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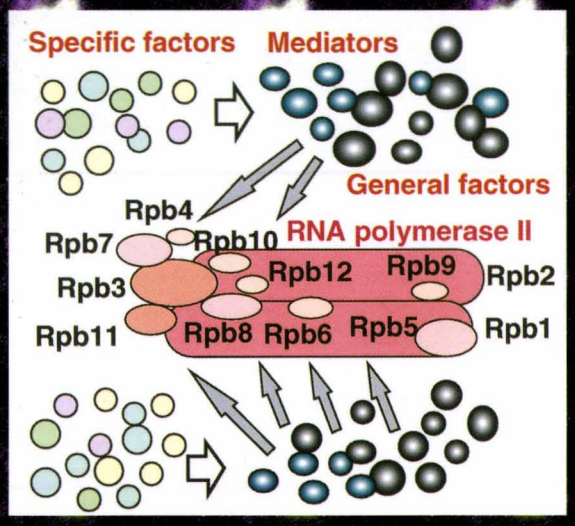

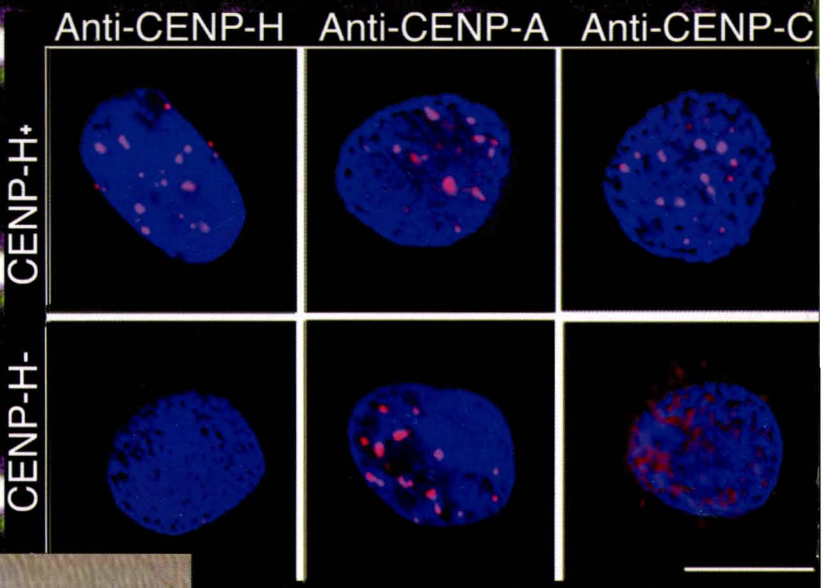

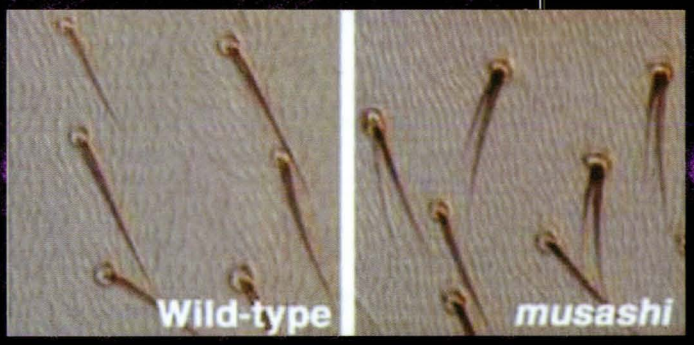

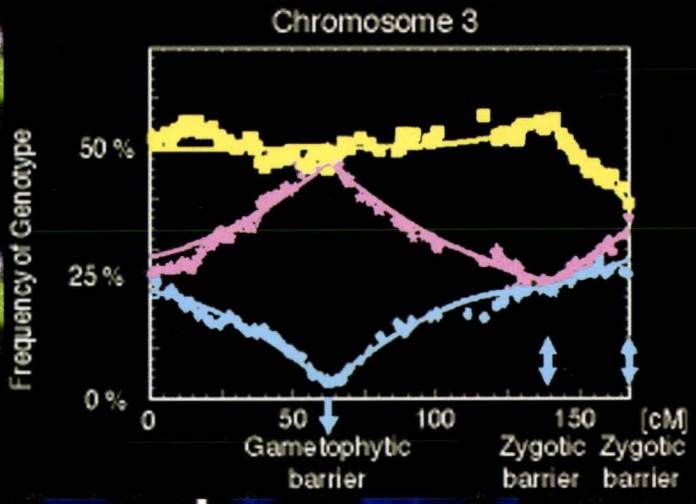

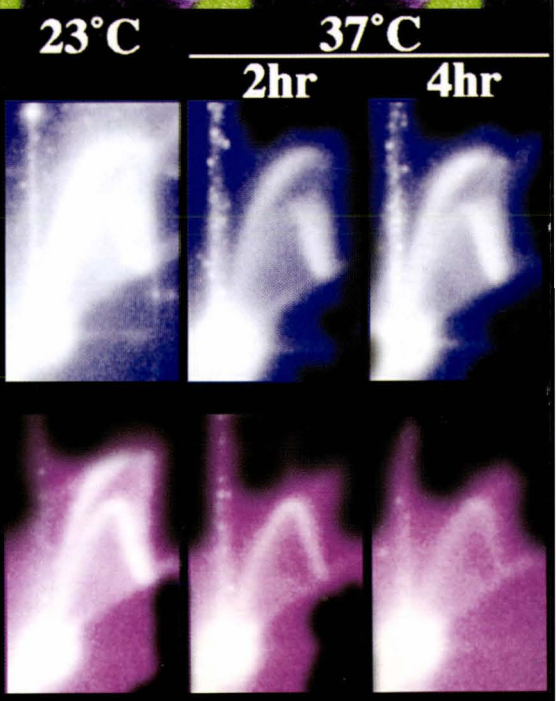

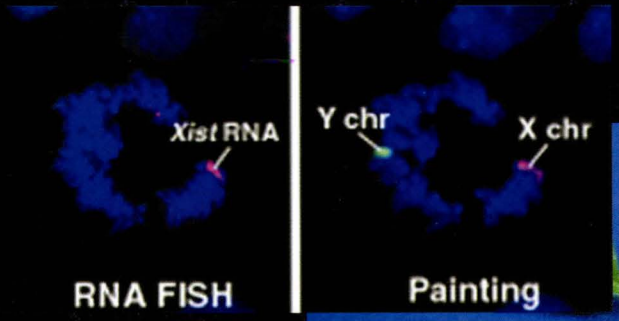

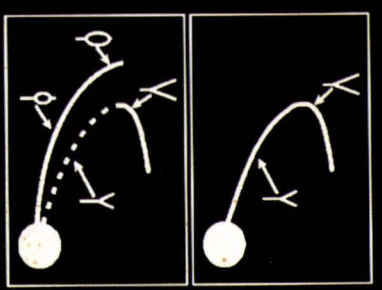

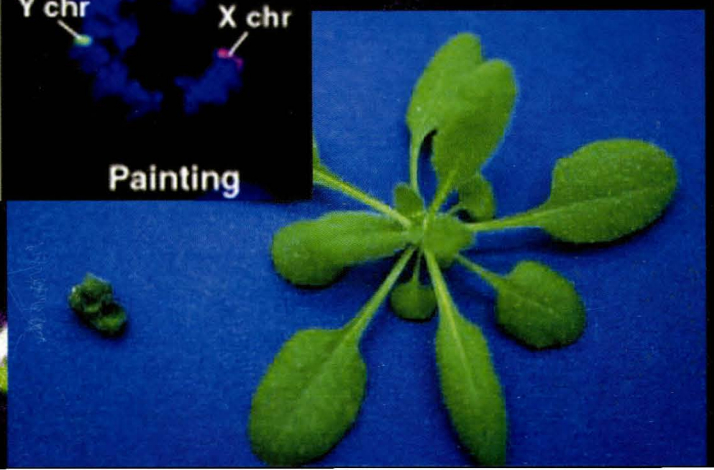

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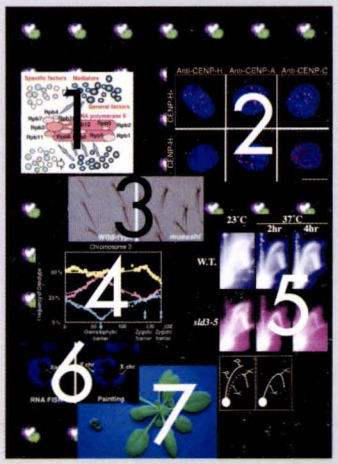

1. **Picky RNA polymerases**

Intracellular contents and assembly states of all twelve subunits of the RNA polymerase II in the fission yeast Schizosaccharomyces pombe.
Kimura, M., Sakurai, H., Ishihama, A.
Eur. J. Biochem., *268*, 612-619 (2001).

2. **Centromere assembly in vertebrate cells**

CENP-H, a constitutive centromere component, is required for centromere targeting of CENP-C in vertebrate cells.
Fukagawa, T., Mikami, Y., Nishihashi, A., Regnier, V., Haraguchi, T., Hiraoka, Y., Sugata, N., Todokoro, K., Brown, W., Ikemura, T.
EMBO J., *20*, 4603-4617 (2001).

3. **Translational repression determines neuronal potential**

Translational repression determines a neuronal potential in Drosophila asymmetric cell division.
Okabe, M., Imai, T., Kurusu, M., Hiromi, Y., Okano, H.
Nature, *411*, 94-98 (2001).

4. **A genome-wide survey of reproductive barriers**

A genome-wide survey of reproductive barriers in an intraspecific hybrid.
Harushima, Y., Nakagahra, M., Yano, M., Sasaki, T., Kurata, N.
Genetics, *159*, 883-892 (2001).

5. **Initiation of eukaryotic DNA replication**

Sld3, which interacts with Cdc45 (Sld4), functions for chromosomal DNA replication in Saccharomyces cerevisiae.
Kamimura, Y., Tak, Y.-S., Sugino, A., Araki, H.
EMBO J., *20*, 2097-2107 (2001).

6. **Regulation of imprinted X-inactivation by Tsix**

Regulation of imprinted X-chromosome inactivation in mice by Tsix.
Sado, T., Wang, Z., Sasaki, H., Li, E.
Development, *128*, 1275-1286 (2001).

7. **Taming the genome by DNA methylation**

Mobilization of transposons by a mutation abolishing full DNA methylation in Arabidopsis.
Miura, A., Yonebayashi, S., Watanabe, K., Toyama, T., Shimada, H., Kakutani, T.
Nature, *411*, 212-214 (2001).

CONTENTS

B. DEPARTMENT OF CELL GENETICS

C. DEPARTMENT OF DEVELOPMENTAL GENETICS

Introduction

This is the 52nd annual report of the National Institute of Genetics (NIG). The planning of administrative reform, aimed at reorganizing research institutes and national universities into "agencies", has continued from the previous year. In September, the Council on the Administrative Reforms for National Universities issued a preliminary report, showing the future reform path for research institutes such as NIG. I believe this reform should not be just a restructuring for the purpose of efficiency, but one that will, in the future, aid the improvement of academic research and higher education. In order to achieve this, we need to further improve the quality of our research and create a solid justification for our existence.

I'm happy to report here that we continued to produce many excellent research achievements this year. Large projects supported by NIG, such as the DNA Data Bank of Japan, have also progressed smoothly. Thanks to improvements to our home page and the completion of the "Internet Museum of Genetics", our activities to popularize knowledge and research results of genetics have also started. This year is memorable for the study of genetics because of the elucidation of human genome draft sequence. We have already obtained full knowledge of genome sequences about many organisms, including many bacteria, nematode and fruit fly. By adding human genome to this list, we have really entered a new era. We would like to seriously consider about its influence on our future research.

The following personnel changes of the faculty took place during 2001. Professor Tomoko Ogawa (Vice-Director and the head of the Department of Cell Genetics) who for many years contributed to the development of NIG became emeritus reaching the mandatory retirement age. Professor Akira Ishihama (Department of Molecular Genetics) succeeded Prof. Ogawa as the Vice-Director. The Center for Information Biology was reorganized as the Center for Information Biology and DNA Data Bank of Japan, adding a new laboratory: the Laboratory of Gene-Expression Analysis. We made two new recruitments this year: Assistant Professor Tetsu Kinoshita has joined the Division of Agricultural Genetics and Assistant Professor Hiroki Kokubo (Genetic Strains Research Center) transferred from Okazaki National Institute for Basic Biology, valuable additions to the research staff. On the other hand, Professor Hiroyuki Takeda and assistant professor Atsushi Kawakami (Early Development) left NIG to form a new group at the Graduate Course in the University of Tokyo. Assistant Professor Toshihiko Hosoya (Developmental Genetics), Assistant Professor Shigeo Tanaka (Cell Genetics) and Assistant Professor Tadashi Imanishi (Center for Information Biology) have left the institute to extend their career in other institutions.

The following personnel changes of the administrative staff took place: in April, Akira Kobayashi, Head of the General Affairs Section, was transferred to Shinshu University as Head of General Affairs, and was replaced by Yukio Tomiyama. In October, Kiyotaka Uezumi, Head of Administration, was transferred to Chiba University as Head of Student Affairs and was replaced by Kenji Ishikawa.

As the Genetics Department of the Graduate University for Advanced Studies, 41 students studied in our PhD program. Eleven students entered the program this year, and seven have obtained the degree of Doctor of Science. Five foreign researchers and eight graduate students were supported by the COE (Center of Excellence) Program.

Yoshiki Hotta, Director-General

STAFF (as of December 31, 2001)

Director-General

HOTTA, Yoshiki, D. Med.

Vice-Director

ISHIHAMA, Akira, D. Sc.

Members

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Department of Molecular Genetics

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MITSUZAWA, Hiroshi, D. Sc.
KIMURA, Makoto, D. Sc.

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SEINO, Hiroaki, D. Sc.

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Division of Microbial Genetics

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YASUDA, Seiichi, D. Sc., Associate Professor
KAMIMURA, Yoichiro, D. Med.

Division of Cytoplasmic Genetics

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YANAGIDA, Mitsuhiro, D. Sc., Adjunct Professor

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OKABE, Masataka, D. Med., D. Sci.

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MINATO, Kiyoshi, M. Sc.
YAMADA, Masa-aki, D. Ag.

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KIYAMA, Ryoiti, D. Sc., Adjunct Professor

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TAKANO, Toshiyuki, D. Sc.
Division of Evolutionary Gentics
IKEMURA, Toshimichi, D. Sc., Professor
SAITOU, Naruya, Ph. D., Associate Professor
TENZEN, Toyoaki, D. Ag.
FUKAGAWA, Tatsuo, D. Sc.
Division of Theoretical Genetics
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TAKAGI, Toshihisa, D. Eng., Adjunct Profesor

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SASAKI, Hiroyuki, D. Med., Head of the Department
Department of Human Genetics
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FUJIYAMA, Asao, D. Sc., Associate Professor
SADO, Takashi, D. Sc.
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KAKUTANI, Tetsuji, D. Sc., Associate Professor
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Division of Brain Fanction
HIRATA, Tatsumi, D. Med., Associate Professor
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TAKAGI, Nobuo, D. Sc., Adjunct Professor

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KOIDE, Tsuyoshi, D. Med.
Mammalian Development Laboratory
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KOKUBO, Hiroki, D. Sc.
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KURATA, Nori, D. Ag., Associate Professor
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Microbial Genetics Laboratory
NISHIMURA, Akiko, D. Ag., Associate Professor

Invertebrate Genetics Laboratory
HAYASHI, Shigeo, D. Sc., Professor
GOTO, Satoshi, D. Sc.

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KOHARA, Yuji, D. Sc., Head of the Center
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FUJITA, Masaya, D. Eng.
Genome Biology Laboratory
KOHARA, Yuji, D. Sc., Professor
ANDACHI, Yoshiki, D. Sc.

8. Structural Biology Center
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TOKUNAGA, Makio, D. Sc., Professor
SHIINA, Nobuyuki, D. Sc.
Molecular Biomechanism Laboratory
SHIMAMOTO, Nobuo, D. Sc., Professor
SOGAWA, Kumiko, D. Sc.
NAGAI, Hiroki, D. Sc.
Multicelluar Organization Laboratory
KATSURA, Isao, D. Sc., Professor
ISHIHARA, Takeshi, D. Sc.
Biomolecular Structure Laboratory
SHIRAKIHARA, Yasuo, D. Sc., Associate Professor
MAENAKA, Katsumi, D. Eng.
Gene Network Laboratory
IMAMOTO, Naoko, D. Med., Associate Professor
KOSE, Shingo, D. Med.

9. Center for Information Biology and DNA Data Bank of Japan
GOJOBORI, Takashi, D. Sc., Head of the Center
Laboratory for DNA Data Analysis
GOJOBORI, Takashi, D. Sc., Professor
IKEO, Kazuho, D. Sc.
Laboratory for Gene-Product Informatics
NISHIKAWA, Ken, D. Sc., Professor
OTA, Motonori, D. Sc.
Laboratory for Gene Function
TATENO, Yoshio, Ph. D., D. Sc., Professor
FUKAMI-KOBAYASHI, Kaoru, Ph. D.
Laboratory for Molecular Classification
SUGAWARA, Hideaki, D. Eng., Professor
MIYAZAKI, Satoru, D. Sc.

10. Radioisotope Center
NIKI, Hironori, D. Med., Head of the Center

11. Experimental Farm

KURATA, Nori, D. Ag., Head of the Farm
NONOMURA, Ken-ichi, D. Ag.

12. Technical Section

ISHII, Yuriko, Chief of the Section

13. Department of Administration

ISHIKAWA, Kenji, Head of the Department
TOMIYAMA, Yukio, Chief of the General Affairs Section
TAKAHASHI, Shouji, Chief of the Finance Section

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Vice-chairman

OSAWA, Shozo; Adviser, Biohistory Research Hall

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KATSUKI, Motoya; Director-General, National Institute for Basic Biology
KODAIRA, Keiichi; President, The Graduate University for Advanced Studies
KURODA, Reiko; Professor, Graduate School of Arts and Science, The University of Tokyo
KYOGOKU, Yoshimasa; Director-General, Biological Information Research Center, National Insttute of Advanced Industrial Science and Technology
MATSUBARA, Ken-ichi; President, DNA Chip Research Inc.
MATSUO, Minoru; President, Nagoya University
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MOHRI, Hideo; President, Okazaki National Research Institutes
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TSUNEWAKI, Koichiro; President, Fukui Prefectural University
YAMANOUCHI, Kazuya; Senior Scientific Staff, Nippon Institute for Biological Science

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Vice-chairman

SEKIGUCHI, Mutsuo; Professor, Fukuoka Dental College

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HANAOKA, Fumio; Professor, Institute of Molecular and Cellular Biology, Osaka University
ISONO, Katsumi; Professor, Faculty of Science, Kobe University
ITO, Koreaki: Director-General, Institute for Virus Research, Kyoto University
MATUURA, Etsuko; Professor, Faculty of Science, Ochanomizu University
OGAWA, Tomoko; Vice-President, Iwate College of Nursing
SASAZUKI, Takehiko; Professor, Medical Institute of Bioregulation, Kyusyu University
SHINOZAKI, Kazuo; Chief Scientist, RIKEN Tsukuba Institute
TAJIMA, Fumio; Professor, Graduate School of Science, The University of Tokyo

Inside Menbers (Alphabetical order)

ARAKI, Hiroyuki; Professor, National Insutitute of Genetics
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HIROMI, Yasushi; Professor, National Insutitute of Genetics
HIROSE, Susumu; Professor, National Insutitute of Genetics
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KATSURA, Isao; Professor, National Insutitute of Genetics
KOHARA, Yuji; Professor, National Insutitute of Genetics
SASAKI, Hiroyuki; Professor, National Insutitute of Genetics
SHIMAMOTO, Nobuo; Professor, National Insutitute of Genetics
SHIROISHI, Toshihiko; Professor, National Insutitute of Genetics

A. DEPARTMENT OF MOLECULAR GENETICS

A-a. DIVISION OF MOLECULAR GENETICS

(1) Intracellular levels of RNA polymerase sigma subunits and transcription factors in *E. coli*

Akira ISHIHAMA, Etsuko KOSHIO, Hiroto MAEDA[1], Akira IWATA[2], Katsunori YATA[3] and Kaneyoshi YAMAMOTO ([1]Kagoshima University, [2]Nippon Institute of Biological Science, Tokyo, and [3]Radioisotope Center)

The RNA polymerase core enzyme with the structure $\alpha_2\beta\beta'$ is converted into holoenzymes by binding one of seven molecular species of the σ subunit. Each holoenzyme recognizes and transcribes a set of *E. coli* genes. The major factor affecting the gene expression pattern in *E. coli* is the intracellular concentrations of these seven σ subunits. We then determined the intracellular concentrations of the subunits at various phases of *E. coli* W3110 growing in various media and at various temperatures. In exponential growth phase, σ^{70} (the principal σ) is the most abundant species, but under certain growth conditions, the level of minor σ subunits exceeds that of the major σ subunit. The levels of functional σ subunits are, however, controlled by anti-sigma factors with inhibitory activity of the σ subunits. We then measured the intracellular levels of known anti-sigma factors such as Rsd (anti-σ^{70}), FlgM (anti-σ^{F}), and DnaK (anti-σ^{H}).

The specificity of RNA polymerase holoenzymes are further modulated by interaction with transcription factors. *E. coli* contains a total of about 260 species of the DNA-binding protein (N. Fujita, unpublished), which all seem to function as transcription factors. We therefore extended our measurement of the intracellular concentrations for all these transcription factors. So far we produced specific antibodies against over-expressed and purified transcription factors, including AcrR, AhpC, AhpF, ArcA, CbpA, CbpB (Rob), Cra (FruR), CRP, CueR, CusR, DeoR, DnaA, DnaK, Dps, EF-Tu, FadR, Fir, Fis, Fur, GreA, GroEL, H-NS, HepA, Hfq, H-NS, Hu, HydG, IciA, IclR, IHF, IlvY, InfB; KdpE, LacI, LeuO, Lrp, MerR, MntR, NusA, OmpR, OxyR, PhoB, PhoP, PspF, RecA, Rho, RpsB, RpbF, Rsd, SdiA, SoxR, SoxS, Ssp, StpA, ThiI, ZntR and Zur. The determination of intracellular levels of these factors is in progress by using quantitative Western blotting for lysates of *E. coli* cells growing at various phases and at various temperatures. For details see refs. 1, 2, 3, 5, 6, 18, 20, 21.

