Joseph G.Culotti,Samuel Lunenfeld Research Institute Mt.Sinai Hospital,Canada
Mar. 30-Apr. 11	Michael P.Sarras Jr,University of Kansas Medical Center,U.S.A.
Apr. 1-	Zhangyi Qu, Harbin Medical University, China
Apr. 4-5	Antonio Gercia-Bellido,Universidad Autonoma de Madrid,Spain
Apr. 7	Gerald Selzer,National Science Foundation,Arlington, U.S.A.
Apr. 7	Machi F Dilworth,National Science Foundation,Arlington, Virginia,U.S.A.
Apr. 10-11	Walter J.Gehring,University of Basel,Switzerland
Apr. 14-15	Delill S.Nasser,National Science Foundation Program,

	U.S.A.
Apr. 24-May. 21	Goonnapa Fucharoen,Khon Kaen University,Thailand
May 18-June. 13	J.Bown,University of Birmingham,School of Biochemistry,UK
May 20-21	Andrew G.W.Leslie,MRC Laboratory of Biology,UK
May 22-June. 15	Kan Ohno,Beckman Research Institute of the City of Hope,U.S.A.
June 18-Aug. 14	Dan Graur,Tel Aviv University,Israel
June 21-25	Matthew Bellgard,Murdoch University,Australia
July 9-10	Alisa S.W.Shum,The Chinese University of Hong Kong, Hong Kong
July 28-Aug. 3	Dipankar Chatterji,Center of Cellular and Molecular Biology,India
July 28-Sep. 7	Kakoli Mukherjee,Center of Cellular and Molecular Biology,India
Aug. 4-26	Giorgio Bernardi,Laboratoire de Genetique Molecularie Institut Jacques Monod, France
Aug. 18-20	Alberto Bernardi,Laboratoire de Genetique Moleculaire Institut Jacques Monod, France
Sep. 9-Dec. 7	Taciana Kostionkovitch,Edinburg University,UK
Sep. 9-Oct. 3	Richard Hayward,Edinburg University,UK
Sep. 24-Oct. 7	Juncai Ma,Chinese Academy of Sciences,The Institute of Microbiology,China
Oct. 4-Nov. 3	Noboru Sueoka,University of Colorado at Boulder, U.S.A.
Oct. 14-15	Akira Chiba,University of Illinois,U.S.A.
Oct. 27	Holling Worth,R.,University of Wisconsin Madison, Madison,Wisconsin,U.S.A.
Nov. 4-5	Susan Strome,Indiana University,U.S.A.
Nov. 5-6	Lev Kisselev,Laboratory of Molecular Bases of Oncogenesis Engelhardt Institute of Molecular Biology, Moscow
Nov. 12-14	Paul Lasko,Mcgill University,Canada
Nov. 19-20	Hiroyuki Matsumoto,University of Oklahoma Health Sciences Center,Oklahoma,U.S.A.
Nov. 21-22	Yasuhide Furuta,Vanderbilt University School of

	Medicine,Nashville,TN,U.S.A.
Nov. 25-28	Lynn M.Riddiford,University of Washington,Seattle, WA, U.S.A.
Nov. 25-28	James W.Truman,University of Washington, Seattle, WA, U.S.A.
Dec. 3-4	Steve D.M.Brown,MRC Mouse Genome Centre, Harwell, OX,ORD, UK
Dec. 8	Barry Honig,Columbia University,U.S.A.
Dec. 8-9	Tom Blundell,University of Cambridge,U.K
Dec. 10-11	Michael Waterman,University of Southern California, U.S.A.
Dec. 15	Sherman M.Weissman,Yale University school of medicine, CT,U.S.A.

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MORIWAKI, Daigoro; Professor emeritus, Tokyo Metropolitan University

AUTHOR INDEX

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Tel. 0559-81-6718
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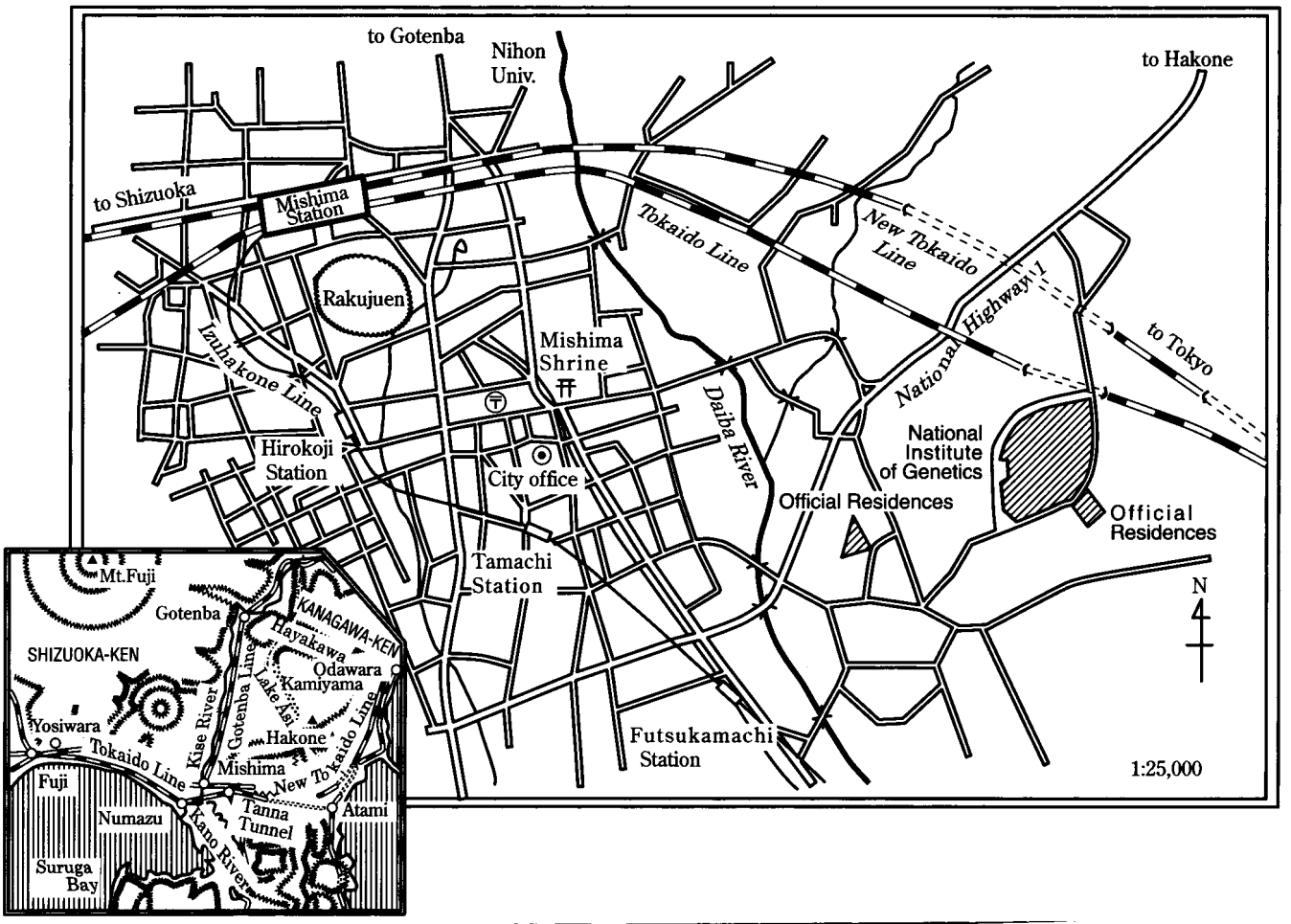
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