(2) Transcription activation by SdiA, an *E. coli* homolog of the quorum-sensing regulator

Kaneyoshi YAMAMOTO, Etsuko KOSHIO, Nobuyuki FUJITA and Akira ISHIHAMA

Transcription factors have been classified as activators or repressors. Recent studies, however, indicate that both activators and repressors have dual functions, showing activation and repression of transcription depending on the site of their DNA binding. *Escherichia coli* is considered to have more than 200 transcription factors, which interact with the RNA polymerase and modulate its activity and promoter selectivity. Since the mode of transcription regulation by a transcription factor is closely correlated with the contact site on the RNA polymerase, we proposed to classify these transcription factors based on their contact subunit(s) of RNA polymerase, *i.e.*, class-I (α contact), class-II (σ contact), class-III (β contact), and class-IV (β' contact). Most class-I factors bind to upstream of the promoter, interact with the C-terminal domain (CTD) of the α subunit, and thereby support the RNA polymerase-binding to the promoter. On the other hand, the class-II factors contact with the C-terminal region (CTD) of σ subunit and stimulate the promoter opening. Many class-III and-VI factors influence various steps of transcription from initiation, elongation to termination.

SdiA, an *E. coli* homologue of the quorum-sensing regulator, regulates the expression of *ftsQAZ* operon for cell division. Transcription *ftsQ* is under of two promoter, upstream *ftsQ*P2 and downstream *ftsQ*P1, which are separated by 125 bp. SdiA activates transcription from *ftsQ*P2 *in vivo*. The purified SdiA activated transcription *in vitro* of *ftsQ*P2 by reconstituted RNA polymerase containing the major

σ subunit (σ^D). Gel shift and DNase I footprinting assays indicated that the binding center of SdiA was located at -34 of the *ftsQ*P2 promoter, and that the binding of RNA polymerase to *ftsQ*P2 promoter was stimulated by SdiA. *In vitro* transcription studies using the mutant RNA polymerase holoenzymes containing a CTD-deleted α subunit (α235) or a CTD-deleted σ^D (σ529), however, indicated that the σCTD, but not αCTD, is required for recruitment of the RNA polymerase and SdiA-dependent transcription activation. Thus we proposed such a novel mode of transcription activation as that: (i) SdiA supports the RNA polymerase binding to *ftsQ*P2 as in the case of class-I activators; but (ii) this recruitment of RNA polymerase by SdiA depends on the presence of intact σCTD as in the case of class-II factors. In addition to the P2 activation, SdiA inhibited RNA polymerase binding to the *ftsQ*P1 promoter and thereby repressed transcription from P1. Neither αCTD nor σCTD were required for this inhibition. Thus, the transcription repression of P1 by SdiA may result from competition with the RNA polymerase in binding to the *ftsQ*P1 promoter.

The measurement of SdiA protein by Western blotting indicates that the intracellular level of SdiA stays constant throughout the growth phase transition from exponential to stationary phase. However, the activity of SdiA seems to be controlled, because the transcription activation *in vitro* by SdiA increased in the presence of acetyl homoserine lactones (AHLs), the bacterial hormones which play as the ligands for activation of most LuxR family proteins. For details see ref. 21.

(3) Systematic Search for Zinc-binding Proteins in *Escherichia coli*

Akira KATAYAMA, Takeshi NISHINO[1] and Akira ISHIHAMA ([1]Nippon Medical School, Tokyo)

Zinc is an essential trace element, but virtually non-toxic, in contrast to iron, copper and mercury. Over 300 enzymes or proteins have been identified to require zinc for functions. In zinc-containing enzymes or proteins, zinc has two major functions, *i.e.*, catalytic and structural. Up to the present time, the structural and functional roles of zinc have been analysed in details with zinc-containing proteins from higher eukaryotes, but little is known about the zinc-binding proteins in prokaryotes. A systematic search for *Escherichia coli* proteins with the zinc-binding activity was performed using the assay of radioactive Zn(II) binding to total *E. coli* proteins fractionated by two methods of two-dimensional gel electrophoresis, *i.e.*, the widely used O'Farrell system and the newly developed radical-free and highly reducing (RFHR) method. A total of at least 30 to 40 radioactive spots were identified, of which 14 have been assigned from amino-terminal sequencing. Besides 5 hitherto known zinc-binding proteins, the newly identified zinc-binding proteins of *E. coli* include acetate kinase (AckA), molecular chaperone (DnaK), serine hydroxymethyltransferase (GlyA), transketolase isozymes (TktA/TktB), translation elongation factor Ts (Tsf), ribosomal proteins L2 (RplB), L13 (RplM) and one of S15 (RpsO), S16 (RpsP) or S17 (RpsQ). Together with about 20 known Zn(II)-binding proteins, the total number of Zn(II)-binding proteins in *E. coli* increased up to more than 30 species or probably more than 3% of about 1,000 proteins expressed under laboratory culture conditions. The specificity and affinity of zinc-binding were analysed for some of the Zn(II)-binding proteins.

(4) Discontinuous transition of *E. coli* phenotypes during transition from exponential growth to stationary phase

Hideki MAKINOSHIMA and Akira ISHIHAMA

Upon sensing an impending saturation level of the population density, *Escherichia coli* cells enter into the stationary phase. During the transition from exponential growth to stationary phase, marked changes take place in the morphology and physiology of *E. coli* cells. Approximately 1,000 genes highly expressed in the exponentially growing cells are mostly turned off or markedly repressed in the stationary-phase cells and instead a set of 50-100 genes that are repressed in the growing cells begins to be expressed. To understand the molecular events underlying the growth phase-coupled changes in gene expression pattern, attempts have been made to physically separate the cells of different growth phases. By using Percoll gradient centrifugation, we succeeded to separate *E. coli* cultures into more than 15 cell populations, each forming a discrete band. The cell separation was found to result from the difference in buoyant density but not the size difference. The cell

density increases upon transition from exponential growth to stationary phase. Exponential-phase cultures formed at least 5 discrete bands with lower densities, whereas stationary-phase cultures formed more than 10 bands with higher densities. Two molecular markers characterizing each cell population were identified: the functioning promoter species, as identified by measuring the expression of green fluorescent protein under the control of test promoters; and the expressed protein species, as monitored by quantitative immunoblotting. These findings together suggest that the growth phase-coupled transition of *E. coli* phenotype is discontinuous. For details see ref. 10.

(5) Compilation, classification and phylogenetic analysis of bacterial transcription factors

Nobuyuki FUJITA and Akira ISHIHAMA

Complete genomic sequences of 36 bacterial, 11 archaeal and 2 eukaryotic species were searched for bacteria-type transcription factors based on sequence similarity to the known factors. Transcription factors could be classified into more than 50 protein families primarily according to the structure of DNA-binding domain. All bacterial species with medium to large genome size, irrespective of their taxonomic group, share most of the transcription factor families, suggesting that these protein families have ancient phylogenetic origins. In contrast, bacteria with small genome size, typically less than 1.5 Mbp, retain only a few transcription factors. This result, together with detailed phylogenetic analysis of each protein family, suggests that the transcription factors have decayed very rapidly after the establishment of parasitic life cycle of these bacterial species. Archaea share limited numbers of transcription factor families with bacteria, and virtually no bacterial transcription factor was conserved in eukaryotes. Possible horizontal gene transfer events were inferred by several methods including massive principal component analysis of the codon usage pattern. An integrated Web-based database of bacterial transcription factors, with identification and classification system for newly determined sequences, is being constructed.

(6) Intracellular level of the RNA polymerase II in the fission yeast stays constant but the level of CTD phosphorylation varies depending on the phase and rate of cell browth

Hitomi SAKURAI, Makoto KIMURA and Akira ISHIHAMA

The RNA polymerase II (Pol II) of the fission yeast *Schizosaccharomyces pombe* consists of twelve Rpb subunits, of which four (Rpb1, Rpb2, Rpb3 and Rpb11) form the assembly and catalytic core of Pol II and five (Rpb5, Rpb6, Rpb8, Rpb10 and Rpb12) are shared among RNA polymerases I, II and III. The intracellular levels of three RNA polymerase forms should be interrelated, but the control of RNA polymerase formation remains mostly unknown. To reveal the physiological role and the synthesis control of each Rpb subunit, the intracellular levels of Rpb proteins were determined in *S. pombe* growing at various phases under various conditions. Results indicate that the intracellular concentrations of Rpb proteins stay constant at levels characteristic of the rate and phase of cell growth, and the relative level between twelve Rpb subunits also remain constant, together implying that the intracellular concentration of Pol II stays constant as in the case of prokaryotic RNA polymerase.

As an attempt to get insights into the activity control of Pol II, we also analyzed the phosphorylation level of the carboxyl-terminal domain (CTD) of the largest subunit Rpb1. Phosphorylated forms of Tyr1 and Thr4 within 29 repeats of the YSPTSPS heptapeptide were detected in both slow-migrating IIo and fast-migrating IIa forms of Rpb1 on SDS-PAGE. However, phosphorylated Ser2 and Ser5 were identified only in the IIo form, indicating that Ser phosphorylation contributes to the conformational change of CTD. The phosphorylation levels of Ser, Thr and Tyr all vary depending on the cell culture conditions. Taken together we conclude that the intracellular level of Pol II stays constant, but the amount of Pol II engaged in transcription cycle varies depending on the culture conditions as estimated from the sites and levels of phosphorylation of Rpb1 CTD. For details see refs. 8, 15, 16.

(7) Interaction between RNA polymerase II and the carboxy-terminal domain (CTD) phosphatase Fcp1

Makoto KIMURA, Hisako SUZUKI and Akira ISHIHAMA

In transcriptional regulation, RNA polymerase II (pol II) interacts and forms complexes with a number

of protein factors. To isolate and identify the pol II-associated proteins, we constructed a *Schizosaccharomyces pombe* strain carrying a FLAG-tag sequence fused to the *rpb3* gene encoding the pol II subunit Rpb3. By immuno-affinity purification with anti-FLAG antibody-resin, we isolated a pol II-complex containing the Rpb1 subunit with a non-phosphorylated form of the carboxyl-terminal domain (CTD). In addition to the pol II subunits, this complex was found to contain three subunits of the transcription factor TFIIF (TFIIFα, TFIIFβ, and Tfg3) and the CTD-phosphatase *Fcp1*. The same type of pol II-complex could also be purified from another *S. pombe* strain carrying a tagged Fcp1 gene. The isolated Fcp1 showed the CTD-phosphatase activity *in vitro*. The *fcp1* gene was found to be essential for cell viability. Direct interaction between purified Fcp1 and pol II was observed *in vito*. By chemical cross-linking, GST-pull down, and affinity chromatography, the Fcp1-interacting subunit of pol II was identified to be Rpb4, which plays regulatory roles in transcription. We also constructed a thiamine dependent *rpb4* shut-off system of *S. pombe*. On repression of the *rpb4* expression, the cell produced more non-phosphorylated form of Rpb1, but the pol II-complex isolated with the anti-FLAG antibody contained less Fcp1 and more phosphorylated form of Rpb1 with the concomitant reduction of Rpb4. This result indicates the importance of Fcp1-Rpb4 interaction for the formation of Fcp1/TFIIF/pol II complex *in vivo*. For details see ref. 9.

(8) Identification and characterization of *S. pombe* proteins interacting with the Rpb7 subunit of RNA polymerase II

Hiroshi MITSUZAWA, Emi KANDA and Akira ISHIHAMA

Rpb7, an RNA polymerase II (Pol II) subunit that is not shared by RNA polymerase I or III, is required for accurate transcription initiation from the core promoter but is unnecessary for RNA synthesis itself. To further understand the function of Rpb7 in transcription by Pol II, we screened for proteins that interact with *S. pombe* Rpb7 using the yeast two-hybrid system. One of the isolated clones encodes the *S. pombe* homolog of the *S. cerevisiae* Nrd1 (nuclear pre-mRNA down regulation) protein. *S. cerevisiae* Nrd1 has recently been shown to be involved in poly(A)$^+$-independent 3'-end formation of snRNAs and snoRNAs. We found that the *S. cerevisiae* Rpb7 interacts with Nrd1 as in the case of the *S. pombe* counterparts, indicating that the interaction have been conserved throughout evolution and thus suggesting that the interactions has physiological relevance. To test for direct interaction between the *S. pombe* Rpb7 and Nrd1-related protein *in vitro*, we carried out GST pull-down assays with recombinant proteins expressed in *E. coli*. Rpb7 was produced as a His-tagged protein while the C-terminal region of the Nrd1-related protein was produced as a fusion to GST. Because both proteins were found to be insoluble, each protein was solubilized in a denaturing buffer containing urea and then renatured by dialysis followed by GST pull-down assays. As expected, Rpb7 bound to the Nrd1-related protein. Gene disruption revealed that the Nrd1-related protein is essential for cell viability. We also identified amino acid residues of Rpb7 that is important in the interaction with the Nrd1-related protein. Our results suggest the possibility that the Rpb7 subunit is involved not only in transcription initiation but also in 3'-end formation of transcripts by Pol II.

(9) Identification and characterization of *S. pombe* TBP-associated factors (TAFs)

Hiroshi MITSUZAWA and Akira ISHIHAMA

TFIID, one of general transcription factors required for accurate transcription initiation by RNA polymerase II (Pol II) *in vitro*, comprises the TATA-binding protein (TBP) and a set of TBP-associated factors (TAFs). To date most TAF studies have been carried out with human, *Drosophila*, and *S. cerevisiae*. We have been analyzing the TAF function using the fission yeast *S. pombe* as another model organism to which genetic methods are applicable. We have reported the identification of two distinct WD repeat-containing TAFs, spTAF72 and spTAF73, from *S. pombe*. spTAF72 but not spTAF73 is shared by another transcription complex that contains the *S. pombe* homolog of the Gcn5 histone acetyltransferase. Interestingly, overexpression of spTAF72 or spTAF73 suppresses the cell cycle arrest during mitosis caused by mutations in genes involved in ubiquitin-dependent proteolysis, suggesting a role for these WD repeat-containing TAFs in the expression of genes involved in progression through the M phase of the cell cycle. As an attempt to understand the role of the WD repeat domain in spTAF72, we screened for proteins that interact with

the carboxyl-terminal WD repeat-containing region of spTAF72 using the yeast two-hybrid system. We identified another *S. pombe* TAF, spTAF50, a homolog of histone H4-like TAFs such as human TAF80, *Drosophila* TAF60, and *S. cerevisiae* TAF60. Gene disruption revealed that spTAF50 is essential for cell viability. *In vitro*, spTAF50 binds to spTAF72 but less efficiently to spTAF73. Immunoprecipitation experiments demonstrated that spTAF50 was present in both the TFIID and SAGA-like complexes as in the case of spTAF72. These results indicate that the carboxyl-terminal region of spTAF72, which largely consists of WD repeats, interacts with spTAF50 in the TFIID and SAGA-like complexes, suggesting a role for the WD repeat domain in the interaction between TAFs. For details see ref. 12.

(10) An RNA effector in activation of influenza viral RNA polymerase

Ayae HONDA, Kiyohisa MIZUMOTO[1] and Akira ISHIHAMA ([1]Division of Nucleic Acid Chemistry and Kitasato University)

The genome of influenza virus is composed of eight negative-strand viral RNA (vRNA) segments. The viral RNA polymerase is composed of one molecule each of three viral proteins, PB1, PB2 and PA, and is involved in both transcription [vRNA-directed mRNA synthesis] and replication [vRNA-directed synthesis of complementary RNA (cRNA) and cRNA-directed vRNA synthesis]. In transcription, the RNA polymerase catalyzes not only viral RNA synthesis but also the cleavage of capped host cell RNA to generate capped RNA primers for viral mRNA synthesis and polyadenylation at the 3'-termini of mRNA. For the molecular anatomy of this multi-functional enzyme, we have established a simultaneous expression of three P proteins in cultured insect cells using recombinant baculoviruses. For purification of P protein complexes, the PA protein was expressed as a fusion with a His tag added at its N-terminus. By using affinity chromatography, a complex consisting of the three P proteins was isolated from nuclear extracts of virus-infected cells. The affinity-purified 3P complex showed the activities of capped RNA-binding, capped RNA cleavage, viral model RNA-binding, model RNA-directed RNA synthesis, and polyadenylation of newly synthesized RNA. For the endonucleolytic cleavage of host cell mRNA, its interaction with viral RNA is not required, but the capped RNA cleavage by the isolaed 3P complex was found to take place in the presence of vRNA but not of cRNA. Thus we concluded that the vRNA functions as an RNA effector for activation of the assembled but inactive viral RNA polymerase. We also analyzed the structural elements for the RNA effector funciton using variant forms of the model RNA templates. For details see ref. 4.

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A-b. DIVISION OF MUTAGENESIS

(1) Ubiquitin conjugating enzyme affecting recombination at *mat1* locus in fission yeast

Park JOON-HYUN and Fumiaki YAMAO

The mating type of *S. pombe* is determined by the

mat1 locus on chromosome 2. In homothallic cells, mating type switching occurs when the DNA cassette at *mat1* is replaced with cassette containing the opposite mating type information at *mat2-P* or *mat3-m*. The heterothallic h^{+N} cell carries intact *mat2-P* and *mat3-m* information together with an aberrant structure at *mat-1* locus, tandem-duplicated *mat1*-p and *mat2-m* sequences. The abnormal *mat-1* structure might be resulted during resolution of intermediate in the mating type switching via DNA recombination, preventing its further conversion. We report here that UbcP3, one of 14 ubiquitin-conjugating enzymes in fission yeast, was necessary to maintain the heterothallism of h^{+N} cells since *ubcP3*-disruption resulted in efficient conversion to homothallic phenotype. Dysfunction of UbcP3 promoted to loop out the tandem-duplicated segments in *mat1* of h^{+N} cells, converting the *mat1* structure to that of h^{90}. These suggested a role of ubiquitin in recombination event during mating type switching. Together with effects of UbcP3-dysfunction on recombination efficiencies at other loci than *mat1* and on gene silencing in *mat* locus, a possible roles of ubiquitin in regulating chromatin structure and functions would be implicated.

(2) Analysis of Rad6 postreplicative repair (PRR) pathway in fission yeast

Park JOON-HYUN and Fumiaki YAMAO

In the budding yeast, the RAD6 postreplicative repair (PRR) system falls into at least two sub-branches, an error-prone and an error-free pathway. Recently, it has been found that a heterodimeric complex consisting of UBC13 and MMS2 functions in the assembly of K63 polyubiquitin chains for error-free repair. However, little is known about the functional link between the ubiquitin system and PRR pathway in the fission yeast. We reported the evidence that UbcP7/UBC13 functions together with a noncanonical ubiquitin-conjugating enzyme, Mms2 in the PRR pathway defined by Rhp6/RAD6, indicating that *ubcP7* and *mms2* are epistatic to the *rhp6* group like the budding yeast. This observation supports the fact that functionality of the PRR pathway has been highly conserved in the two distantly related yeast species. We also showed through a yeast two-hybrid library screen that Mms2 interacts with the WD protein Cpc2/mammalian RACK1, as well as UbcP7. This finding reinforces the possibility that they might function together in the same PRR pathway. Epistasis analysis reveals that cpc2 also belongs to the rhp6 pathway, the functional analysis of which is now undergoing.

(3) Ubiquitin-conjugating enzymes involved in degradation of mitotic cyclin

Hiroaki SEINO

Cell cycle events are regulated by activation and inactivation of Cdk kinases. Inactivation of Cdk kinases is mainly achieved by degradation of cyclins. Especially degradation of mitotic cyclin is important for cell cycle progression. Mitotic cyclin is degraded by ubiquitin/proteasome system. Ubiquitin ligase involved in ubiquitylation of mitotic cyclin is APC/C (anaphase promoting complex / cyclosome). Ubiquitin-conjugating enzymes were revealed to be two proteins, Ubc4 and Ubcx/E2-C in biochemical analysis of Xenopus and clam oocyte extract. However, relationship between these two ubiquitin-conjugating enzymes were not elucidated.

I found that fission yeast homologues of these two ubiquitin-conjugating enzymes, UbcP1/Ubc4 and UbcP4/Ubc11 directly function for ubiquitylation of mitotic cyclin in non-redundant fashion *in vivo*. Recently I elucidated the functional differences of these two ubiquitin-conjugating enzymes *in vivo*. These enzymes clearly have distinct functions for degradation of mitotic cyclin.

(4) Characterization of SCF^{GRR1} that ubiquitinates G1 cyclin Cln2 in Saccharomyces cerevisiae

Tsutomu KISHI

In budding yeast, G1 cyclin Cln2 is rapidly degraded by ubiquitin/proteasome system when yeast cells enter S phase. Upon degradation, Cln2 is first phosphorylated by its cognate Cln2/Cdc28, and then the phosphorylated Cln2 is ubiquitinated by SCF ubiquitin ligase, which is composed of Skp1, Cdc53 and Grr1. Finally, ubiquitinated Cln2 is targeted to proteasome, where Cln2 is degraded.

To isolate genes that regulate the degradation of Cln2, I have employed genetic screen by selecting for stable Cln2-LacZ fusion protein. One of the mutant

dog75 (Degradation of G1 cyclin) was characterized. Phosphorylation of Cln2 is not affected in dog75 mutants, indicating that Dog1 in involved in either the ubiquitination of Cln2 or the degradation of ubiquitinated Cln2. I next examined whether Cln2 is ubiquitinated in dog75 mutants. Cln2-HA was immunoprecipited, and then immunoblotted with anti-poly-ubiquitin antibody. Ubiquitinated Cln2 was decreased in dog75 mutants. This indicates that Dog75 is involved in the ubiquitination of Cln2. Dog75 codes for protein that has homology with protein kinase. I am now studying whether Dog75 protein kinase directly activates SCF ubiquitin ligase.

Publication

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A-c. DIVISION OF NUCLEIC ACID CHEMISTRY

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

Division of Nucleic Acid Chemistry
Kiyohisa MIZUMOTO (School of Pharmaceutical Sciences, Kitasato University)

Our previous studies indicated that the SeV transcription-stimulatory activity from bovine brain extract was resolved into at least two complementary fractions, one of which may be tubulin (K. Mizumoto *et al., J. Biochem.*, **117**, 527-534, 1995). We have recently purified the host factor activity complementary to tubulin, and shown that it contains at least two components, and one of them is phosphoglycerate kinase (PGK), a glycolytic enzyme (Ogino *et al. J. Biol. Chem.* **274**, 35999-36008, 1999). Neither a substrate for PGK, 3-phosphoglycerate, nor a competitive inhibitor against 3-phosphoglycerate, DL-glycerol-3-phosphate, affected the transcription-stimulatory activity of PGK, suggesting that the enzymatic activity *per se* of PGK does not seem to be required for its activity. Cellular tubulin, which stimulates both mRNA synthesis and leader RNA synthesis, was found to be integrated into the transcription initiation complex (Takagi *et al., J. Biochem.* **118**, 390 396, 1995, Takagi *et al., Arch. Virol.* **141**, 1623-1635, 1996). On the other hand, PGK stimulates viral mRNA synthesis at the elongation step, probably through its interaction with tubulin that has been integrated into the initiation complex.

Here we further purified the host factor activity from bovine brain extract and demonstrated that the activity consists of four complementary factors, tubulin, PGK, p52, and an unknown factor. Surprisingly, the third host factor, p52, was also identified as a glycolylic enzyme, enolase (Ogino *et al., Biochem. Biophys. Res. Commun.* **285**, 447-455, 2001). We also demonstrated that p52 as well as a recombinant human α-enolase binds to tubulin, as PGK does. Our findings suggest interesting relationships between tubulin and glycolylic enzymes that may function as coactivators for SeV transcription through their interactions with tubulin integrated in the transcription initiation complex.

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(2) Research Activities

Mechanisms of transcription regulation by eubacteria-type RNA polymerase: Kan TANAKA

1) Gene silencing at the stationary growth phase

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

2) Mechanisms of nitrogen-assimilatory gene expression in cyanobacteria

NtcA is a generally found transcription factor in cyanobacteria, and regulates nitrogen assimilatory genes, such as nitrate/nitrite reductases, nitrate/nitrate transporter, and glutamine synthetase. Using a recombinant NtcA protein and a cyanobacterial RNA polymerase, we reconstituted an in vitro transcription system for the glnA and ntcA promoters. We have found that 2-oxoglutarate, a carbon skeleton for the nitrogen assimilation, enhances the NtcA-DNA binding and activates the transcription initiation. Since this activation occurs even in the condition where NtcA binding sites are fully occupied by NtcA, it was suggested that 2-oxoglutarate activated the NtcA-DNA binding as well as a later step during the transcription initiation.

3) Regulation of plastid DNA transcription by nuclear-encoded sigma factors

Plastids of higher plants are organelle, which have been originated from a symbiosis of ancestral cyanobacteria. Plastids still have their own genome of c.a. 150-kb, and the transcription is performed by eubacteria-type RNA polymerase. While subunits for core RNA polymerase are encoded by the plastid genome, sigma subunits are encoded by the nuclear genome, and dominate the plastid transcription under the control of the nucleus. Six sigma factor genes were found in *Arabidosis*, and we recently isolated a T-DNA tagged sig2 mutant line. This mutant contains poorly developed chloroplasts of low chlorophyll contents. Further analysis revealed that the amounts of several chloroplast tRNA species including $tRNA^{glu}$ were severely decreased in the mutant line. Thus, the SIG2 sigma factor was suggested to activate transcription of a series of plastid tRNA genes. Since $tRNA^{glu}$ is a well-known cofactor for the tetrapyrrole biosynthesis, SIG2 appears to regulate both protein and chlorophyll synthesis through the tRNA transcription during the chloroplast development.

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B. DEPARTMENT OF CELL GENETICS

B-a. Division of Cytogenetics

(1) Recombinogenic activity of chimeric recA genes (Pseudomonas aeruginosa/Escherichia coli): a search for RecA protein regions responsible for this activity

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In the background of weak, if any, constitutive SOS function, RecA from Pseudomonas aeruginosa (RecAPa) shows a higher frequency of recombination exchange (FRE) per DNA unit length as compared to RecA from Escherichia coli (RecAEc). To understand the molecular basis for this observation and to determine which regions of the RecAPa polypeptide are responsible for this unusual activity, we analyzed recAX chimeras between the recAEc and recAPa genes. We chose 31 previously described recombination- and repair-proficient recAX hybrids and determined their FRE calculated from linkage frequency data and constitutive SOS function expression as measured by using the lacZ gene under control of an SOS-regulated promoter. Relative to recAEc, the FRE of recAPa was 6.5 times greater; the relative alterations of FRE for recAX genes varied from approximately 0.6 to 9.0. No quantitative correlation between the FRE increase and constitutive SOS function was observed. Single ([L29M] or [I102D]), double ([G136N, V142I]), and multiple substitutions in related pairs of chimeric RecAX proteins significantly altered their relative FRE values. The residue content of three separate regions within the N-terminal and central but not the C-terminal protein domains within the RecA molecule also influenced the FRE values. Critical amino acids in these regions were located close to previously identified sequences that comprise the two surfaces for subunit interactions in the RecA polymer. We suggest that the intensity of the interactions between the subunits is a key factor in determining the FRE promoted by RecA in vivo. For details see Ref. 1.

(2) Domain structure and dynamics in the helical filaments formed by RecA and Rad51 on DNA

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Both the bacterial RecA protein and the eukaryotic Rad51 protein form helical nucleoprotein filaments on DNA that catalyze strand transfer between two homologous DNA molecules. However, only the ATP-binding cores of these proteins have been conserved, and this same core is also found within helicases and the F1-ATPase. The C-terminal domain of the RecA protein forms lobes within the helical RecA filament. However, the Rad51 proteins do not have the C-terminal domain found in RecA, but have an N-terminal extension that is absent in the RecA protein. Both the RecA C-terminal domain and the Rad51 N-terminal domain bind DNA. We have used electron microscopy to show that the lobes of the yeast and human Rad51 filaments appear to be formed by N-terminal domains. These lobes are conformationally flexible in both RecA and Rad51. Within RecA filaments, the change between the "active" and "inactive" states appears to mainly involve a large movement of the C-terminal lobe. The N-terminal domain of Rad51 and the C-terminal domain of RecA may have arisen from convergent evolution to play similar roles in the filaments. For details see Ref. 2.

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(1) Integrative study on chromosome evolution of mammals, ants and wasps based on the minimum

interaction theory

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There is well-known evidence that in many eukaryotes, different species have different karyotypes (e.g. n=1-47 in ants and n=3-51 in mammals). Alternative (fusion and fission) hypotheses have been proposed to interpret this chromosomal diversity. Although the former has long been accepted, accumulating molecular genetics evidence seems to support the latter. We investigated this problem from a stochastic viewpoint using the Monte Carlo simulation method under the minimum interaction theory. We found that the results of simulations consistently interpreted the chromosomal diversity observed in mammals, ants and wasps, and concluded that chromosome evolution tends to evolve as a whole toward increasing chromosome numbers by centric fission. Accordingly, our results support the fission hypothesis. We discussed the process of chromosome evolution based on the latest theory of the molecular structure of chromosomes, and reconfirmed that the fission burst is the prime motive force in long-term chromosome evolution, and is effective in minimizing the genetic risks due to deleterious reciprocal translocations and in increasing the potential of genetic divergence. Centric fusion plays a biological role in eliminating heterochromatin (C-bands), but is only a local reverse flow in contrast to the previously held views. For details see Ref. 1.

Publication

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B-b. Division of Microbial Genetics

(1) Sld3, which interacts with Cdc45 (Sld4), functions for chromosomal DNA replication in *Saccharomyces cerevisiae* (ref. 1)

Yoichiro KAMIMURA, Yon-Soo TAK and Hiroyuki ARAKI

Chromosomal DNA replication in eukaryotic cells initiates from multiple origins which fire sequentially throughout the S phase; some fires early and others late. The pre-replicative complex (pre-RC) starts to assemble at origins from late M phase and DNA polymerases are recruited onto origins to initiate DNA synthesis during the S phase. The Dpb11 protein which forms a complex with essential DNA polymerase ε (Polε) is required for association of DNA polymerases with origins in DNA replication and for the control of the S-phase checkpoint. To identify factors interacting with Dpb11, we have isolated 10 *sld* (synthetic lethality with *dpb11-1*) mutations which fall into 6 complementation groups. We have cloned *SLD1*~*6* and found that *SLD1* is identical to *DPB3* encoding the third largest subunit of Polε, *SLD4* is identical to *CDC45* required for the initiation and elongation step of chromosomal DNA replication and *SLD6* is identical to *RAD53* which has crucial role for the cell cycle checkpoint. The *SLD2, 3, 5* genes were new genes essential for the cell growth (Kamimura et al., Mol. Cell. Biol., **18**, 6102-6109, 1998).

The Cdc45 protein, which binds to the minichromosomal maintenance (Mcm) proteins, has a pivotal role for the initiation and elongation steps of chromosomal DNA replication in eukaryotes. We show that throughout the cell cycle in *Saccharomyces cerevisiae*, Cdc45 forms a complex with a novel factor, Sld3. Consistently, Sld3 and Cdc45 associate simultaneously with replication origins in the chromatin immunoprecipitation assay: both proteins associate with early-firing origins in G1 phase and with late-firing origins in late S phase. Moreover, the origin associations of Sld3 and Cdc45 are mutually dependent. The temperature-sensitive *sld3* mutation confers defect of DNA replication at the restrictive temperature and reduces an interaction not only between Sld3 and Cdc45 but also between Cdc45 and Mcm2. These results suggest that the Sld3-Cdc45 complex associates with replication origins through Mcm proteins. At the restrictive temperature in *sld3-5* cells, RF-A, a single-strand DNA binding protein, does not associate with origins. Therefore, the origin association of Sld3-Cdc45 complex is prerequisite for origin unwinding in the initiation of DNA replication.

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

Hiroshi MASUMOTO, Sachiko MURAMATSU, Yoichiro KAMIMURA and Hiroyuki ARAKI

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

Since Sld2, which form a complex with Dpb11 (Kamimura et al., Mol. Cell. Biol., **18**, 6102-6109, 1998), has six conserved amino acid sequences for Cdk-phosphorylation, we examined whether Sld2 is phosphorylated by S-Cdk. As expected, in SDS-polyacrylamide gel Sld2 migrated slower in S phase than in G1 phase. Phosphatase treatment converted slow mobility species of Sld2 obtained from S phase to fast mobility specie as obtained from G1 phase, suggesting that Sld2 is phosphorylated in S phase.

The partially purified Cdk phophorylated the Sld2 protein purified from *E. coli* cells expressing it. This in vitro phosphorylation depends on Cdk-phosphorylation motif since the Cdk did not phosphorylate the All-A protein (see below). These results suggest that Sld2, at least in in vitro reaction, is phosphorylated by Cdk. Then, we examined whether Sld2 is phosphorylated by S-Cdk in in vivo. When we expressed Sic1, an inhibitor of S-Cdk, phosphorylated form of Sld2 did not appear. Further, when we examined S-cyclins-deleted cells that are viable since M-phase-cyclins compensate for them while the start of DNA synthesis delays, the slow migrating form of Sld2 appeared later than wild-type cells. Thus, in vivo phosphorylation of Sld2 in S phase depends on S-Cdk activity.

Next, we substituted the serine or threonine residues in six S-Cdk phosphorylation sites by alanine. The mutant protein containing six alanine substitutions (All-A) did not support cell growth but also onset of S phase while three combinations of five alanine substitutions supported cell growth. Furthermore, slow mobility species of the mutant protein containing six alanine substitutions did not appear. Moreover, when we substituted three of replaced alanine residues of All-A by an asparatate, a phosphomimeric residue, the resultant mutant allele support cell growth. These results suggest that S-Cdk dependent phosphorylation of Sld2 is essential for DNA replication.

Since Sld2 and Dpb11 form a complex, we investigated their complex formation by tagging them and using co-immunoprecipitation assay. Co-immunoprecipitation of Sld2 and Dpb11 was detected in S phase but not in G1 phase and Dpb11 immunoprecipitated only with phosphorylated form of Sld2. When we expressed wild-type and six-alanine substitution mutant proteins of Sld2 in the *sld2* mutant cells defective in complex formation between Sld2 and Dpb11, complex formation between the mutant protein and Dpb11 did not be detected while the wild type protein coprecipitated with Dpb11. These results suggest that phosphorylation of Sld2 is required for complex formation between Dpb11 and Sld2.

Temperature-sensitive mutants of the *SLD2* gene defective in formation of the Sld2-Dpb11 complex is suppressed by the increased dosage of *DPB11*. We found that increased dosage of *DPB11* also suppresses the growth defect of the All-A mutant. This result further suggests that inefficient formation of the Sld2-Dpb11 complex in the All-A mutant cells gives rise to defect of the cell growth.

Since Dpb11 is required for associations of DNA polymerases with replication origins (Masaumoto et al., Mol. Cell. Biol., **20**, 2809-2817, 2000), it seems likely that S-Cdk regulates complex formation between Sld2 and Dpb11 and consequently loading of DNA polymerases to replication origins.

(3) Functional analysis of Sld5 and Psf1

Yoichiro KAMIMURA and Hiroyuki ARAKI

The *SLD5* gene isolated as a synthetic lethality with *dpb11-1* (see section (1)) encodes an essential 34 kDa protein. We also isolated the *PSF1* (Partner of *Sld Five*) and *PSF2* genes, respectively as a multicopy supperssor of *sld5-12* and *psf1-1* mutations. In co-immunoprecipitation experiment, Sld5 forms a complex with Psf1 and Psf2. This complex is unstable in *psf1-1* cells. This result suggests that complex formation is important for the function of these proteins. *Psf1-1* mutant cells arrested with a dumbbell shape and were defective in DNA replication at the restrictive temperature as did *sld5-12* cells. To date, many replication related proteins including Dpb11 associate ARS regions using chromatin immunoprecipitation assay (CHIP). Indeed, Psf1 associated with replication regions in S phase at the same time as Dpb11 and Polε. This association with origins

depended on Sld3 and Dpb11. Two-hybrid assay showed the interaction between Psf1 and Dpb11 of Sld3. These data suggest that Sld5-Psf1-Psf2 complex intermediates association between Sld3-Cdc45 and Dpb11-Pol ε.

Publications

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B-c. Division of Cytoplasmic Genetics

(1) Linkage analysis and linkage disequilibrium mapping of functional psychoses

Takeo YOSHIKAWA (Lab. for Molecular Psychiatry, RIKEN Brain Science Institute)

We have been collecting pedigrees of schizophrenia and mood disorders. As of December 2001, these pedigrees amounted to 132 families and 411 individuals including probands and their relatives. Among them, 119 families (357 individuals) comprise the schizophrenia group, and 13 families (54 individuals), the affective disorder group. In 2001, we started a whole genome scan of the schizophrenia pedigree panel, and have finished analyzing all chromosomes at a 10 cM density, and chromosomes 6, 11 and 18 at a 5 cM density, by means of Pedigree Disequilibrium Test. In the second stage, the markers which showed a significant linkage/association to the disease were analyzed by using trio families (parents and their affected offsprings) and Extended Transmission Disequilibrium Test statistics. The markers which still remained to be significant after the second stage screening were those mapped onto chromosomes 11 and 18. We are now testing these genetic loci by using the third sample panel consisting of cases (unrelated schizophrenics) and controls.

(2) QTL mapping of mouse models of depressive disorder

Takeo YOSHIKAWA

The forced swimming test (FST) and tail suspension test (TST) have been widely used for the screening of anti-depressant efficacy. Clinically effective drugs reduce immobility times in both tests. The pharmacological action is thought to prevent the animals from experiencing despair in stressful conditions. Thus, immobility times are considered to reflect a propensity to depression in human. We found that the C3H/He mouse strain showed much shorter immobility periods than the C57BL/6 strain in both tests, suggesting that the C3H/He mice are genetically more resistant to such stress conditions. In 2001, we have prepared 560 F2 intercross mice for the QTL mapping and performed whole genome scanning of these mice using 120 microsatellite markers. We have detected FST-QTLs on mouse chromosomes 6, 8, 11 and 17, and TST-QTLs on chromosomes 4, 8, 11 and 14 (Publicaton #8). These QTLs controlling immobility times in FST and TST may harbor genes which correspond to susceptibility genes in human depression.

(3) Gene expression analysis of a model animal for depression using GeneChips

Takeo YOSHIKAWA

Learned Helplessness (LH) is a good animal model for the chronic depression. We have examined several parameters to efficiently produce LH rats, and succeeded in stably making these rats. We removed the frontal cortex and hippocampus from the brains of LH rats, antidepressant-treated LH rats and control rats, and extracted RNA, and compared their expression levels using the GeneChips. These analyses revealed that a number of receptor genes for neurotransmitters were down-regulated in both brain regions of LH rats, and their expression levels were resumed to control levels by antidepressant treatments.

(4) Candidate gene approaches

Takeo YOSHIKAWA

The target genes are selected for both positional

and functional relevance. The analyzed genes in 2001 include *myo*-inositol monophosphatase 2 (*IMPA2*) (for mood disorders and schizophrenia; Publication #1), cholecystokinin (for panic disorder; Publication #3), cholecystokinin B receptor (for panic disorder and schizophrenia; Publication #4,5), adenosine A2a receptor (for panic disorder; Publication #4), and adenylate cyclase type 9 (*ADCY9*) (for mood disorders; Publication #6,7). We have identified genetic polymorphisms of these genes, and examined their roles in the developments of psychiatric disorders.

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C. DEPARTMENT OF DEVELOPMENTAL GENETICS

C-a. Division of Developmental Genetics

(1) Translational regulation of zygotic gene expression

Masataka OKABE, Takao IMAI[1], Mitsuhiko KURUSU[2], Hideyuki OKANO[1] and Yasushi HIROMI
([1]Osaka Univ. Graduate School of Medicine, Keio Univ. School of Medicine, [2]University of Tsukuba)

Development is controlled by expressing genes in appropriate place and at appropriate timing. Although most gene expressions are regulated at the transcriptional level, translational control has its unique advantages; for example, in rapidly supplying large quantities of proteins after fertilization using maternal mRNAs stored in the oocyte. We found that a cell-fate decision during sensory organ formation involved a translational control of zygotic gene expression. Each mechanosensory bristle in Drosophila is composed of four support cells and one neuron, which are generated through asymmetric cell divisions of a precursor cell. In the first asymmetric division, Notch-signaling directs the asymmetry between neuronal and non-neuronal lineages, and a zinc finger transcriptional repressor Tramtrack69 (TTK69) acts downstream of Notch as a determinant of non-neuronal identity. We found that TTK69 protein expression in the neuronal lineage is repressed translationally rather than transcriptionally. This translational repression was achieved by a direct interaction between the 3' untranslated region of *ttk69* mRNA and an RNA binding protein Musashi. In one of the daughter cells Notch-signaling inhibits Musashi activity allowing translation of *ttk69* mRNA in the non-neuronal progeny. We propose that the constitutive translational repression and its release by upstream signaling pathway enable rapid gene expression even in post-embryonic development. For details see Ref. 1.

(2) A common molecular basis required for Drosophila sensory organ formation

Nao NIWA, Masataka OKABE and Yasushi HIROMI

Sensory organs such as eye, nose and ear have diverse morphological features that are specialized for the particular physical stimulus that each organ has to receive. Although three sensory organs in Drosophila, compound eye in the head, Johnston's organ (ear) in the antenna, and chordotonal organ (stretch receptor) in the leg, have no apparent morphological similarities, they share at least one property during its formation; they all require the bHLH transcription factor encoded by the *atonal* gene for their formation. This suggests that these diverse sensory organs may have a common origin and share a molecular basis for their formation. We analyzed the spatio-temporal conditions in which diverse sensory organs form. We found that these segment-specific sensory organs form under the same molecular environment that is defined by Dpp and ecdysone signals. Both signals act through the 3' enhancer region of the *atonal* gene and direct its expression in a specific region of each segment. Since Atonal expression endows a neurogenic activity, all segment-specific sensory organs form within a neurogenic field that is common to all segments. This suggests that diverse sensory organs may have evolved from an ancestral organ that was present in all segments. Previous models on organogenesis assumed that a master regulator of organ formation, such as the *eyeless* gene, directed all aspects of the formation of a particular sensory organ, e.g., an eye. We found that even in an *eyeless* mutant, a cluster of Atonal expressing cells are present in the eye imaginal disc when apoptosis is blocked. Therefore, the function of the *eyeless* gene is not the formation of a sensory organ *per se*, but rather a modification of the proto-sensory organ by adding segment-specific features to the common neurogenic field. Vertebrate homologues of *dpp* and *atonal* gene are also involved in the formation of the eye and ear, suggesting that the molecular basis for sensory organ formation was already established in a common ancestor of protostomes and deuterostomes.

(3) Function of nuclear receptor Seven-up in the Drosophila central nervous system

Makoto KANAI, Masataka OKABE, Yasushi HIROMI

A hallmark of the nervous system is the huge diversity in the neuronal and glial cell types that it contains. Even in a rather simple system such as a neuromere of the Drosophila embryonic central nervous system (CNS), each unit contains more than 250 neurons that likely consist of over a hundred cell types. These neurons are generated through sequential divisions of ca. 30 neuronal precursors in the CNS, called neuroblasts. The identity of cells that each neuroblast generates depends on their birth order from founder neuroblasts. A recent study revealed that the temporal information of the birth order is provided by sequential expression pattern of a group of transcription factors called "gap proteins". Since each neuroblast has its unique lineage pattern, factors that link neuroblast identity and birth order identity must exist. Nuclear receptor Seven-up is expressed in most neuroblasts in diverse subsections of each lineage. In *seven-up* mutant the number of neurons that express particular identity markers is altered, accompanied by a change in the expression pattern of gap proteins. Seven-up may be involved in coordinating neuroblast identity and birth order identity.

(4) Identification of the down stream factors of a nuclear receptor, Seven-up

Motomi MATSUNO, Yasushi HIROMI

Seven-up, one of the Drosophila nuclear receptor, functions as a genetic switch between two different photoreceptor subtypes during compound eye development. We are trying to understand what Seven-up target genes are and how Seven-up regulates them. Since Seven-up ligand binding domain has a transcriptional repression activity, we searched for genes whose expression was up-regulated in *seven-up* mutant eye imaginal disc. So far we have identified 53 candidate genes, including those encoding transcriptional factors, cytoskeleton molecules, and cell adhesion molecules.

(5) The functional analyses of TFIIH p52 subunit

Motomi MATSUNO, Yasushi HIROMI

Basal transcriptional factor TFIIH is a multi-functional protein complex that is involved not only in transcription but also in DNA repair and cell cycle regulation. Among its nine subunits are those that have enzymatic functions such as kinase and helicase activities, which are likely to be used differentially depending on the state of the cell. Since most studies concerning TFIIH have so far been done *in vitro* or in unicellular organisms, it is not known how TFIIH function is regulated in a multicellular environment where three functions operate simultaneously. We are approaching this problem by focusing on the p52 subunit, which was previously identified as a Seven-up interacting protein in our laboratory. We identified nine loss-of-function alleles of Drosophila p52 gene, and showed that all alleles show growth delay and abnormal feeding behavior, and die before reaching the mature third instar larval stage. Furthermore, mutant clones in the eye imaginal discs have abnormal cell cycle progression phenotypes and cannot be maintained to the adult stage, although defect in transcription is minor. The cell cycle phenotype of p52 mutants was different from that seen in mutations in the Cdk activating kinase subunit of TFIIH, and were similar to defects observed upon blocking nucleotide excision repair. We propose that p52 has essential roles in DNA repair during development and its loss impacts upon cell cycle regulation indirectly.

(6) Differentiation of Drosophila longitudinal glia

Yoshihiro YUASA, Masataka OKABE, Yasushi HIROMI

Just as neurons comprise of diverse cell types, glia also contain many subtypes that differ in morphology, migration patterns and specificity in neuronal recognition. In Drosophila, transcription factor Glial cells missing (GCM) has been identified as the most upstream regulator of gliogenesis, providing the distinction between neuron and glia. Since GCM is expressed in all glia, glial subtype determination must depend on other regulatory proteins. We have shown that the homeodomain protein REPO directs the glial differentiation pathway initiated by GCM. Mutant phenotypes of *repo* suggest that REPO is required for the migration and differentiation of embryonic glial cells. In order to further understand how REPO functions in glial terminal differentiation, we have analyzed the mechanism of gene regulation by REPO.

In the ventral nerve cord, a specific glial subtype called longitudinal glia is needed for the formation and maintenance of longitudinal axon tracts. We identified transcription factor DRI that is expressed specifically in longitudinal glia as a new downstream target gene of REPO. DRI cooperates with transcription factor PointedP1, another downstream target of GCM, in regulating expression of longitudinal glia specific marker genes. We propose that cooperation of these transcription factors with REPO plays a key role in the longitudinal glial differentiation.

(7) FGF signal is required for glial ensheathment by redistributing Neuroglian protein

Kaz TAKIZAWA, Emiko SUZUKI[1], Yoshiki HOTTA and Yasushi HIROMI ([1]Institute of Medical Science, University of Tokyo)

In the development of Drosophila CNS, longitudinal glia migrate medially and associate with axon tracts to be aligned in two rows, medial and lateral, before ensheathing the longitudinal axon bundle. Molecular mechanisms underlying these processes are poorly understood. We focused our study on the expression of a cell surface protein Neuroglian that is expressed in glial cells and is known to mediate cell adhesion *in vitro*. The distribution of Neuroglian in longitudinal glia changes dramatically during glial morphogenesis;. while initially on the cell surface, Neuroglian accumulates at the medial edge of the medial row and at the interface of the two rows, concomitant with the extension of the glial process. At stage 16, filopodia-like structure appear and eventually connect with each other to form a compartment structure that covers the dorsal surface of the medial half of each hemisegment. Electron microscopic analysis revealed that the compartment border has a different morphology of cell contacts from those within the compartment. Mutations in FGF receptor *heartless* failed to redistribute the Neuroglian protein, while *Neuroglian* mutant showed a malformation of the compartment structure. These data indicate that FGF signal mediates glial ensheathment by directing Neuroglian relocalization.

(8) Genetic dissection of Spred, a novel negative regulator of ras signaling

Shu KONDO, Yasushi HIROMI

Ras signaling is implicated in various aspects of cell differentiation and proliferation in developing animal tissues. The human Spred protein is a potent negative regulator of ras signaling in tissue culture cells, but its physiological function is not clear. The Spred protein has a cystein-rich domain in the N terminus that is also present in another negative regulator of ras signaling, Sprouty, which was identified in our laboratory. The Drosophila genome contains one Spred homolog, and we are analyzing its physiological role in Drosophila. We found that Drosophila Spred is expressed in photoreceptor cells R2, R5 and R8, the central nervous system and the embryonic muscle attachment sites. We created a Spred mutant by imprecise excision of the P-element AE33 which is inserted in an intron of the Spred gene. To our surprise, the Spred mutant flies are fully viable and fertile, showing no apparent visible phenotypes. Analysis of transgenic flies overexpressing Spred in specific tissues and a genome-wide modifier screen in the Spred mutant background to identify functional redundancy with other genes are underway.

(9) Receptors as presenters of positional information

Masaki HIRAMOTO, Yasushi HIROMI

The basic model that is currently employed to explain the axon patterning by secreted guidance molecules is the Chemotropic Hypothesis proposed by Cajal. It proposes that positional information is expressed by the distance from the source. We found new way to create positional information utilizing intra-cellular compartmentalization. Netrin is an evolutionary conserved guidance molecule, which is recognized and captured by its receptor Frazzled. We found Frazzled creates positional information for other cells by rearranging the distribution of Netrin (Capture/ Relocation system). In this process, intra-axonal localization of Frazzled provides positional cue for guidance of other axons. We propose that this system is the basic mechanism for general axonal pattering by secreted ligands and their receptors. To test this idea, we are investigating the patterning mechanism by another secreted ligand Slit and its receptor Robo. We focused on two longitudinal pioneer axons whose medio-lateral positioning depends on Slit

and Robo. We found that Slit receptors also exhibit compartmentalized localization within the axon, and can provide medio-lateral positional information through a non-cell autonomous effect. These results suggest that the presentation of positional information by intra-axonal localization of receptors is a general strategy in axonal guidance.

(10) GCM-motif protein function in mammalian hematopoiesis

Toshihiko HOSOYA, Yasuno IWASAKI[1], Motomi OSATO[2], Tomomasa YOKOMIZO[2], Chieko HIGASHI-TAKIZAWA, Yoshiaki ITO[2], Kazuhiro IKENAKA[1], Yasushi HIROMI, Yoshiki HOTTA ([1]National Institute for Physiological Sciences, [2]Institute for Virus Research, Kyoto University)

In Drosophila, Gcm-motif transcription factors govern binary cell-fate decisions in both neurogenesis and hematopoiesis. Just as GCM acts as a switch between glia and neuron during neurogenesis, expression of GCM and GCM2 during hematopoiesis bisects blood cell population into plasmatocytes (macrophages) and melanin-producing crystal cells. GCM and GCM2 are expressed and required specifically in plasmatocytes, and their misexpression in crystal cells converts them to the plasmatocyte fate. We investigated whether GCM-motif proteins have conserved functions in mammals. We found that a mammalian Gcm-motif protein mGCMa is expressed in the embryonic blood cells. When misexpressed in the Drosophila blood system, mGCMa exhibited the same cell-fate transformation activity as the Drosophila GCM-motif proteins. Thus mGCMa possesses molecular characteristics required for the function as a genetic switch in hematopoiesis. mGCMa inactivation by gene knock-out caused defects in macrophage development, suggesting an evolutionarily conserved role of Gcm-motif proteins in hematopoiesis.

(11) Presentation methods that are friendly to color-blind people

Masataka OKABE and Kei ITO[1] ([1]National Institute for Basic Biology)

In scientific presentations and publications, "color" has become a significant vehicle for information and presentation effect. However, color perception varies greatly among individuals; in particular, red-green color blindness are found in 4-9% of males in various populations, a frequency comparable to that of the AB blood type. Thus inappropriate color choices can cause unexpected difficulty in understanding color figures. We are examining how color and color combinations are perceived by various color vision types, to develop a method for presenting color information that can convey maximal information to most color vision types including color blindness. We introduced this method in talks entitled "How to make figures and presentations that are friendly to color-blind people" at several academic meetings in Japan and USA, and also on our web site: http://www.nig.ac.jp/labs/DevGen/shikimou.html.

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(1) Hydra Peptide Project: An epitheliopeptide Hym-301 is involved in head formation

Toshio TAKAHASHI[1], Hiroshi SHIMIZU, Masayuki HATTA[2], Osamu KOIZUMI[3], Yoshitaka KOBAYAKAWA[4] and Toshitaka FUJISAWA ([1]University of California, Irvine, [2]Ochanomizu University, [3]Fukuoka Women's University, [4]Kyushu University)

During the course of systematic screening of peptide signaling molecules from *Hydra magnipapillata*, we identified a 14 amino acid peptide with an internal disulfide bond and amidation at the C-terminus. In situ hybridization and immunohistochemistry showed that the peptide is synthesized and localized in the ectodermal epithelial cells in the hypostome region. During a budding process, both the gene and the peptide are expressed in the presumptive hypostome region before tentacle formation. The exogenous application of the peptide at 10^{-6} M enhanced the tentacle formation, whereas RNAi inhibited it. This is the first report on the amidated

peptide derived from epithelial cells that is involved in morphogenesis in *Hydra*.

(2) Diffuse nerve net in hydra body column represents a primitive enteric nervous system

Hiroshi SHIMIZU and Toshitaka FUJISAWA

Digestive movements in vertebrates' intestine such as peristalsis involve three basic structures, longitudinally and circumferentially running muscle layers and the net-like nervous system termed myenteric plexus between them. Interestingly, the digestive tract of *Hydra* also has two muscle layers and the net-like nervous system termed nerve net in between. Despite the similarities, *Hydra*'s digestive process has been thought to be a non-dynamic event. Here we show evidence that *Hydra* exhibits dynamic digestive movements similar to peristalsis and mass peristalsis (defecation) in vertebrates' intestine. Peristalsis moves the contents back and forth in the body cavity while mass peristalsis ejects fecal contents from the mouth. Nervous system is primarily responsible for these movements since animals with no nerve cells showed only a weak peristalsis. Body column tissue excised from an animal showed the two types of movement by itself indicating that nerve net in the body column is responsible for these movements. These observations suggest that *Hydra*'s digestive tract is structurally and functionally similar, although primitive, to vertebrates' intestine and that nerve net in *Hydra* body column represents enteric nervous system.

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C-b. Division of Gene Expression

(1) Segmentation gene product Fushi tarazu is an LXXLL motif-dependent coactivator for orphan receptor FTZ-F1

Taiga SUZUKI[1], Haruhisa KAWASAKI, Ruth T. YU[1], Hitoshi UEDA and Kazuhiko UMESONO[1] ([1]Institute for Virus Research, and Graduate School for Biostudies, Kyoto University, Kyoto, 606-8507, Japan)

Orphan receptors for whom cognate ligands have not yet been identified form a large subclass within the nuclear receptor superfamily. To address one aspect of how they might regulate transcription, we analyzed the mode of interaction between the *Drosophila* orphan receptor FTZ-F1 (NR5A3) and a segmentation gene product Fushi tarazu (FTZ). Strong interaction between these two factors was detected by use of the mammalian one- and two-hybrid interaction assays without addition of ligand. This interaction required the AF-2 core and putative ligand binding domain of FTZ-F1 and the LXXLL motif of FTZ. The requirement of these elements was further confirmed by examination of their target gene expression in *Drosophila* embryos and observation of a cuticle phenotype in transgenic fly lines that express mutated factors. In *Drosophila* cultured cells, FTZ is required for FTZ-F1 activation of a FTZ-F1 reporter gene. These results reveal a resemblance in the mode of interaction between FTZ-F1 and FTZ and that of nuclear receptor-coactivator and indicate that direct interaction is required for regulation of gene expression by FTZ-F1. Thus, we propose that FTZ may represent a novel category of LXXLL-motif dependent coactivators for nuclear receptors. For details, see Ref. 1.

(2) Molecular cloning of human squamous cell carcinoma antigen 1 gene and characterization of its promoter

Katsuyuki HAMADA[1], Hiroto SHINOMIYA[2], Yoshihiro ASANO[2], Toshimasa KIHANA[1], Mari IWAMOTO[1], Yasushi HANAKAWA[3], Koji HASHIMOTO[3], Susumu HIROSE, Satoru KYO[4] and Masaharu ITO[1] ([1]Department of Obstetrics and Gynecology, [2]Department of Bacteriology, and [3]Department of Dermatology, School of Medicine, Ehime University, Shitsukawa, Shigenobu, Onsen-gun, Ehime 791-0295, Japan and [4]Department of Obstetrics and Gynecology, Kanazawa University, School of Medicine, Ishikawa 920-0934, Japan)

The squamous cell carcinoma antigen (SCCA) serves as a serological marker for squamous cell carcinomas. Molecular cloning of the SCCA genomic region has revealed the presence of two tandemly arrayed genes, SCCA1 and SCCA2, which are 95% identical in nucleotide sequence. SCCA1 is a papain-like cysteine proteinase inhibitor, while SCCA2 is a chymotrypsin-like serine proteinase inhibitor. We analyzed here the sequence and the promoter activity of the 5'-flanking region of the SCCA1 gene. Deletion analysis of SCCA1 and SCCA2 promoter identified a 471-bp core promoter region upstream of the transcription start site. The transcriptional activity of SCCA1 promoter was up-regulated in squamous cell carcinoma cells, compared with keratinocyte and adenocarcinoma cells. The ratios of SCCA1 to SCCA2 promoter activity in squamous cell carcinoma, keratinocyte and adenocarcinoma cells were respectively 1.6, 5.3 and 2.8. Position -50 of SCCA1 and SCCA2 promoters played an important role in determining the promoter activities of SCCA1 and SCCA2. These findings suggest that the transcriptional regulation of SCCA1 and SCCA2 might differ among squamous cell carcinoma, keratinocyte and adenocarcinoma cells, and that SCCA1 promoter might be a potential target of gene therapy for squamous cell carcinoma. For details, see Ref. 2.

(3) Gene expression of human squamous cell carcinoma antigens 1 and 2 in human cell lines

Katsuyuki HAMADA[1], Yasushi HANAKAWA[2], Koji HASHIMOTO[2], Mari IWAMOTO[1], Toshimasa KIHANA[1], Susumu HIROSE, Masahiko NAKAMURA[3] and Masaharu ITO[1] ([1]Department of Obstetrics and Gynecology, and [2]Dermatology, School of Medicine, Ehime University, shitsukawa, Shigenobu, Onsen-gun, Ehime 791-0295, and [3]Division of Physiology, Institute for Protein Research, Osaka University, Yamadaoka, Suita, Osaka 565-0871, Japan)

The squamous cell carcinoma antigen (SCCA) serves as a serological marker for squamous cell carcinomas. Molecular cloning of the SCCA genomic region has revealed the presence of two tandemly arrayed genes, SCCA1 and SCCA2. SCCA1 is a papain-like cysteine proteinase inhibitor, while SCCA2 is a chymotrypsin-like serine proteinase inhibitor. Little is known concerning how expression of the SCCA1 and SCCA2 genes is regulated in human cell lines. The purpose of this study was to determine whether the SCCA1 gene or SCCA2 gene is more strongly expressed in human cell lines. Squamous cell carcinoma cell lines secreted respectively 4 times and 50 times as much SCCA proteins into medium as normal human keratinocyte and non-squamous cell carcinoma cell lines, as measured by enzyme-linked immunosorbent assay. Quantitative RT-PCR ELISA digoxigenin-labeling assay demonstrated that SCCA1 mRNA expression in squamous cell carcinoma cell lines was respectively 2.8 times and 42 times that in keratinocyte and non-squamous cell carcinoma cell lines. The ratio of SCCA1 to SCCA2 mRNA expression differed distinctly among squamous cell carcinoma, keratinocyte and non-squamous cell carcinoma cell lines (2.8, squamous; 24.1, keratinocyte; 11.0, non-squamous). These findings suggest that SCCA1 is mainly expressed in squamous cell carcinoma, keratinocyte and non-squamous cell carcinoma cell lines and that the ratio of SCCA1 to SCCA2 expression might be a novel marker for the detection of squamous cell carcinoma. For details, see Ref. 3.

(4) A novel growth-related nuclear protein binds and inhibits rat aldolase B gene promoter

Tomoko YABUKI[1], Satoru MIYAGI[1], Hitoshi UEDA, Yasushi SAITOH[1] and Ken-ichi TSUTSUMI[1] ([1]Cryobiosystem Research Center, Faculty of Agriculture, Iwate University, Ueda, Morioka, Iwate 020-8550, Japan)

The promoter of the rat aldolase B (AldB) gene that confers liver-specific transcription has an additional

role. It functions in vivo as an origin region of DNA replication in the cells in which the gene is repressed (Zhao, Y., Tsutsumi, R., Yamaki, M., Nagatsuka, N., Ejiri, S., Tsutsumi, K., 1994. Initiation zone of DNA replication at the rat aldolase B locus encompasses transcription promoter region. Nucleic Acids Res. **22**, 5385-5390). This promoter/origin region has multiple protein-binding sites and, thus, binding of a particular set of protein factors in AldB-expressing or non-expressing cells seems to correlate with functional switch of this promoter/ origin region. In the present study, we characterized two closely related proteins, termed AlF-C1 and AlF-C2, which are assumed to be involved in repression of the AldB gene. These two proteins share an identical amino acid sequence except for a 47-residue-insertion in AlF-C1, and are members of a gene family including heterogeneous nuclear ribonucleoprotein (hnRNP) and CCAAT-binding factor subunit A (CBF-A) genes. Bacterially expressed AlF-C1 can bind sequence-specifically to the AldB gene promoter, whereas AlF-C2 can only weakly. Transfection experiments using mammalian expression vectors showed that AlF-C1 down-regulates the AldB gene promoter in rat hepatoma cells, while AlF-C2 had no or little effect. Expressions of mRNAs encoding these two proteins are enriched in fetal livers and in regenerating livers. These results implied that AlF-C1 and/or C2 is involved in growth-regulated repression of the AldB gene. For details, see Ref. 4.

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C-c. Division of Early Embryogenesis

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

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

Mesoderm induction and its dorsoventral specification are important processes of vertebrate early development. When transplanted onto the animal-pole region of host blastula embryos, the zebrafish yolk cells induce ectopic formation of the mesoderm and the organizer, indicating that the yolk cell, especially yolk syncytial layer (YSL), is responsible for mesoderm induction and its dorsoventral patterning. Recently, genetic studies with mice and zebrafish demonstrated that Nodal could be an endogenous mesoderm inducer. However, we still do not know how the expression of nordal-related genes are regulated in the YSL and/ or it's neighboring blastomeres. To address these questions, we carried out a large scale screening of genes expressed in the zebrafish YSL. By combination of a subtracted cDNA library with our *in situ* screening method, we have successfully obtained more than 10 independent-positive clones out of 200 clones tested (>5%). Sequencing analyses suggest that they encode some enzymes, amino acid transporting proteins, plasma membrane binding protein, transcription factor, RNA binding protein, and many novel genes. Recently, we found that one of the isolated genes, *226D7*, encodes a novel sox gene that acts downstream of Nodal signaling to specify endoderm precursors (Sakaguchi et al., 2001). Functional analyses of other isolated genes are now underway.

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

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

The telencephalon is formed in the most anterior part of the central nervous system (CNS) and is organized into ventral subpallial and dorsal pallial domains. In mice, it has been demonstrated that Fgf signaling has an important role in induction and patterning of the telencephalon. However, the precise role of Fgf signaling is still unclear, due to overlapping functions of Fgf family genes. To address this, we examine, in zebrafish embryos, the activation of Ras/ MAPK, one of the major downstream targets of Fgf signaling. Immunohistochemical analysis reveals that an extracellular signal-regulated kinase (ERK), a vertebrate MAPK, is activated in the anterior neural boundary (ANB) of the developing CNS at early segmentation stages. Experiments with Fgf inhibitors reveal that ERK activation at this stage is totally dependent on Fgf signaling. Interestingly, a substantial amount of ERK activation is observed in *ace* mutants in which *fgf8* gene is mutated. We then examined the function of Fgf signaling in telencephalic development by use of several inhibitors to Fgf signaling cascade including dominant-negative form of Ras (Ras^{N17}) and Fgf receptor (Fgfr), and a chemical inhibitor of Fgfr, SU5402. In treated embryos, the induction of telencephalic territory normally proceeds but the development of the subpallial telencephalon is suppressed, indicating that Fgf signaling is required for the regionalisation within the telencephalon. Finally, antisense experiments with morpholino-modified oligonucleotides suggest that zebrafish *fgf3*, which is also expressed in the ANB, co-operates with *fgf8* in subpallial development (Shinya et al., 2001).

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

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

Somite formation, a process in which reiterated epithelial structures are progressively demarcated from the mesenchymal presomitic mesoderm (PSM) in a rostrocaudal sequence, is thought to be governed by a clock-and-wavefront mechanism. Through Notch signalling, the segmentation clock (oscillation in the PSM cells) is coordinated and translated into a cyclic wave of expression of *hairy*-related and other genes, sweeping caudorostrally through the PSM. This so-called kinematic wave is proposed to operate in conjunction with a maturation wavefront that gradually moves posteriorly, resulting in arrest of the cyclic wave and initiation of segment furrow formation in the anterior PSM. In zebrafish these two essential components of an evolutionalry conserved somite-making mechanism "clock and wavefront" were shown to be Notch- and *fused somites* (*fss*)- dependent, respectively. By experimental manipulation and analyses in zebrafish somitogenesis mutants, we found a novel component, Fgf/ MAPK signaling, involved in segmentation. Fgf signaling activated in the posterior PSM is a crucial positional cue in locating the maturation wavefront in the anterior PSM and in maintaining posterior PSM cells in immature state in an *aei/ deltaD*-and *fss*-independent manner (Sawada et al., 2001; Saga & Takeda, 2001).

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

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

Midline tissues, including the floor plate and notochord, produce inducing signals and pattern the neural tube and somites. A group of zebrafish mutants (midline mutants) has common defects in the ventral neural tube, somites and dorsal aorta. We are now focusing on one of the midline mutants, *chameleon* (*con*). To identify the *con* gene, we mapped the mutation on LG20. We also mapped and compared the positions of candidate genes that may play roles in the Shh pathway. Genomic walking is now underway.

(5) EST project in medaka fish

Takanori NARITA, Tetsuaki KIMURA, Tomoko JINDO and Hiroyuki TAKEDA

Medaka (*Oryzias latipes*) has several advantages to zebrafish. For examples, medaka has about half size of genome (800 Mb; zebrafish-1700 Mb) and the number of genes are expected to be lower than that of zebrafish. Tolerance to cold temperature makes it easy to obtain a temperature-sensitive mutant which would be a

powerful tool for developmental genetics. To gain insight into genetic system of medaka, we perform a large scale isolation of genes expressed in developing medaka embryos.

(6) Genetic screening for mutations affecting early embryogenesis in medaka fish

Daisuke KOBAYASHI, Tadao KITAGAWA, Shigeo TAKASHIMA, Takahiro KAGE, Hayato YOKOI, Atsuhi SAWADA[1], Takanori NARITA, Tetsuaki KIMURA, Tomoko JINDO, Kazuo ARAKI[2], Yuji ISHIKAWA[3] and Hiroyuki TAKEDA ([1]Nagoya University, [2]National Research Institute of Aquaculture, [3]National Institute of Radiological Sciences)

To understand the genetic pathways that govern vertebrate ealy embryogensis, we are conducting a mutant screen in medaka fish. In our screen, male fish mutagenized with ethylnitrosourea (ENU) were outrcossed to wild-type female and the F1 offspring was used to establish F2 families. F2 siblings were intercrossed and the F3 progeny was scored 24 and 48 hours after fertilization for morphological alterations affecting neural, mesodermal and endodermal development.

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C-d. Division of Physiological Genetics

Functional and structural analyses of mammalian Polycomb group complexes

Haruhiko KOSEKI

In order to address the functions of mammalian Polycomb group (PcG) complexes, we intended to identify components of the complexes by employing biochemical and genetic approaches. For this purpose, we focused on following three topics, (1) identification of additional components of mammalian PcG complexes, (2) search for target loci of mammalian PcG and (3) Binding of PcG complexes onto Hox cluster genes.

(1) Involvement of 155kDa spliceosome associated protein (SAP155), Ring1B and Mph2 in mammalian PcG complexes

We have identified SAP155 protein as an interactor of mammalian PcG proteins, Mel18 and Ring1B by yeast two hybrid system. Immunoprecipitation analyses revealed that SAP155 was co-immunoprecitated by anti-Mel18 and anti-Ring1B antibodies from 11.5 dpc mouse embryonic extracts. Further evidences indicating the association of SAP155 with PcG complexes were provided through genetic analyses using *Sap155*-deficient mice. *Sap155*-/- embryos die at morula stage. *Sap155*+/- mice exbihited posterior transformations of the axial skeleton. These alterations were enhanced by *Mel18* mutation while repressed by *Mll* mutation. In addition to SAP155, we identified Ring1B and Mph2 as interactors of Mel18. Ring1B- and Mph2-deficient mice exhibited posterior transformations as well as *Mel18*-, *Bmi1*- and *Sap155*-deficient mice suggesting their inclusion into

PcG complexes.

(2) Generation of cDNA microarray including 15K independent clusters

For systematic screening of downstream genes for PcG, we intended to generate representative cDNA microarray. From full-length cDNA libraries derived from tail bud, fetal gut and adult thymus, we have idolated 15K clusters. In the first preliminary screening using 2K microarray, we have found 10 clusters, whose expressions were affected in Mel18/Bmi1 double homozygotes.

(3) Mapping of binding sites for Ring1B and Mph1 within Hoxb cluster locus

To map PcG responsive elements on mouse Hox cluster locus, we have developed chromatin immunoprecipitation (ChIP) method by using mouse mid-gestation embryos. By using monoclonal antibodies against Ring1B and Mph1, we have successfully immunoprecipitated chromosomal DNA from purified chromatin and binding sites for Ring1B and Mph1were determined with 500 base pairs resolution. Interestingly, Ring1B was shown to tether transcriptionally inactive region exclusively suggesting repressive functions of Ring1B.

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(1) Functional nucleosomal phases over HS2 of the human β-LCR

Ryoiti KIYAMA (National Institute of Advanced Industrial Science and Technology)

High-level, tissue-specific expressions of the β-globin genes require the presence of an upstream locus control region (β-LCR) which coincides with chromatin segments displaying erythroid-specific DNase I hypersensitivity. The tandem NF-E2 binding sites within the DNase I hypersensitive site 2 (HS2) of

the β-LCR are important for the strong enhancer activity. Previously, we reported that the translational phases of the nucleosomes over HS2 of the human β-LCR were arranged by the nucleosome located at curved DNA acting as a key nucleosome and influenced on the enhancer activity (1). Here, we investigated the chromatin structure of HS2 region in further details. The results of gel shift assay and micrococcal nuclease digestion experiments using in vitro reconstituted chromatin showed that only the thirty bp fragment containing the tandem NF-E2 binding sites had less nucleosome forming activity than those from the neighboring sequences, indicating that this region has a specific chromatin structure suitable for function. The results of hydroxyradical footprinting suggested that the nucleosomal phase of HS2 region was rotationally unique while there were multiple translational phases and that the tandem NF-E2 binding sites were always located outside but not in the histone side, facilitating interaction with NF-E2. Finally, we examined the binding of NF-E2 to the sites in the packed chromatin over HS2 region by ligation-mediated PCR and a chromatin immunoprecipitation assay using non-erythroid HeLa cells, where we provided evidence for direct NF-E2 binding on the nucleosome *in vivo*. These results support a crucial role of NF-E2 in the regulation of β-globin gene expression through a direct interaction to the cognate motifs located on the chromatin before chromatin remodeling.

(2) Functional genotyping based on DNA rearrangements and its application

Ryoiti KIYAMA (National Institute of Advanced Industrial Science and Technology)

We focus upon the mechanism of tumorigenesis by loss of heterozygosity (LOH) which results in loss of function of tumor suppressor genes. For this, we first obtained candidate genetic loci by means of genomic subtraction between the normal and tumor (the mixed cell type and the clear cell type of renal cell carcinoma) cell DNA from the same individual which was followed by cloning the DNA fragments derived from the sites of DNA arrangements. We obtained a total of 44 such loci, which showed 10% to 90% of frequencies of LOH among approximately 100 patients (1). They included *VHL* (von Hippel Lindau), *APC* and *IRF-1* and other previously reported loci as well as new loci including 5q32-q34, 6q21-q22 and 8p12. Four such loci had minimum distances of less than 10 mega-bases (Mb). We obtained a candidate tumor suppressor gene from one of these loci and functional analysis of the gene is now under way. Using the information of candidate loci, we fabricated DNA microarrays for high throughput genotyping of the candidate genes.

Publications

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D. DIVISION OF POPULATION GENETICS

D-a. Division of Population Genetics

(1) Local changes in mutation pressure in Drosophila genomes

Toshiyuki TAKANO-SHIMIZU

The genomes of warm-blooded vertebrates, particularly humans, are compartmentalized into the so-called isochores that are DNA segments of sizes varying from 200 kb to more than 1 Mb and relatively homogeneous in their DNA content. The genome-composition organization is assumed to be determined and maintained by the action of mutation and selection, but the degree to which these two factors have contributed has been the subject of much controversy. Regardless of the validity of the mutation and selection hypotheses, both hypotheses are based on the finding that local environments along chromosomes affect DNA composition of genes.

By contrast, Drosophila genomes have been considered to be much more homogeneous with respect to G+C content along chromosomes. However, I found evidence of remarkable local changes in GC/AT substitution biases on Drosophila chromosomes. The substitution pattern at ten loci in the telomeric region of the *X* chromosome was studied for four species of the *D. melanogaster* species subgroup. *D. orena* and *D. erecta* are clearly the most closely related species pair (the *erecta* complex) among the four species studied; however, the overall data at the ten loci revealed a clear dichotomy in the silent substitution patterns between AT-biased-substitution *melanogaster* and *erecta* lineages and GC-biased-substitution *yakuba* and *orena* lineages, suggesting two or more independent changes in GC/AT substitution biases. More importantly, the results indicated a between-locus heterogeneity in GC/AT substitution bias in this small region independently in the *yakuba* and *orena* lineages. Indeed, silent substitutions in the *orena* lineage were significantly biased toward G and C at the consecutive *yellow, lethal of scute,* and *asense* loci, but they were significantly biased toward A and T at *sta.* The substitution bias toward G and C was centered in different areas in *yakuba* (significantly biased at *EG:165H7.3, EG:171D11.2,* and *suppressor of sable*). The similar silent substitution patterns in coding and noncoding regions, further, suggested mutational biases as a cause of the substitution biases.

In sum, this study offered evidence of heterogeneous and species-specific (fluctuating) mutation pressure along Drosophila chromosomes.

For details, see Ref. 1.

(2) Genetic study of evolutionary changes in the genes involved in Drosophila bristle formation

Toshiyuki TAKANO-SHIMIZU

Interspecific cross is a powerful means to uncover hidden within- and between-species variation in populations. One example of such hybrid anomaly is a bristle loss phenotype of hybrids between *Drosophila melanogaster* and *D. simulans,* though both the pure species have exactly the same pattern of bristle formation on the notum. This means that the genetic architecture of bristle formation can change in local populations in the absence of any obvious phenotypic alternation. There exists a large amount of genetic variability in the *simulans* populations with respect to the number of missing bristles in hybrids, and the variation is largely attributable to *simulans X* chromosomes. I screened the *simulans X* chromosome for genetic factors that were responsible for the differences between a pair of *simulans* lines with high (H) and low (L) missing bristle numbers. A single major quantitative locus was mapped to the 14-16 region. This indeed explained nearly all difference between the two parental strains and also accounted for most of the differences between H and L lines in three other independent pairs.

We took a further fine mapping experiment to identify the QTL: a construction of about 2500 recombinant strains between the H and L *simulans* lines. They were studied for the number of missing bristles in hybrids with *D. melanogaster* and for genotypes at several marker loci covering the 14-16 region. This ends up identification of 15 recombinants

in an about 500 kb region studied. These enable a subsequent fine mapping of the QTL.

(3) Genetic and molecular dissection of the within- and between-species variation in the sex comb teeth number in Drosophila

Toshiyuki TAKANO-SHIMIZU

Sexual dimorphic characters are very often among the first characters to change in the evolution of new species, and they provide a number of advantages for studying the genetic and molecular basis of species differences in morphology. Males of some species of the subgenus *Sophophora* including *D. melanogaster* posses sex-specific bristles on their first legs, the so-called sex comb. The sex comb is a highly variable character between species and within species.

We analyzed the within-species sex-comb-teeth number variation by the composite interval mapping for F2 samples between high and low sex-comb-teeth number lines. We did this with an aim to identify quantitative trait loci and to estimate their effects, finding four significant QTL, two on each major autosomes. The sign of the QTLs differed among QTLs, and one of them showed an overdominant effect. Because of a lack of male recombination, the F2 analysis cannot study all possible genotypes even in a very large number of samples; for instance, a double homozygote at two loci on a single chromosome never emerges in F2 populations. Then, the chromosome substitution or recombinant inbred strains were constructed, and they can now be used for our subsequent fine mapping and for studying epistasis between QTLs.

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

Publication

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D-b. Division of Evolutionary Genetics

(1) Functional analyses of centromere of higher vertebrate cells

Tatsuo FUKAGAWA, Atsushi OKAMURA, Ai NISHIHASHI, Yoshikazu MIKAMI, Toshimichi IKEMURA

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

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

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

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

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

IV) ZW10 is required for a spindle checkpoint function in vertebrate cells.

V) We identified a novel constitutive centromere protein, CENP-I, which shows sequence similarity with fission yeast Mis6 protein, and we showed that CENP-I is a constitutive component of the centromere that colocalizes with CENP-A, -C and -H throughout the cell cycle in vertebrate cells. To determine the precise function of CENP-I, we examined its role in centromere

function and assembly by generating a conditional loss-of-function mutant in the chicken DT40 cell line. In the absence of CENP-I, cells accumulated BubR1 at kinetochores and arrested at prometaphase with mis-aligned chromosomes for long periods of time. Eventually, checkpoint function appeared to be lost and cells reentered the cell cycle without undergoing cytokinesis. Immunocytochemical analysis of CENP-I-deficient cells demonstrated that both CENP-I and CENP-H are necessary for CENP-C, but not CENP-A, lo alization to the centromere, indicating that CENP-I plays an essential role in centromere assembly in vertebrate cells. These experiments define an assembly pathway for the vertebrate kinetochore in which binding of CENP-A is followed by mutually interdependent targeting of CENP-H and CENP-I, and then by binding of CENP-C.

For detail, Fukagawa et al., EMBO J., 20, (2001), Fukagawa et al., Nucl. Acids Res., (2001). Okamuta et al., Gene, 283, (2001), and Sonoda et al., Dev. Cell, 1, (2001)

(2) Chromosome-wide measurement of replication timing for human chromosomes 11q and 21q: disease-related genes and genome-synteny breakage in timing- witch regions

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The completion of the human genome sequence provides fundamental knowledge to a wide field of biology. We used the sequence information to measure replication timing of the entire lengths of human chromosomes 11q and 21q focusing on a total of 450 STSs. Mb-sized zones that replicate early or late in S phase (thus early/ late transition) were defined at the sequence level. Early zones were more GC-rich and gene-rich than were late zones, and early/ late transitions occurred primarily at positions identical to or near GC% transitions. In the early/ late transition regions, concentrated occurrence of cancer-related genes that include *CCND1* encoding cyclin D1, *FGF4*, *TIAM1*, and *FLI1* was observed. The transition regions contained other disease-related genes including *APP* associated with familial Alzheimer's disease, *SOD1* associated with familial ALS, and *PTS* associated with phenylketonuria. We also found the single nucleotide polymorphism (SNP) frequency was high in transition regions. These findings are consitent with the prediction that increased DNA damage occurs in replication-transition regions. We also found that breakage of synteny between the human and mouse genomes occurred primarily at or near the replication-transition regions.

For detail, Science, 294, 2282, 2001; and Human Molecular Genetics, **11**, 13-21, 2002.

(3) Analysis of codon- and oligonucleotide-composition diversity of bacterial genomes with a self-organizing map (SOM)

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With increases in the amounts of available DNA sequence data, it has become increasingly important to develop tools for comprehensive systematic analysis and comparison of species specific characteristics of genome sequences for a wide range of species. In the present study, we used a novel neural-network algorithm, a self-organizing map (SOM), to efficiently and comprehensively analyze codon usage in approximately 60,000 genes from 29 bacterial species simultaneously. This SOM makes it possible to cluster and visualize genes of individual species separately at a higher resolution than can be obtained with principal component analysis. The organization of the SOM can be explained by the tRNA compositions and genome G+C% of the individual species. Using SOM, we examined codon usage heterogeneity in the *E. coli* O157 genome, which contains "O157-unique segments (O-islands)", and showed that the SOM is a powerful new strategy for characterization of horizontally transferred genes. For details, see Kanaya et al., Gene (2001). With SOM, we also analyzed di-, tri-, and tetra-nucleotide frequencies of the 65 bacterial genomes and obtained good intra- and inter-species separation, which could not be obtained with conventional multivariate methods.

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(1) Murine equivalent of the human histo-blood group ABO gene is a cis-AB gene and encodes a glycosyltransferase with both A and B transferase activity

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We have cloned murine genomic and complementary DNA that is equivalent to the human ABO gene. The murine gene consists of at least 6 coding exons and spans at least 11 kilobase pairs (kbp). Exon-intron boundaries are similar to those of the human gene. Unlike human A and B genes that encode two distinct glycosyltransferases with different donor nucleotide-sugar specificities, the murine gene is a *cis*-AB gene that encodes an enzyme with both A and B transferase activities, and this *cis*-AB gene prevails in the mouse population. Cloning of the murine AB gene may be helpful in establishing a mouse model system to assess the functionality of the ABO genes in the future. For details, see ref. 1.

(2) CAMUS DB for amino acid sequence data

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DDBJ/ EMBL/ GenBank International Nucleotide

Sequence Database is still increasing, keeping doubling time only slightly longer than one year for the last these years. This situation affects the increase of DDBJ Amino acid sequence Database (DAD), which is made from translation of nucleotide sequences in CDS regions annotated in DDBJ, and makes computation time for homology search of DAD longer as well as that of DNA database. We therefore created compressed sequence database, consisting of highly homologous sequence clusters in multiple aligned form with representative sequences, the DAD version of CAMUS (Compressed database for homology searches And MUltiple aligned Sequence database). Website URL of CAMUS DB is http://hypernig.nig.ac.jp/camus/. For details, see ref. 2.

(3) Haplotype analysis of human alpha2-HS glycoprotein (fetuin)

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Alpha2-HS glycoprotein (AHSG), which is equivalent to fetuin in other species, is a protein found in human plasma. AHSG is polymorphic with two common alleles and many variants. To examine the intragenic haplotypes and their diversity at this locus, a contiguous genomic DNA sequence (10.3 kb) was analyzed in 20 samples (40 chromosomes), and haplotypes were determined for 309 subjects. Judging from the aligned nucleotide sequences and the conserved amino acid residues comparing human and chimpanzee AHSG, it was concluded that the type 1 allele is probably older and has evolved into four major suballeles. The type 2 allele was generated from one branch of the type 1 allele. AHSG*3 and *5 variants were each found to have a single nucleotide change in exon 7, resulting in the change of an amino acid residue from Arg299 to Cys and from Asp258 to Asn, respectively. It was noted that the AHSG*3 mutation gives rise to an additional cysteine residue, which possibly affects the conformation of the protein. The AHSG gene was found to have a low mutation rate and no apparent recombination events. Furthermore, the detected substitutions were nonhomogeneously distributed at this locus. In particular, four nonsynonymous substitutions were concentrated in the carboxyl-terminal domain. For details, see ref. 3.

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D-c. Division of Theoretical Genetics

(1) Autonomous formation of spatial pattern in development

Shigeru KONDO

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

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

The formation of periodic patterns in animal skins has been explained by the reaction-diffusion (RD) system, a hypothetical chemical reaction proposed by A. Turing1. However, the model alone cannot explain the directionality of stripes. To investigate the mechanism regulating the direction of stripes, we have studied differences in the pattern formation of two species of Genicanthus that share almost identical morphological properties except for their stripe direction. In both species, stripes are formed by the rearrangement of the characteristic transient pattern that suggest the underlying mechanisms. Computational analysis shows that adding diffusion anisotropy to the standard RD model can explain all of the features of pattern formation in Genicanthus. As only a weak anisotropy is required for the effect, a small change in the skin structure may cause a marked change in stripe direction, which might have implications for evolution and initiating species divergence.

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(1) Development of ontology and database for the cell signaling system

Takako TAKAI[1] and Toshihisa TAKAGI ([1]Human Genome Center, Institute of Medical Science, University of Tokyo)

In the post-genome sequencing era, the most significant issue is the reconstruction ofliving organisms in computer, based on their genome information. Reconstruction and analysis of molecular interactions among gene products, pathways, and networks could be addressed as its first step. We analyzed conceptual structure of the cell signaling system and specified the concepts as SIGNAL-ONTOLOGY.

The ontology consists of concepts of molecules, molecular interactions, pathway motifs, and cellular functions. We also developed a database for the cell signalling system, SPARK, based on the ontology. The database system is constructed by XML-database, compound graph representation, and ontology. All the data contained in the database are collected from literatures according to controlled vocabulary in the ontology. SIGNAL-ONTOLOGY and SPARK will be opened soon from http://ontology.ims.u-tokyo.ac.jp/.

(2) Knowledge representation of Signal transduction pathways and logical inferences

Ken-ichiro FUKUDA[1] and Toshihisa TAKAGI ([1]Computational Biology Research Center, National Institute of Advanced Industrial Science and Technology)

Our group focuses on providing methods required to develop Signal Transduction Pathway (STP) databases. The problem is broken down into two subproblems, i.e., knowledge representation design of STPs and its utilization to infer relevant biological hypothesis. The knowledge representation model that we developed is based on a Compound graph model and can cope with knowledge fragmentation, complex hierarchies and various levels of details on specific bodies of knowledge (heterogeneous knowledge granularity). Equipped with the ontologies for STPs, the model is able to formalize the knowledge described

with natural language or drawings of diagrams. Then, the inference procedure that a biologist performs is modeled as a hypothetical reasoning framework on a case-base, and a prototype knowledge base was implemented, which infers cross-talk of pathways.

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E. DEPARTMENT OF INTEGRATED GENETICS

E-a. Division of Human Genetics

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

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Genomic imprinting, an epigenetic gene-marking phenomenon, causes parental-origin-specific monoallelic expression of a subset of mammalian genes. Imprinted genes tend to form clusters in specific regions of the genome, which may be related to the mechanism of imprinting or the reason for the evolution of imprinting. As a step to know the structural and functional characteristics of the imprinted genome domains, we study the mouse chromosome region 7F4/ F5, which contains at least 12 imprinted genes. This region is syntenic to human 11p15.5, which contains genetic loci responsible for Beckwith-Wiedemann syndrome and some types of tumors. We have determined the complete DNA sequence of this mouse domain of 1.0 Mb and the corresponding chicken region of 0.5 Mb. A more detailed analysis of the sequence is now ongoing. Since chicken does not appear to have imprinting, comparisons of the mouse and chicken sequences should reveal regulatory elements specifically involved in imprinting.

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

Hiroyuki SASAKI, Ko ISHIHARA, Hiroyasu FURUUMI, Yuzuru KATO and Wolf REIK[1] ([1]Babraham Inst., UK)

The imprinted mouse 7F4/ F5 domain contains two linked imprinted genes *Igf2* and *H19* near its centromeric boundary: *Igf2* is paternally expressed and *H19* maternally expressed. It is known that the paternal-specific methylation of the differentially methylated region (DMR) located upstream of *H19* is the primary cause for the *Igf2/ H19* imprinting. We have investigated the temporal and spatial changes in *Igf2/ H19* imprinting during mouse development (ref. 2, 4 and 9). We have also identified an evolutionarily conserved CTCF-dependent insulator element at the centromeric boundary of the *Igf2/ H19* sub-domain (submitted). Lastly, we have characterized a mouse mutant called *minute*, which has an inversion breakpoint at about 20-kb downstream of *H19* (ref. 10).

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

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

DNA methylation works as an important gene-marking mechanism for the discrimination of the parental alleles of imprinted genes. To understand how the primary imprints are established in the male and female germ cells, we study the expression and localization of the *de novo* DNA methyltransferases *Dnmt3a* and *Dnmt3b* in the male and female gonads. We have found that Dnmt3b and a specific form of Dnmt3a are present in the nuclei of prospermatogonia (also called gonocytes) and growing oocytes (in preparation), in which the primary imprints are established. We also characterized the enzymatic properties of *Dnmt3a* and *Dnmt3b in vitro* (ref. 6).

(4) DNA methylation, imprinting and human disorders

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

It is known that tumor suppressor genes are methylated and inactivated in a number of cancers. We have found that acute meyelogenous leukemia cases with methylated p15 tumor suppressor gene have higher levels of all three DNA methyltransferases *DNMT1, DNMT3A* and *DNMT3B* (ref. 1). We have

also studied three Japanese cases with ICF (immunodeficiency, centromeric instability, facial anomalies) syndrome, an autosomal recessive disorder with aberrant DNA methylation, and identified three novel mutations within the coding region of *DNMT3B* (submitted). Lastly, we have reported that paternal disomy for chromosome 14 causes a distinctive malformation syndrome with abdominal muscular defects, skeletal anomalies and characteristic facies (ref. 8).

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

Hiroyuki SASAKI, Takaaki YOKOMINE, Asato KUROIWA[1], Yoichi MATSUDA[1] and Masaoki TSUDUKI[2] ([1]Hokkaido Univ.; [2]Hiroshima Univ.)

Although it is generally thought that genomic imprinting has evolved only in mammals, this has not been tested in other vertebrate species. We have asked whether the homologues of the mammalian imprinted genes show parental-origin-specific monoallelic expression in chicken. Using DNA polymorphisms identified between various breeds and strains, it was shown that both *IGF2* and *MPR1* are expressed biallelically in chicken embryos (ref. 5). We also found that chicken Z chromosome is not subject to inactivation for gene dosage compensation in ZZ males (in preparation). We are currently studying the structure and function of chicken DNA methyltransferases, in order to know whether the differences in DNA methylation machinery can be the cause for the evolution of genomic imprinting and dosage compensation of sex chromosomes.

(6) Development of a universal DNA chip system applicable for any organism

Hiroyuki SASAKI, Hisao SHIROHZU, Shin-ichi MIZUNO[1], Tadafumi IINO[1], Hidetoshi OZAWA[1], Teruhisa OTSUKA[1] and Kosuke TASHIRO[1] ([1]Kyushu Univ.)

We have started a collaborative research project to develop a universal DNA chip system that can be used to study the expression of all genes from any organism.

(7) Role for *Tsix* in X chromosome inactivation

Takashi SADO, En LI[1] and Hirosyuki SASAKI ([1]Harvard Med. Sch.)

The *Xist* gene is essential for the initiation of X inactivation. It is known that antisense transcription occurs at the *Xist* locus to produce *Tsix* RNA. We have generated *Tsix*-deficient mouse and shown that *Tsix* plays an important role to regulate *Xist* expression. Available evidence suggests that *Tsix* RNA itself is critical rather than antisense nature of transcription. Using ES cells deficient for either *Tsix* or *Xist*, we are currently trying to elucidate how *Tsix* RNA exert its effect on *Xist* expression.

(8) Role for *Dnmt3a* and *Dnmt3b* in X chromosome inactivation

Takashi SADO, Masaki OKANO[1], En LI[2] and Hiroyuki SASAKI ([1]RIKEN, CDB, [2]Harvard Med. Sch.)

To study the role for *de novo* methyltransferases in X inactivation, we examined expression of *Xist* in male ES cells doubly deficient for Dnmt3a and Dnmt3b, in which the promoter region of the *Xist* gene is extensively demethyated. RNA FISH revealed that *Xist* becomes ectopically expressed and accumulates on the X chromosome upon induction of differentiation. It appeared, however, that the X chromosome coated with *Xist* RNA did not become transcriptionally inactivated, suggesting that these *de novo* methyltransferases are involved in the process of X inactivation following accumulation of *Xist* on X chromosome.

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

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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. This simple notion is often misunderstood by many people, even

including many scientists. The scientific importance of the human genome project both for science and humanity is well proven through many publications in major journals. The Nature paper by the International Human Genome Consortium is the most frequently cited paper in life-science field. Since most of human chromosomes can be purified by means of dual-laser cell sorting system, such isolated chromosomes are good resources for the studies to understand biological functions retained in individual chromosome. Using purified chromosomes, we have constructed human mono-chromosomal cosmid libraries (except for CM#9-12), CM#9-12, #21, CM-Y fosmid libraries, and BAC libraries. In addition, we are in the process of constructing primate libraries including chimpanzee and gorilla. Using these resources, BAC-end sequencing of chimpanzee clones proved that the difference between human and chimpanzee sequence is less than 1.5%.

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

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In fission yeast, *Schizosaccharomyces pombe,* deficiency of ras1 gene causes abnormal cell shape and abolishes mating ability. However, the signaling pathway in the cell and its target genes are largely unknown because of the lack of appropriate analysis system. To overcome this problem, we categorized genes based on their expression levels in the presence or absence of the ras1 gene product under different growth conditions. We utilized micro-arrays of clones covering entire genome of the fission yeast. Y-S. B. earned his Ph.D. degree through this work.

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E-b. Division of Agricultural Genetics

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

Tetsuji KAKUTANI, Tetsu KINOSHITA, Asuka MIURA, Koichi WATANABE and Masaomi KATO

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

In Arabidopsis, genome sequencing has been completed for the first case in plants. In addition, many trans mutations affecting epigenetic states have been isolated in this plant. Arabidopsis *ddm1* (*decrease in DNA methylation*) mutation results in decrease in methylation and transcriptional de-repression in genomic repeat sequences. The *DDM1* gene encodes a protein similar to the chromatin-remodeling factor SWI2/SNF2 (Jeddeloh *et al.*, Nat. Genet. 12, 1714-) The most striking feature of *ddm1* mutation is that it induces a variety of developmental abnormalities by causing heritable change in other loci (Kakutani *et al.*, PNAS 93, 12406-). The molecular basis has been clarified in two of the loci directly causing the developmental abnormalities.

One of them, *clam*, is characterized by lack of elongation in leaves, roots and shoots. This phenotype is heritable, but somatic sectors with normal phenotype were occasionally observed. The size and frequency of the sector differ from plant to plant. The phenotype was stabilized in some of the progeny families; no reversion sector was observed in such family. We mapped the locus responsible for the clam phenotype at high resolution. By genotyping 926 chromosomes, we identified the gene responsible for the *clam* phenotype. It is *DWF4* gene, which involved in synthesis of brassinolide, a plant growth regulator necessary for cell elongation. The unstable *clam* phenotype was induced by insertion of a novel endogenous Arabidopsis transposon, which we named *CACTA1*. This transposon transposes and increases in the copy number specifically in *ddm1* mutant background. These results suggest that gene silencing associated with DNA methylation is important for suppression of transposons (ref.1).

Another developmental abnormality, late flowering trait, was induced by ectopic over expression of *FWA* gene associated with hypomethylation of tandem repeat upstream of the coding region. Interesting thing is that change in nucleotide sequence was also not observed in *fwa-1* and *fwa-2* alleles isolated by conventional mutagenesis. In both cases, over-expression associated with the hypomethylation resulted in the phenotypes (Soppe et *al*,, Mol. Cell 6, 791-). An interesting remaining problem is function of *FWA* gene product during normal development.

(2) DNA hypomethylation mutation in rice

Tetsuji KAKUTANI, Koichi WATANABE and Asuka MIURA

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

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E-c. Division of Brain Function

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

Tatsumi HIRATA

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

(2) Mosaic development of the olfactory cortex with *Pax6*-dependent and -independent components

Tatsumi HIRATA, Yoshiko TAKAGI, Yasufumi SATO

The olfactory cortex is the target area of olfactory bulb axons and suggested to be derived from neuroepithelial progenitors of various ventricular domains during development. We examined the development of the olfactory cortex, using the newly developed monoclonal antibody (mAb) 9-4c, which recognizes reticulon 1-A and -B. The mAb labeled neuroepithelial progenitors at the pallio-subpallial boundary and their putative descendants in the deep layers of the olfactory cortex. In the *Pax6* mutant embryo, labeling at the pallio-subpallial boundary was specifically lacking, and the number of immunopositive cells in the olfactory cortex was markedly reduced. In contrast, the guidepost neurons of olfactory bulb axons, lot cells, developed relatively normally in the superficial layer of the olfactory cortex in the mutant embryo. These guidepost neurons have been recently shown to originate in the pallium and eventually guide the initial projection of olfactory bulb axons. The olfactory bulb projection in the *Pax6* mutant embryo

also suggested the dualistic nature of the olfactory cortex development; the initial projection of olfactory bulb axons developed relatively normally, whereas the final projection of their collateral branches was severely defective.

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E-d. Division of Applied Genetics

(1) The rice retrotransposon *Tos17* prefers low-copy-number sequences as integration targets

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The rice retrotransposon *Tos17* is highly activated by tissue culture. To evaluate the impact of transposition of *Tos17* on the rice genome and examine its utility for insertional mutagenesis, more than 100 sequences flanking newly transposed *Tos17* copies were characterized. The 5-bp target-site duplications flanking *Tos17* did not show any consensus sequence, and preferred nucleotides, A/T and G/C, were only found at the second and third nucleotides from both ends of the target site duplications, respectively, indicating that *Tos17* has relatively low target-site specificity at the nucleotide sequence level. Integration targets were widely distributed over the chromosomes; however, preferential integration into the sucrose synthase 2 gene and into *Tos17* itself was demonstrated by PCR screening using pooled DNA prepared from the mutant population. Hybridization studies indicated that *Tos17* preferentially integrates into low-copy-number regions of the genome. In agreement with this result, about 30% of flanking sequences examined showed significant homology to known genes. Taken together, these results show that *Tos17* can have a significant impact on the rice genome and can be used as a tool for efficient insertional mutagenesis.

(2) Screening of the rice viviparous mutants generated by endogenous retrotransposon *Tos17* insertion. Tagging of a zeaxanthin epoxidase gene and a novel *OsTATC* gene

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The rice retrotransposon *Tos17* is one of a few active retrotransposons in plants and its transposition is activated by tissue culture. Here, we present the characterization of viviparous mutants of rice induced by tissue culture to demonstrate the feasibility of the use of retrotransposon *Tos17* as an endogenous insertional mutagen and cloning of the tagged gene for forward genetics in unraveling the gene function. Two mutants were shown to be caused by the insertion of *Tos17*. *Osabal*, a strong viviparous mutant with wilty phenotype, displayed low abscisic acid level and almost no further increase in its levels upon drought. The mutant is shown to be impaired in the epoxidation of zeaxanthin. On the other hand, *Ostatc*, a mutant with weak phenotype, exhibited the pale green phenotype and slight increase in abscisic acid levels upon drought. Deduced amino acids of the causative genes of *Osabal* and *Ostatc* manifested a significantly high homology with zeaxanthin epoxidase isolated from other plant species and with bacterial Sec-independent translocase TATC protein, respectively. This is the first example of transposon tagging in rice.

(3) Isolation and characterization of rice phytochrome A mutants

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To elucidate phytochrome A (*phyA*) function in rice, we screened a large population of retrotransposon (*Tos17*) insertional mutants by polymerase chain reaction and isolated three independent *phyA* mutant lines. Sequencing of the *Tos17* insertion sites confirmed that the *Tos17s* interrupted exons of *PHYA* genes in these mutant lines. Moreover, the *phyA* polypeptides were not immunochemically detectable in these *phyA* mutants. The seedlings of *phyA* mutants grown in continuous far-red light showed essentially the same phenotype as dark-grown seedlings, indicating the insensitivity of *phyA* mutants to far-red light. The etiolated seedlings of *phyA* mutants also were insensitive to a pulse of far-red light or very low fluence red light. In contrast, *phyA* mutants were morphologically indistinguishable from wild type under continuous red light. Therefore, rice *phyA* controls photomorphogenesis in two distinct modes of photoperception--far-red light-dependent high irradiance response and very low fluence response--and such function seems to be unique and restricted to the deetiolation process. Interestingly, continuous far-red light induced the expression of CAB and RBCS genes in rice *phyA* seedlings, suggesting the existence of a photoreceptor(s) other than *phyA* that can perceive continuous far-red light in the etiolated seedlings.

(4) Linear DNA intermediates of the *Ttol* retrotransposon in Gag particles accumulated in stressed tobacco and *Arabidopsis thaliana*

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The active transcription of some plant retrotransposons under diverse stress conditions suggests active transposition. However, transposition has been demonstrated only during tissue/cell culture. To examine whether transposition is activated under conditions other than tissue/cell culture, DNA intermediates for retrotransposition of the tobacco retrotransposon *Ttol* were analyzed. Using transgenic Arabidopsis callus expressing high levels of *Ttol* RNA in a *ddm1* hypomethylation mutant background, the existence of extrachromosomal *Ttol* linear DNA molecules in a Gag-particle fraction was demonstrated. By combination with ligation-mediated PCR amplification, we detected *Ttol* linear DNA molecules in particle fractions from callus and methyl jasmonate-treated leaves of tobacco, but not from non-stressed leaves. *Ttol* DNA intermediates could not be detected in the tobacco corolla where *Ttol* is expressed. These results indicate that the transcriptional activation of *Ttol* by defense-related stresses leads to the synthesis of DNA intermediates, whereas post-transcriptional suppression of *Ttol* activity is suggested in the corolla.

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(1) Control of X-chromosome inactivationin early mouse embryogenesis

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

One of two X chromosomes is transcriptionally silenced in early embryonic cells of female mammals to compensate for the two-fold differences in X-linked gene dosage difference occurring between XY males and XX females. It has been shown that the paternally inherited X chromosome (Xp) is selectively inactivated in such extraembryonic tissues as trophectoderm and primitive endoderm which differentiate earlier than epiblast tissues giving rise to three definitive germ layers of the entire fetus. Only Xp is inactivated in trophectoderm of normally fertilized female embryos, since the paternal *Xist* allele is solely expressed due to genomic imprinting. In spite of such imprinting, we found random inactivation in XpXp androgenetic embryos not only in all epiblast but extraembryonic tissues. Thus, we went on to further study on the control of the paternal *Xist* allele in mouse embryos making use of Xp0 embryos produced by females heterozygous for a large paracentric X chromosome inversion, In (X) 1H. *Xist* RNA FISH study revealed that the paternally derived *Xist* allele is highly expressed in every cell of the embryo from the 4-cell stage onward, but it is terminated by the blastocyst stage without inactivating the only X chromosome in a cell. The present and earlier findings suggest that the paternal *Xist* allele is expressed by default at cleavage stages. *Xist* expression is terminated in Xp0 embryos as a result of counting the number of X chromosome in a cell occurring at the morula/blastocyst stage. Most probably, therefore, X-inactivation in fertilized XX embryos takes place in the following manner: After counting the number of X chromosome in a cell, high-level *Xist* expression is continued in XX embryos. The high level *Xist* expression becomes irreversible in differentiating trophectoderm cells, whereas *Xist* expression reverts to the initial low level in undifferentiated ICM. Choice of future inactive X becomes random since Xp and Xm somehow becomes epigenetically equivalent during this period.

(2) Search for genes involved in X chromosome inactivation using murine T (X;16) 16H translocation

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

The morphologically normal X chromosome is inactivated in all cells of female mice heterozygous for Searle's T (X;16) 16H translocation. Cytogenetic and biochemical studies suggest that the initial choice of the future inactive X chromosome is non-random in favor of the normal X chromosome in heterozygous embryos. Since this non-random inactivation could be due to breakage or position effect of a gene (s) involved in random choice due to Searle's translocation, we decided to characterize the translocation breakpoint for ultimate cloning of the candidate gene. The X chromosomal breakpoint has been placed in the 4cM region between *G6pdx* and *Pola1*. Since the breakpoint could not be found in a YAC clone containing *Pola1*, we switched our endeavor to screening of BAC library. We successfully isolated several BAC clones containing DNA markers known to be present between *G6pdx* and *Pola1*. By FISH using these clones as probes, we found that the breakpoint is between two loci M-02832 and M-00766. Analysis is now under way to locate the breakpoint in YAC clones containing M-02832 and M-00766, respectively. At the same time we are trying to make a complete physical map around the breakpoint including M-02832 and M-00766.

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F. DEPARTMENT OF GENETIC STRAINS RESEARCH CENTER

F-a. Division of Mammalian Genetics Laboratory

(1) Male-specific reproductive failure caused by X-chromosomal substitution between two mouse subspecies

Ayako OKA, Nobuo TAKAGI[1], Kiyotaka TOSHIMORI[2], Toshiyuki TAKANO-SHIMIZU[3], Akihiko MITA, Yoichi MIZUSHINA, Noriko SAKURAI, Hiromi YAMAMOTO and Toshihiko SHIROISHI ([1]Res. Cent. For Mol. Genet. And Grad. Schl. Of Env. Earth Sci. Hokkaido Univ., [2]Department of Anatomy and Cell Biology, Miyazaki Medical College, [3]Division of Population Genetics, NIG)

In the course of constructing a consomic mouse strain, B6.MSM-ChrX, by replacing X-chromosome of a standard laboratory strain C57BL/6J (*Mus musculus domesticus*) with that of a strain MSM (*Mus musculus molossinus*), we observed declining fecundity only in the males that carry the MSM-derived X chromosome (X-MSM/Y) as the backcross generations progressed. It suggested that epistatic interaction of X-linked gene(s) and autosomal and/or Y-linked gene(s) controls fecundity of the male mice. The observed infertility is attributable to disruption of the interaction between MSM-derived X-linked gene(s) and autosomal and/or Y-linked gene(s) of C57BL/6J.

In this study, we carried out in-depth phenotype characterization of the X-MSM/Y males. we also conducted mapping of the X-linked genes responsible for the phenotypes by quantitative trait loci (QTL) analysis. The results of the phenotype characterization indicated that the X-MSM/Y males failed to fertilize, or have some defects in very early development after the fertilization. The X-MSM/Y males had the reduced testis weights and various severities of degeneration at the histological level in the testis. Furthermore, spermatozoa of the X-MSM/Y males showed malformation in their heads and low motility. Much severe abnormality of sperm heads was observed in the advanced backcross generations. The QTL analyses for the reduced testis weight and the abnormal spermatozoal morphology detected distinct QTLs for each phenotype, which is located to the distal and the central region of X chromosome respectively. This study provided genetic basis of the initiation process of reproductive isolation between the two subspecies, *M. m. domesticus* and *M. m. molossinus*.

(2) Genetic analysis of a mouse mutant, X-linked polydactyly (*Xpl*)

Yukari YADA[1], Shigeru MAKINO, Sadao ISHIWA[1] and Toshihiko SHIROISHI. ([1]Ochanomizu Univ.)

X-linked polydactyly (*Xpl*) is a spontaneous mouse mutation that exhibits preaxial polydactyly only on the hindlimb. To study the molecular basis underlying *Xpl* phenotype, we analyzed the expression patterns of several marker genes in the limb buds of *Xpl* embryos by *in situ* hybridization. At E11.5, ectopic expression of genes such as *Shh, Fgf4, ptc, Gre* and *Hoxd11* were observed at the anterior margin of the hindlimb buds. These markers were not detected before the initial expression of *Shh* gene at the posterior margin of the limb buds. However, at E10.5, *Gli*, which is a downstream gene of *Shh*, was expressed at the anterior side of the *Xpl* hindlimb prior to the ectopic *Shh* expression. All results suggested that, in the normal limb development, *Xpl* gene acts in the downstream pathway of *Shh* signaling cascade.

To isolate the actual *Xpl* gene, we carried out linkage analyses of *Xpl* in crosses with wild mice-derived strains. As a result, *Xpl* was mapped to a 0.83cM interval between the microsatellite markers, *DXMgc39* and *DXMit32*. Since in the human syntenic region, Xp22, a congenital face and limb deformity, Oral-facial-digital syndrome type 1 (OFD1: OMIM311200), has been mapped, *Xpl* was thought to be a mouse model for OFD1. We mapped a murine homologue for OFD1, *cXorf5*, using our cross panel, and we excluded it from the critical region of *Xpl*.

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

Shigeru MAKINO and Toshihiko SHIROISHI

A recessive mouse mutation, mesenchymal dysplasia (*mes*), causes some morphological defects including mild preaxial polydactyly. Genetic analysis and molecular characterization in our previous study indicated that *mes* is caused by a 32 bp-deletion in the C-terminal cytoplasmic domain of the patched (*ptc*) gene, which encodes a transmembrane receptor protein for Shh.

Previously, we showed that compound heterozygotes of knockout allele of *ptc* (*ptc*$^-$) and *mes* allele, which survive up to birth and die neonatally, exhibit normal neural tube development in contrast to *ptc* homozygotes which die around 10 dpc with severe neural tube defects. This suggested that the C-terminal cytoplasmic domain is dispensable for inhibition of Shh signaling in early embryogenesis.

In contrast, the same compound heterozygotes show ectopic expression of *Gli, Shh, Gre* and *Fgf4* at the anterior mesenchyme and AER. This suggested that the C-terminal cytoplasmic domain of Ptc has a role in the negative regulation of Shh signaling at the anterior mesenchyme of the limb buds.

(4) Genetic analysis of hot taste and pain sensitivity in the Mishima battery of mouse strains

Tamio FURUSE[1], David A. BLIZARD[2], Kazuo MORIWAKI[3], Kazumi YAGASAKI[1], Toshihiko SHIROISHI and Tsuyoshi KOIDE ([1]Tokyo University of Agriculture and Technology, [2]The Penn. State University, [3]Bio Resource Center, RIKEN)

Capsaicin is the major component of red pepper, which causes hot taste. An experiment on knockout mice for the receptor of capsaicin, VR1, showed that the sensation for both capsaicin and thermal pain mediate the same receptor. In order to approach the underlying genetic mechanism for diversity of preference for red pepper, we conducted a 12-hr 1-bottle fluid intake test of capsaicin solution using animals from the following inbred strains: 10 wild-derived inbred strains (PGN2, BFM/2, BLG2, NJL, CHD, HMI, CAST/Ei, SWN, KJR and MSM), 1 strain derived from the so-called fancy mouse (JF1), and 3 domesticated strains (C57BL/6J, DBA/1J and BALB/cAnN). Relative to baseline water intake, C57BL/6J and DBA/1J consumed 10 percent while KJR and MSM ingested approximately 60 percent of the 15uM capsaicin solution. The results of 1-bottle test are similar to that displayed by these strains in the hot plate test that we had done previously. In the 1-bottle test, F1 progeny of KJR and C57BL/6 consumed capsaicin solution approximately same as KJR. This result indicates that the genes involved in capsaicin tolerance in KJR are dominant. In order to map the loci for capsaicin preference and pain sensation, analyses of 1-bottle test and hot plate test on F2 progenies are currently under way.

(5) A quantitative genetic analysis of spontaneous activity in the wild-derived mouse strains

Tamio FURUSE[1], Toshiyuki TAKANO-SHIMIZU[2], Kazuo MORIWAKI[3], Toshihiko SHIROISHI and Tsuyoshi KOIDE ([1]Tokyo University of Agriculture and Technology, [2]Division of Population Genetics, NIG, [3]RIKEN BioResource Center)

Spontaneous locomotor activity is a behavior that is greatly influenced by the forces of natural selection in the process of evolution. Locomotor activity of animals in nature plays a crucial role in resource acquisition, territory defense and migratory behavior. When the resources are limited or the territory is large, animals have to move long distances to acquire food and to protect their territory. In the laboratory environment, mice have not been selected for their ability of spontaneous locomotion. Thus, the genetic variability for behavioral performance including spontaneous locomotor activity is still maintained as a distinct genetic feature of each mouse strain.

We previously conducted multi-phenotype behavioral characterizations using a series of inbred strains derived from wild mice in addition to the common laboratory strains, the Mishima battery of mouse strains. In the study of spontaneous locomotor activity in the habituated normal home cages using infra-red sensor, we found that mice of KJR strain was hyperactive in contrast to mice of BLG2 strain, which showed a low level of spontaneous locomotion. To unravel the genetic loci involved in this behavioral phenotype, we conducted a QTL analysis on the backcross population of a cross between KJR and BLG2 strains. In the QTL analysis with 376 backcross progeny, the maximum LOD score, 4.8, was found between the markers *D3Nig1* and *D3Mit128* and significantly exceeds the threshold of LOD score 3.3 for BC1. We named this most telomeric region of

chromosome 3 as *Loco1*. Further linkage analysis using the selected progeny which carrying the allele from KJR at the *Loco1* locus suggested the presence of another locus, *Loco2*, on chromosome 17. An analysis showed that *Loco1* and *Loco2* interacted epistatically.

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F-b. Division of Mammalian Development Laboratory

(1) Molecular mechanism of somite segmentation

Yu TAKAHASHI[1], Aki ISHIKAWA, Aya KITABAYASHI[1] and Yumiko SAGA ([1]National Institute of Health Sciences)

The somites are the first morphologically distinct segmental units formed in vertebrate embryo and give rise to metameric structures such as vertebrae, ribs and skeletal muscles. The somites are formed by segmentation from the anterior end of the presomitic mesoderm (PSM), which is unsegmented and posterior to the most recently formed somite. Each somite is subdivided into anterior (rostral) and posterior (caudal) compartments that differ in their properties and gene expression. The rostro-caudal polarity of a somite is established within the PSM prior to segmentation. However, molecular mechanisms underlying the formation of rostro-caudal polarity and the specification of segmental boundaries are totally unknown. Our aim in this study is to determine the mechanisms underlying the generation of a segmental pattern within the PSM. Previously, we cloned *Mesp2*, a gene coding a novel bHLH transcription factor, MesP2. *Mesp2* is expressed in the rostral PSM. *Mesp2*-null mice exhibited defective somitogenesis due to the lack of rostral somitic compartment. By the genetic analysis, we have shown that MesP2 play a critical role to establish rostro-caudal polariy of somite by regulating Dll1 expression via Notch signaling pathways.

Recently, we have established a hypomorphic *Mesp2* allele by an introduction of zebrafish Mesp2-homologue, *mesp-b*. Introduced *mesp-b* almost rescued the Mesp2 deficiency in the homozygous *mesp-b* knockin mouse, indicating that *mesp-b* is a functional homologue of mouse *Mesp2*. Segmented somites were clearly observed although the partial fusion of vertebral columns remained. Interestingly, however, the nature and dosage of the *mesp-b* gene affected the rescue event. A mouse line, which has a hypomorphic *Mesp2* allele generated by the introduction of *neo-mesp-b*, gave rise to an epithelial somite without normal rostrocaudal (RC) polarity. RC polarity was also lacking in the presomitic mesoderm. The defects in RC polarity were determined based on disturbed expressions of *Uncx4.1* and *Dll1* in the segmented somites and presomitic mesoderm, respectively. In contrast, the expression of

EphA4, lunatic fringe or *protocadherin*, implicated in the segment border formation, was fairly normal in hypomorphic mutant embryos. These results suggest that the Mesp family of transcription factors is involved in both segment border formation and establishment of RC polarity through different genetic cascades.

(2) Molecular clock and somitogenesis

Hiroki KOKUBO and Yumiko SAGA

Recently, several genes, including *hairy1* in chick and *lunatic fringe* in chick and mouse, have been identified and shown to be expressed in the presomitic mesoderm in a cyclic fashion with temporal periodicity corresponding to the formation time of one somite. Therefore these are assumed to link to a molecular clock which governs temporal control of somitogenesis. We have cloned novel mouse *hairy* and *E(spl)* related subfamily genes, called *hesr -1, -2,* and *-3,* using homology search. Comparison of sequences shows that presence of three functional domains, bHLH, orange and C-terminal domain, that are conserved among *Drosophila,* mouse and human, but obviously distinct from the HES family members. *hesr-1* is expressed in the caudal part of each somites and presomitic mesoderm. In Delta-1 homozygous mutant embryo, *hesr-1* expression is not observed in somites and presomitic mesoderm. In *lunatic fringe* and *delta-3 (pudgy)* homozygous mutant mice, *hesr-1* is expressed in a broader domain in presomitic mesoderm. These observations implicate that *hesr-1* might play role(s) in regulating somitogenesis through the Notch signaling pathway. As an approach to elucidate the function of *hesr-1* during somitogenesis, we generated a knockout mouse. However, no difference between wild type and mutant is observed so far. We speculate that there could be functional redundancy among the hesr proteins. We have generated knockout mice for hesr-2 and -3. We are trying to generate the compound mice to reveal the coordinated function of these three genes.

(3) The lineage analysis of *Mesp1*-expressing cells

Satoshi KITAJIMA[1] and Yumiko SAGA ([1]National Institure of Health Sciences)

MesP1 and MesP2 are transcription factors containing an almost identical bHLH motif. Cells lack both genes can not contribute to the heart formation. A lineage study using cre-lox system revealed that *Mesp1* is the earliest molecular marker expressed in the heart precursor cells. We have conducted a detail lineage analysis using Cre-reportor line, Rosa-26. The heart is exclusively composed of cells derived from Mesp1 expressing mesoderm. However, we found some cells are deprived of the β-gal staining, which means that these are derived from *Mesp1*-nonexpressing cells. Among them, neural crest cells have been shown to contribute to the conotrucus septum prior to and during overt septation of the outflow tract. In addition, we found several cell clusters in between the primitive ventricles and trabecular component of both right and left ventricles, which distribution is reasonably similar to the cells defined as the cardiac conduction system. We are now trying to define the identity of the cells using several marker specific to the cardiac conduction system. Since the lineage of these cells has been controversial, we might be able to demonstrate their origin is different from other cardiac cells at the beginning of the birth.

(4) Cloning and functional analysis of mouse *Nanos* genes

Masayuki TSUDA, Yumiko SASAOKA, Makoto KISO, Seiki HARAGUCHI[1] and Yumiko SAGA ([1]Shiga university of Medicine)

A *Drosophila* gene *nanos* encodes RNA binding zinc finger protein, which are shown to be involved in the germ cell development by suppressing somatic gene expression in the germ cells. To know the possible involvement of Nanos-homologue protein in mouse germ cell development, we tried to clone mouse *nanos* genes. The first *nanos* gene we cloned (*mNos-1*) was maternally expressed in an unfertilized egg and the zygotic expression was observed in the neuronal cell lineage especially in adult hippocampus, but it was not expressed in the developing germ cells. The knockout mouse developed without any significant abnormalities so far. The second and third genes (*mNos-2* and *mNos-3,* respectively) were then cloned by the use of homology with human *nanos* genes predicted by human genome project. Interestingly, these two genes are co-expressed in a developing male gonad but not in a female gonad. To know the function of these genes

in germ cell development, we are generating the gene-knockout mice.

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F-c. Division of Genetic Strains Resarch Center, Plant Genetics Laboratory

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

A. Regulation of expression of *KNOX* family class 1 homeobox genes of rice

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

To elucidate genetic programmes that control embryogenesis and regeneration of rice, we conducted cloning and structural and functional analyses of genes which encode transcription factors and protein kinases. We previously identified five *KNOX* family class 1 homeobox genes and analysed their expression patterns during early embryogenesis and regeneration by RT-PCR and *in situ* hybridization. We also found that constitutive expression of these genes are sufficient to maintain cells in a meristematic undifferentiated state (Ref.2). Since specific expression of *KNOX* family class 1 homeobox genes in a shoot meristem is essential for normal development of plants, we started to study its regulatory mechanisms. An *OSH1* promoter was shown to be active in a leaf in addition to the shoot meristem, indicating that the promoter region is not sufficient to confer specific expression in the shoot meristem and other regions are necessary for its precise expression. Introduction of an *OSH1* cDNA into rice caused ectopic expression of *OSH1* in the leaf and resulted in altered leaf morphology. Introduction of the cDNA with a frame shift mutation also caused altered leaf morphology similar to the wild type cDNA but with reduced degrees. These effects were independent of direction of the cDNA and presence or absence of the promoter. These results indicate that an extra copy of the *OSH1* exons somehow induced the ectopic expression of endogenous *OSH1* in the leaf and resulted in the altered leaf morphology. We obtained similar results for other genes such as *OSH6, OSH15* and *OSH71.* These results suggest that both of the promoter and exon regions are not sufficient for the restricted expression of these genes in the shoot meristem in rice.

B. Genetic analysis of sporogenesis using sterile mutants of rice

Ken-Ichi NONOMURA, Kazumaru MIYOSHI, Mitsugu EIGUCHI, Akio MIYAO*, Hirohiko HIROCHIKA* and Nori KURATA (*Natl. Inst. Agrobiol. Sci.)

Genetic analyses for microsporogenesis and megasporogenesis is important to understand the system of sexual plant reproduction. We selected thirty-eight of rice sterile mutants defective in reproductive organ development and meiotic chromosome behavior. All of these mutant phenotypes segregated in a manner of single recessive genes. These mutations were induced somaclonally by suspension culture of rice calli, where the endogenous retrotransposon *Tos17* easily transposed into multiple genome regions. A part of mutations were known to be caused by the insertional mutagenesis by the *Tos17.* Therefore we examined the gene-tagging events for these mutants. Close linkage of sterile phenotype to a transposed Tos17 insertion was detected in 22 of 38 mutant lines. We are further investigating gene disruption with *Tos17* to isolate genes responsible for these mutations.

In this study, a gene disruption with *Tos17* insertion was already confirmed in *Msp1* locus, which affect sporogenesis in sexual reproductive organs, anther and ovule. *Msp1* gene encodes receptor type serine/ threonine protein kinase. RT-PCR analysis revealed that the expression of this gene was found in young flowers before meiosis. In mutant anther, pollen mother cells increased abnormally and were arrested at meiotic stages, and development of hypodermal anther wall layer was defective, resulting in complete male sterility. In mutant ovule, megaspore mother cells also increased abnormally, but passed

through meiosis successfully. Multiple megaspores occurred growth and/or space competition among themselves during megagametogenesis and often gave rise to an embryo sac composed of multiple gametocytes with irregular number and position of cells or nuclei. Such a mutant megagamete could participate in fertilization with wild type pollens and sometimes set aberrant seeds with twin embryos. We concluded that *Msp1* gene function would basically related to signal transduction for restricting the division of archesporial cells in very early sporogenesis, but permitting extra division only after differentiation into hypodermal anther wall formation (Ref.7).

In addition, to analyze phenotype of meiotic mutants according to the precise sporoe development, we preliminary elucidated the timing of premeiotic DNA synthesis and the relationship of stage progression of germ cell maturation to floret elongation. These analyses revealed that premeiotic S phase undergoes in floret of 2.0- to 2.5-mm length and that sporogeneous cells quickly entered meiosis after brief G2 interphase (Ref. 8).

C. Structural and functional analysis of rice HAP3 family genes

Nori KURATA, Akiko SERIZAWA, Kazumaru MIYOSHI, Yukihiro ITO

One of the HAP3 family genes, LEC1 (LEAFY COTY LEDON 1) in arabidopsis was reported to be one of the central regulators of seed development. HAP3 is a submit of CAATT binding protein complex which confer regulation on promoter activity of multiple genes. To elucide a possibility that a certain HAP3 gene of rice shows a key function in the embryo development, we screened HAP3 family genes of rice from a cDNA library constructed with 3DAP (day after polllination) embryos and from available genomic sequences (about 60% of the total genome) using conserved domains. Finally we obtained ten HAP3 subunit genes, designated OsHAP3-1 to OsHAP3-10. RT-PCR experiment revealed that three clones, OsHAP3-1, 3-2 and 3-3, expressed ubiquitoresly in almost organs including callus, embryo, shoot, leaf and flower, Whereas OsHAP3-4, 3-5, 3-6 and 3-7 showed slight and specific expression in embryogenesis, and OsHAP3-8, 3-9 and 3-10 showed no expression in any organ examined so far.

Several genes showing specific expression in embryo development were selected to make promoter + GUS construct, 35S promoter + cDNA construct or RNAi construct. Transformation and regeneration of rice plants using those constructs are now in progress.

D. Isolation and sequence analysis of receptor-like protein kinase genes of rice

Yukihiro ITO, Nori KURATA

We started to isolate and characterize protein kinase genes as candidates to transduce a positional information which is postulated to be important for plant development. We focused on a receptor-like protein kinase gene such as *SOMATIC EMBRYOGENESIS RECEPTOR KINASE* (*SERK*) of carrot and isolated its homologues from rice. We have cloned two genes and their cDNAs named *ORK1* and *ORK6*. The genome clone of *ORK1* showed unusual structure and detail analysis is in progress. *ORK6* encodes a receptor-like protein kinase with leucin-rich repeats in a receptor domain. The amino acid sequence of ORK6 is highly similar to plant receptor-like protein kinases such as SERK, CLAVATA1 and ERECTA of *Arabidopsis*, which are known to function in a shoot meristem. We also identified four related genes in databases. Overexpression of *ORK6* or introduction of antisense or RNAi constructs of *ORK6* into rice showed no phenotypic change under a normal growth condition. Analysis of regeneration will be carried out using calli derived from these transgenic plants.

(2) Positional cloning of a heterochronic gene, *PLA1*, regulating the plastchron and the duration of vegetative phase in rice

Byoung-Ohg AHN, Kazumaru MIYOSHI, Yukihiro ITO, Jun-Ichi ITOH*, Yasuo NAGATO*, Nori KURATA (*Graduate School of Agricultural Life Science, University of Tokyo)

Heterochronic mutations affecting the timing of developmental events may be of major significance in ontogeny and evolution. In plants, several heterochronic mutations that affect stepwise development of vegetative tissue and therefore alter shoot architecture have been identified. The recessive mutations at the rice *PLASTCHRON1* (*PLA1*) locus cause the short

plastochron and ectopic expression of vegetative programs in the reproductive phase. To understand the molecular aspects of *PLA1* function during plant development, we have started to isolate the *PLA1* gene by map-based cloning.

A genetic and physical map was constructed to isolate a causal gene for *pla1* phenotype. Small-scale mapping was carried out to determine approximate map position of a *pla1* locus and then a high-resolution genetic map was made for *pla1-1*, one of the *pla1* alleles, using an F_2 population comprising 578 *pla1-1* homozygous plants. A high-resolution genetic map showed that the *pla1-1* locus was mapped between RFLP markers C961 and R1738A on chromosome 10, within a 3.6cM genetic distance.

A physical map encompassing the *pla1-1* locus was constructed by overlapping Bacterial Artificial Chromosome (BAC) clones through chromosome walking. PCR-based RFLP markers from BAC-end clones were developed and mapped to place the *pla1* locus between the markers. Physical map construction using BAC clones indicated that a BAC clone, B44A10 (167Kb), contained the *PLA1* locus within 74Kbp that corresponds to a 0.52cM genetic distance.

The B44A10 clone was almost sequenced and published by Cold Spring Harbor Laboratory, USA. Gene prediction analysis of the 74kb region containing *PLA1* locus with GENESCAN program (MIT, USA) predicted several candidate genes for *PLA1*. Comparison of the nucleotide sequences of candidate genes between wild type and *pla1* mutant plants revealed that putative cytochrome P450 gene carries a mutation within the coding region in all three alleles of *pla1* mutants. Expression analysis by RT-PCR indicated that cytochrome P450 gene was expressed only in organ bearing shoot apical meristem (SAM) such as seedling and young panicle, but not in leaves or root. All together, the *PLA1* locus was strongly suggestted to encode cytochrome P450 protein. Molecular complementation analysis using the genomic fragment containing wild type cytochrome P450 gene is in progress.

(3) Construction of rice artificial chromosomes

Tadzunu SUZUKI, Ken-Ichi NONOMURA, Nori KURATA

One of advantages of plant cells for biotechnology is easier induction of reproductive adults than animal cells. Fusing this feature with molecular biotechnology such as construction of stable plant artificial chromosomes will introduce us to tremendous progression of chromosome technology. Rice, a kind of key cereals, is important not only to breeding, but also to basic researches on account of the smallest genome size in monocots. In this study, the candidate DNAs which may have a centromere function were used to create rice artificial chromosomes (RACs). Then methods of transferring the RACs to rice cells have been examined.

The candidate DNA fragments of the artificial chromosomes were constructed using both of the yeast artificial chromosome (YAC) and the bacterial artificial chromosome (BAC) harboring the centromere sequences of rice. Approximate 300 kb insert DNA of the YAC clone is composed of two kinds of centromeric repeats of RCS2 and RCE1, which are a tandem repeat unit of 160 bp and a dispersed repeat of 1.9 kbp locating on all centromeres, respectively. It was suggested that the clone was derived from a functional center of the rice centromere (Ref.1). For transferring the YAC into rice cells, the vector sequences of the YAC clone was retrofitted with rice selection marker genes. For the creation of a BAC based construct, the 100 kb of RCS2 repeats was isolated and introduced into a binary BAC vector.

Because the transfer methods of huge size DNAs into plant cells have not been established, two methods of lipofection and particle bombardment system were examined here. Lipofection can reduce damages of DNAs more than 100 kb length and is usually applied to transfer huge size DNAs as well as small ones into animal cell lines. However, this method had few been used for plant cell transformation. To clarify the usefulness of lipofection method for plant cell transformation, a trial to introduce a 4 kb plasmid having the GFP marker gene into protoplasts isolated from rice cultured cell line Oc was carried out. The efficiency of transformation achieved to 2×10^{-4}. DNAs of 20 kb or less in length also revealed to be lipofected into rice protoplasts, though the efficiency is still low. These results indicated that lipofection method can be applied for plant cell transformation at least for transient expression. Further stable transformation using larger DNAs of BAC and YAC clones remains to be examined.

Particle bombardment method, which has already been used in plant transformation with small plasmids,

is unsuitable for introducing large DNAs into plant cells. However, this method is being examined for transformation with BAC clones, since DNAs less than 100 kb had been already shown to be transferred into rice cells. In addition, modifications of the method are in progress for reducing the damages of DNAs. In the conventional way, DNAs are spread on gold particles and delivered into cells with the particles. In our modified procedure, DNAs are spread on the cell surfaces and introduced into the cells when the particles go into the cells. The preliminary results indicated that usual plasmids could be sufficiently introduced. The adaptation and optimization will make a new procedure more effective than the conventional one.

Generation of several kinds of transgenic rices with artificial chromosomes of different centromere repeats combination will make us possible to elucidate functional role of each centromere components.

(4) Analysis of rice genome configuration in the nucleus; isolation and characterization of rice nuclear protein genes

Kazuki MORIGUCHI, Yukihiro ITO, Yukiko YAMAZAKI, Nori KURATA

The primary aim of this study is to isolate plant nuclear proteins exhaustively to exhibit the whole contents of nuclear proteins using rice, which is one of the model plants whose genome project has progressed. The secondary aim, is to make clear what events and factors are necessary to maintain genome organization and function in nucleus. For this purpose, we isolated and classified nuclear protein genes and examined their characteristics and localization in the nucleus to get a link to their functions.

As the first step, we applied the yeast nuclear transportation trap (NTT) system, which was shown to trap NLS coding cDNAs. Three cDNA libraries derived from two panicle stages (FP: flowers in meiotic stages, YP: flowers before and after pollination) and 4 days callus after regeneration treatment have been used. We have isolated cDNA clones derived from about 430 independent genes and found that the NTT system was much less efficient for trapping nuclear proteins of plant than animal. By a homology search analysis against protein databases, 45% of predicted proteins indicated unknown function or no homology and 31% of those indicated known nuclear proteins or similar ones. We next examined several clones from each group for their intracellular localization in plant by expressing GFP fusion proteins of them in *Allium* epidermal cells. Over 70% of examined proteins, which are classified into unknown function protein, localized in nucleus or both in nucleus and cytoplasm. Several characteristic distribution of expressed proteins were observed as revealed by GFP localization vs. chromatin (DAPI) distribution. Some seemed to exist on the chromatin and others in the nuclear matrix.

Three plans are starting for further examination. The first one is additional isolation of nuclear proteins by introducing more efficient gene trap system for plant. The second one is to analyze alterration of nuclear protein content in some developmental stages. The last one is construction of makers for observing detailed intranuclear structure and localization. Two colored localization analysis using two different fusion-proteins, one with GFP and the other with DsRed2 has been employing.

(5) Genome-wide analysis of reproductive barriers and positional cloning of a reproductive barrier

A. Quantitative Analysis of genotype segregation for reproductive barriers

Yoshiaki HARUSHIMA, Nori KURATA

Genetic mechanisms for isolation of "species" are called as reproductive barriers and these include hybrid incompatibility, hybrid inviability, hybrid sterility, hybrid breakdown, etc. The distortions of allele frequencies from Mendelian expectation in progeny of inter- or intra- species hybrid due to hybrid sterility genes, hybrid breakdown genes and gametophytic competition genes have been often observed. We have developed a new method for detecting the map location and gene action of loci that contribute to the distortions of allele frequencies from Mendelian expectation by regression analysis of allele frequencies of markers covering an entire genome (ref. 3). Mathematica packages for the analysis were published at http://shigen.lab.nig.ac.jp/rice/seganalysis/. Asian rice cultivars, *Oryza sativa*, can be classified into two main types, Japonica and Indica, based on several characteristics. To clarify the state of Japonica-Indica differentiation, all reproductive barriers causing allele

frequency distortions from Mendelian expectation in F_2 populations were mapped and compared among three different Japonica-Indica crosses. The number of reproductive barriers in the three crosses was similar, however most of the barriers were mapped at different loci. Therefore, these reproductive barriers formed after Japonica-Indica differentiation. In the three Japonica-Indica cross combinations, the genetic variations within both Japonica and Indica are small as shown as high similarity of the restriction fragment length of RFLP markers. Considering the high genetic similarity within Japonica cultivars and Indica cultivars, the differences in the reproductive barriers on each cross were unexpectedly numerous. The reproductive barriers of Japonica-Indica hybrids likely evolved more rapidly than other genetic elements (ref. 5).

B. Positional Cloning of a Segregation Distortion Gene Detected in a Progeny of a Cross between japonica and indica rice

Yoshiaki HARUSHIMA, Nori KURATA

The aim of this study is isolation of the most prominent barrier on chromosome 3 detected in F_2 of Nipponbare-Kasalath hybrid by positional cloning, and elucidation of the molecular nature of the individual reproductive barriers. We have clarified the aimed gene was a male gametophyte gene that interact with maternal locus on chromosome 6. In other words, the pollen with Kasalath genotype at the gametophyte gene preferentially fertilized by 94% probability in maternal plant that is heterozygote or Kasalath homozygote at the interactive locus on chromosome 6. For detailed mapping the gametophyte gene, we have selected plants with recombination in 1.9 cM interval from 1300 F_2 and 473 backcross plants and retrieved their genomic DNA of the selected plants from bulked young leaves of their selfed seedling. Considering the genotypes of the interactive locus, the genetic map of the male gametophyte gene is being constructed by the dosage analysis using the selected population. The physical map of this region is also being constructed using Nipponbare BACs that were selected by a cosegregated RFLP marker, C582, with the aimed gene. BAC contig were confirmed by PCR analyses of end sequences of BACs. Same of amplified BAC end fragments were mapped as RFLP markers on the genetic map. Both flanking markers of the male gametophyte gene were mapped at 9 and 2 recombinants in the population and these markers were on the two BAC contig.

(6) Generation and screening of enhancer trap lines of rice

Yukihiro ITO, Mitsugu EIGUCHI, Nori KURATA

To isolate valuable mutants defective in various steps of rice development and to clone the corresponding genes, we are generating enhancer trap lines of rice. We employ an enhancer trap system used in *Arabidopsis* with some modifications. This system is based on the *Agrobacterium*-mediated transformation using *Ac/Ds* transposable elements of maize. The enhancer trap construct contains, in the T-DNA region, the *Ds* element named *Ds-GUS* that harbours a GUS coding region with a CaMV 35S minimal promoter and a hygromycin resistance gene. *Ds-GUS* was flanked by the 35S promoter and a coding region of a selectable chlorsulfuron resistance gene, so that excision of *Ds-GUS* causes connection of the 35S promoter and the coding region and can confer chlorsulfuron resistance.

We also use a 35S-Ac transposes (AcTPase) gene together with a bialaphos resistance gene to supply transposase which is essential and sufficient for transposition of *Ds-GUS*.

We generated forty-two transgenic rice lines with a single copy of *Ds-GUS* and six lines with 35S-AcTPase. We crossed four lines of *Ds-GUS* with six lines of 35S-AcTPase and obtained 675 germinal transposants by PCR screening and 1,564 transposants by the chlorsulfuron and hygromycin resistance at F2 generation. To obtain more transposants we crossed other 13 original *Ds-GUS* lines with the 35S-AcTPase line and obtained 926 transposants out of 3,277 F2 plants screened. We maintain F_1 plants by growth of ratoons and continuously obtain F_2 seeds.

We also started enhancer screening by X-gluc staining at various organs and stages of rice development including germinating seeds, 2-week seedlings, young panicles and all organs at a flowering stage. Out of 675 transposants screened we identified more than 10 lines that showed specific GUS staining in one of following organs such as a root tip, a root hair, a wounded leaf, an inflorescence, an anther, a style or a young tiller.

We will also conduct mapping of original *Ds-GUS* insertion sites on rice chromosomes to select chromosome specific *Ds-GUS*.

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F-d. Division of Microbial Genetics

(1) HscA is involved in the dynamics of FtsZ-ring formation in *Escherichia coli* K-12

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

FtsZ, a homologue of eucaryotic tubulin, localizes throughout the cytoplasm in non-dividing *E. coli*. However, it assembles in cytokinetic rings, however, at the early stages of septation. Factors controlling the dynamics of FtsZ ring formation are unknown, and the molecular mechanism governing these dynamics is yet to be determined. At 42°C, JE10715 mutant bacteria formed multinucleated filaments with a highly reduced number of FtsZ-rings at potential division sites. The JE10715 phenotype resulted from a mis-sense mutation in the *hscA* gene which encodes a heat shock Hsp70 family protein, with a single alanine-to-valine substitution at position 192 within the ATPase domain. Both JE10715 and the *hscA* knockout strain of JE10715 were completely complemented by a plasmid-born, wild type *hscA* gene, but not by a mutant-type *hscA715* gene. An *hscA* conditional knockout of the wild-type strain under non-permissive conditions exhibited longer rod cells with an abnormal localization of FtsZ. The over-expression of *dnaK* partially complemented the JE10715 mutation. *In vitro*, the ATPase activity of the mutant protein, HscA715, was reduced to 63% of the wild-type HscA activity. HscA cosedimented with FtsZ-polymers in the presence of GTP. HscA is involved in FtsZ-ring formation, through a chaperon-like interaction with FtsZ. Defects in *hscA*, however, can partially be compensated for by redundant genes, including the wild-type *dnaK*. (For details, see Ref. 1)

(2) *kdsA* mutations affect FtsZ-ring formation in *Escherichia coli* K-12

Hiroshi FUJISHIMA, Masaaki WACHI and Akiko NISHIMURA

No one has, as yet, addressed the relationship between the nature of the outer membrane and cell division. *kdsA* encodes 3-deoxy-D-manno-octulosonic acid (KDO) 8-phosphate synthetase which catalyses the first step in the synthesis of KDO, the linker between lipidA and oligosaccharide of lipopolysaccharide (LPS). Seven temperature-sensitive mutants containing mis-sense mutations in *kdsA* were affected in the production of KDO and all mutants stopped dividing at 41℃ and formed filaments with either one or no FtsZ ring. All observed defects were reversed by the plasmid-born wild-type *kdsA* gene. Western blotting analysis, however, demonstrated that the amount of FtsZ protein was not affected by the mutation. The mutants were more susceptible to various hydrophorbic materials, such as novobiocine, eosine Y and SDS at 36℃. Methylene blue, however, restored *kdsA* mutant growth. Plasmid-born wild-type *msbA*, encoding a lipid A transporter in the ABC transporter family, partially suppressed *kdsA* mutantation. A mutation of *lpxA*, functioning at the first stage in lipid A biosynthesis, inhibited both cell division and growth, producing short filaments. These results indicate that the instability of the outer membrane, caused by the defect in KDO biosynthesis, affects the FtsZ-ring formation. (For details, see Ref. 2)

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F-e. Laboratory Invertebrate Genetics

(1) UDP-sugar transporter implicated in glycosylation and processing of Notch

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Glycosylation modifies protein activities in various biological processes. We report here functions of a novel UDP-sugar transporter (UST74C) localized to the Golgi apparatus in cellular signaling of *Drosophila*. Genetic analyses demonstrated that UST74C is required for both Wingless and Notch signaling in embryos, and the requirement becomes restricted to Notch signaling in larvae. Larval phenotypes indicate that both Fringe-dependent and independent pathways of Notch are affected. Biochemical analyses demonstrated both glycosylation and proteolytic maturation of Notch are defective in mutant larvae. The results suggest that changes in nucleotide-sugar level can differentially affect Wg, and two distinct aspects of Notch signaling.

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G. CENTER FOR GENETICS RESOURCE INFORMATION

G-a. Genetic Informatics Laboratory

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

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

The Oryzabase is a comprehensive rice science database established in the collaboration with rice researcher's committee in Japan. The database is originally aimedddimededzabase consists of five parts, which are (1) genetic resource stock information, (2) gene dictionary, (3) chromosome maps, (4) mutant images, and (5) fundamental knowledge of rice science.

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

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

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

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

(2) PEC: Profiling of Escherichia coli Chromosome

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

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

Since the whole genome sequence of E.coli was published in 1997, enormous efforts have been made to analyze the functions of the genes/orfs. Further innover innov. Further innovFurther innovbiologists, however, are not always satisfied with such databases. On the other hand, computational scientists face problems on how to annotate their analytical results generated and/or calculated from sequence data.

To resolve the dilemma, we'v developative research is still needed to complete those tasks. Many different E.coli databases are now available through the Internet, but most databases have been constructed by computer scientists with the contents derived from sequence analysis. Experimental e developed the PEC aiming to provide experimental scientists a chance to meet and communicate with computer scientists and vice versa through the Internet. The database includes original deletion mutant data and genetic stock available through ts incorporated into PEC. The database also provides analytical data such as

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

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G-b. Genome Biology Laboratory

(1) NEXTDB: The nematode expression pattern database

Tadasu SHIN-I, Yuji KOHARA

We updatea "NEXTDB" that integrates all the information of ESTs, gene expression patterns and gene functions of *C.elegans* which are being produced and analyzed in this laboratory, and, made it open. Images of whole mount *in situ* hybridization for mRNA were taken by CCD cameras equipped on Zeiss Axioplan microscopes, loaded to the Sun workstation to process and arrange them properly in the database. Then, they were annotated with respect to developmental stages and expression patterns on the database. Images of immunostaining taken on Zeiss LSM510 confocal microscope, and images and descriptions of RNAi phenotypes, were also stored in the database. New version of NEXTDB contains about 120,000 cDNAs that have been classified to 12,000 unique cDNA groups, *in situ* images of about 9,000 cDNA groups, RNAi phenotype images of 160 groups, and immunostaining images of 120 groups. The new version is available over the Internet. URL:<http://nematode.lab.nig.ac.jp/>

(2) Clustering analysis of gene expression patterns in C.elegans

Masahiro ITO, Yohei MINAKUCHI, Michiko SERIZAWA and Yuji KOHARA

In this lab, whole mount in situ hybridization has been performed using the cDNAs that have been classified in our EST project. Thus far, *in situ* analysis for some 7600 genes was finished. Minimal annotation has been given to the *in situ* images; 10 stages for embryogenesis, 4 stages for larval-adult stage, on average 10 patterns (cell(s), tissue, region) per stage, 3 levels of relative intensity of signals per each pattern. Using the information ((10+4)x10x3=420 dimensions), we have done clustering analysis of the *in situ* patterns. The gene expression patterns were roughly clustered by the factor analysis using the Euclid distance. This treatment classified the embryonic patterns of 5064 genes into 39 clusters that contained 5 to 3355 genes per cluster (12 clusters had more than 50 gene members). Then, we focused on several cell/ lineage/ stage-specific clusters; gut specific cluster (910 genes), hypodermis specific cluster (421 genes), and body wall specific cluster (72 genes). The three clusters were further classified by the Ward method. This treatment produced 7 sub-clusters along the developmental time course from the gut cluster and 6 sub clusters from the hypodermis and body wall muscle clusters. This means that we know the time of transcription initiation of the member genes in the individual cell lineages. The same sub-cluster might be regulated by the same mechanisms. Thus, we are now trying to extract common sequence motifs, possible candidates for transcriptional regulatrory signals, in the 5'- upstream region of the genes in the same sub-cluster.

(3) Semi-automatic system for creation of cell shape model in *C.elegans* embryogenesis

Hideaki HIRAKI and Yuji KOHARA

C. elegans is a model organism that allows us to observe details of the developmental process as it is transparent and has a limited number of cells. Because cell to cell interactions have critical roles in early embryogenesis, it is very important to grasp the cell shapes, the arrangement of them and the contacts among them. A polygon model of the early embryogenesis was built in our laboratory from a time series of 3-dimensional images of differential interference contrast (DIC) microscopy. The model has been used as the basis to model the early embryogenesis regarding the regulation of gene expressions or the physical process of cleavages, and as the template superimposed with the images of antibody staining to identify the localization of various proteins. Building such model took long time and much effort because it is difficult to extract automatically the cell boundaries in DIC images and the outlines of the cells had to be traced by hand.

Accordingly, we have been developing a system to build the model of the cell shapes from a time series of confocal microscopic images of the plasma membranes stained with a fluorescent dye automatically by computer. In detail, we developed computer programs to extract the cell shapes with the seeded region growing method on 3-dimensional image and to extract candidates of the seeds with the distance transformation filter. We applied them to a dataset of 24-200 cell stages successfully. The system has not been fully automatic yet as the seeds of the regions have to be selected from the candidates by the operator to obtain a good result. We are improving the system to be more automated and are planning to make 4-dimensional databases consisting of the cell lineages constructed from time series datasets. And then, we will apply the system to compare the cellular arrangements and the cell-to-cell contacts among the relative species of C. elegans and the mutants such as created by RNAi.

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

Ken-ichi OGURA, Norihito KISHIMOTO, Shohei MITANI[1], Keiko GENGYO-ANDO[1], Yuji KOHARA ([1]Tokyo Women's Medical University)

The translation of maternal *glp-1* mRNA is temporally and spatially controlled in *C. elegans* embryos. Its 3' UTR (Untranslated region) is important in this regulation. Here we show that a CCCH zinc finger protein, POS-1, represses *glp-1* mRNA translation by binding to a spatial control region within the 3' UTR. We identified an RNP-type RNA-binding protein as a POS-1-interacting protein, PIP-1. PIP-1 is present in oocytes to early embryos and overlaps with POS-1 in the cytoplasm and P granules of posterior blastomeres. PIP-1 binds to a subregion of the temporal control region in the 3' UTR and is required for the translation of *glp-1* mRNA. We propose that POS-1 cooperates with PIP-1 to control translation of the maternal *glp-1* mRNA.

(5) An isoform of eIF4E is a component of germ granules and is required for spermatogenesis in C. elegans

Anahita Amiri[1], Brett D. Keiper[2], Ichiro KAWASAKI[1], Yuan Fan[1], Yuji KOHARA, Robert E. Rhoads[2] and Susan Strome[1], ([1]Indiana University, [2]Louisiana State University Health Sciences Center)

Control of gene expression at the translational level is crucial for many developmental processes. The mRNA cap-binding protein, eIF4E, is a key player in regulation of translation initiation; appropriate levels of eIF4E are essential for normal cell-cycle regulation and tissue differentiation. The observation that eIF4E levels are elevated during gametogenesis in several organisms suggests that eIF4E might have a specific role in gamete formation as well. We show that one of the five isoforms of C. elegans eIF4E, IFE-1, is enriched in the germline and is a component of germ granules (P granules). The association of IFE-1 with P granules requires the P-granule protein PGL-1. In vitro PGL-1 interacts directly with IFE-1, but not with the other four isoforms of eIF4E. Analysis of animals depleted of IFE-1 by RNAi shows that IFE-1 is required for spermatogenesis, specifically for efficient progression through the meiotic divisions and for the production of functional sperm, in both hermaphrodites and males. The requirement for IFE-1 is highly sensitive to temperature. IFE-1 is not required for oogenesis, as ife-1 (RNAi) hermaphrodites produce viable progeny when normal sperm are supplied. Consistent with a primary role in spermatogenesis, ife-1 mRNA levels are highest in regions of the gonad undergoing spermatogenesis. Our results suggest that C. elegans

spermatogenesis requires either this specific isoform of eIF4E or an elevated level of eIF4E.

(6) Toward understanding of tbx-9

Yoshiki ANDACHI

tbx-9 is a member of the T-box family of transcription factors. T-box genes have been found in various metazoans, and many of the genes play an important role in pattern formation especially in embryogenesis. tbx-9 encodes a transcription activator since the tbx-9 product can induce the transcription of a reporter gene containing the tbx-9 binding sequence upstream of the gene. The expression of tbx-9 is first observed in nucleus of the E cell at the 8-cell stage by in situ hybridization analysis. The expression lasts up to its daughters, Ea and Ep. At this stage, Ca, Cp and some AB-derived cells that seem to be precursors of hypodermal cells start tbx-9 expression. The expression of tbx-9 can also be seen in 4 body wall muscle precursor cells of MS descendants at the 200-cell stage. The latest expression of tbx-9 is in precursor cells of hypodermis that disappears just before morphogenesis. A tbx-9 deletion mutation produced by gene disruption causes disorganization in embryogenesis that occurs predominantly in posterior part of the body. At least one reason for the deformity is a defect in the positioning of body wall muscle cells at the comma stage. Penetrance of the phenotype is low, suggesting the presence of another gene that is functionally redundant with tbx-9. Indeed, all of the tbx-9 mutant embryos given dsRNA of tbx-8, another T-box gene phylogenetically most related to tbx-9, show abnormality in the overall length of the body. In these embryos body wall muscle cells are apparently detached from hypodermis. Although N2 embryos given tbx-8 dsRNA also reveal disorganization in their bodies, penetrance of the phenotype is low and the deformity is observed predominantly in the central part of the body.

To elucidate the nature of transcription regulation responsible for the tbx-9 mutant phenotype, I have been trying to identify target genes of tbx-9 by cDNA microarray analysis. Search for genes whose expression level is altered in embryos of the strains in which the amount of the tbx-9 product has been modified, and tests of these genes by in situ hybridization analysis to know whether the increase or decrease of their expression truly reflects on the change of expression pattern of them are in progress.

(7) The Caenorhabditis elegans unc-32 Gene Encodes Alternative Forms of a Vacuolar ATPase a Subunit

Nathalie PUJOL[1,2], Claire BONNEROT[2], Jonathan J. EWBANK[1], Yuji KOHARA and Danielle THIERRY-MIEG[2] ([1]CNRS, INSERM, France, [2]CRBM, CNRS, France)

Eukaryotes possess multiple isoforms of the a subunit of the V0 complex of vacuolar-type H+-ATPases (V-ATPases). Mutations in the V-ATPase a3 isoform have recently been shown to result in osteopetrosis, a fatal disease in humans, but no function has yet been ascribed to other isoforms. In Caenorhabditis elegans, the unc-32 mutant was originally isolated on the basis of its movement defect. We have isolated four new mutant alleles, the strongest of which is embryonic lethal. We show here that unc-32 corresponds to one of the four genes encoding a V-ATPase a subunit in the nematode, and we present their expression patterns and a molecular analysis of the gene family. unc-32 gives rise via alternative splicing to at least six transcripts. In the uncoordinated alleles, the transcript unc-32 B is affected, suggesting that it encodes an isoform that is targeted to synaptic vesicles of cholinergic neurons, where it would control neurotransmitter uptake or release. Other isoforms expressed widely during embryogenesis are mutated in the lethal alleles and would be involved in other acidic organelles. Our results indicate that V-ATPase a subunit genes are highly regulated and have tissue-specific function.

(8) Gene expression profiles in Ciona intestinalis tailbud embryos

Yutaka SATOU[1], Naohito TAKATORI[1], Lixy YAMADA[1], Yasuaki MOCHIZUKI[1], Makoto HAMAGUCHI[1], Hisayoshi ISHIKAWA[1], Shota CHIBA[1], Kaoru IMAI[1], Shungo KANO[1], Seiko D. MURAKAMI[1], Akie NAKAYAMA[1], Atsuo NISHINO[1], Yasunori SASAKURA[1], Gohki SATOH[1], Taishin SHIMOTORI[1], Tadasu SHIN-I, Eiichi SHOGUCHI[1], Miho M. SUZUKI[1], Norio TAKADA[1], Nanami UTSUMI[2], Natsue YOSHIDA[2], Hidetoshi SAIGA[2], Yuji KOHARA and Nori SATOH[1] ([1]Kyoto University, [2]Tokyo Metropolitan University)

A set of 3423 expressed sequence tags derived from the Ciona intestinalis tailbud embryos was categorized into 1213 independent clusters. When compared with DNA Data Bank of Japan database, 502 clusters of them showed significant matches to reported proteins with distinct function, whereas 184 lacked sufficient information to be categorized (including reported proteins with undefined function) and 527 had no significant similarities to known proteins. Sequence similarity analyses of the 502 clusters in relation to the biosynthetic function, as well as the structure of the message population at this stage, demonstrated that 390 of them were associated with functions that many kinds of cells use, 85 with cell-cell communication and 27 with transcription factors and other gene regulatory proteins. All of the 1213 clusters were subjected to whole-mount in situ hybridization to analyze the gene expression profiles at this stage. A total of 387 clusters showed expression specific to a certain tissue or organ; 149 showed epidermis-specific expression; 34 were specific to the nervous system; 29 to endoderm; 112 to mesenchyme; 32 to notochord; and 31 to muscle. Many genes were also specifically expressed in multiple tissues. The study also highlighted characteristic gene expression profiles dependent on the tissues. In addition, several genes showed intriguing expression patterns that have not been reported previously; for example, four genes were expressed specifically in the nerve cord cells and one gene was expressed only in the posterior part of muscle cells.

This study provides molecular markers for each of the tissues and/ or organs that constitutes the Ciona tailbud embryo. The sequence information will also be used for further genome scientific approach to explore molecular mechanisms involved in the formation of one of the most primitive chordate body plans.

(9) Profiles of Maternally Expressed Genes in Fertilized Eggs of Ciona intestinalis

Takahito NISHIKATA[1], Lixy YAMADA[1], Yasuaki MOCHIZUKI[1], Yutaka SATOU[2], Tadasu SHIN-I, Yuji KOHARA, Nori SATOH[2] ([1]Konan University, [2]Kyoto University)

A set of 1,378 expressed sequence tags (ESTs), both the 5'-most and 3'-most ends, derived from Ciona intestinalis fertilized eggs was categorized into 1,003 independent clusters. When compared with sequences in databases, 452 of the clusters showed significant matches with reported proteins, while 190 showed matches with putative proteins for which there is not enough information to categorize their function, and 361 had no significant similarities to known proteins. Sequence similarity analyses of the 452 clusters in relation to the biological function as well as the structure of the message population at this stage demonstrated that 362 of them have functions that many kinds of cells use, 65 are associated with cell-cell communication, including a candidate cDNA for sonic hedgehog, and 25 are transcription factors. Sequence prevalence distribution analysis demonstrated that the great majority (78%) of the mRNAs are rare mRNAs or are represented by a single clone/cluster. All of the 1,003 clusters were subjected to whole-mount in situ hybridization to analyze the distribution of the maternal mRNAs in fertilized eggs, and a total of 329 genes showed localized distribution of the mRNAs: 16 showed cortical localization, 12 showed mitochondrial-like distribution, 99 crescent-like distribution, 63 partial localization, and 139 weak localization. When the distribution pattern of all the maternally expressed mRNAs was examined in the 8-cell stage embryos, it became evident that 248 genes which have localized mRNA patterns at the fertilized egg stage lose their localized distribution by the 8-cell stage. In contrast, 13 genes newly gain a localized pattern by the 8-cell stage. In addition, a total of 39 genes showed distinct in situ signals in the nucleus of blastomeres of the 8-cell stage embryo, suggesting early zygotic expression of these genes by this stage. These results suggest that complicated cytoplasmic movements are associated with the characteristic distribution of maternal mRNAs, which in turn support proper embryonic axis formation and establishment of the genetic network for embryonic cell specification.

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H. STRUCTURAL BIOLOGY CENTER

H-a. Biological Macromolecules Laboratory

(1) Thin Layer Illumination Microscopy as a Novel Technique for Single Molecule Fluorescence Imaging of the Inside of Cells

Makio TOKUNAGA

Objective-type total internal reflection fluorescence microscopy, which we have developed, enabled us to visualize single molecules on or just below the cell surfaces. However, the technique is applied only when fluorescent molecules are from the glass-medium or glass-cell surface to a depth of less than about 150 nm. To overcome the limitation, I have further developed a new single-molecule microscopy by devising the way of light illumination for fluorescence excitation. Specimens were illuminated with a thin-layered laser beam with the thickness of about 5 to 10 micrometers.

The present microscopy has a significant advantage of low background and high sensitivity, so it allowed us to visualize single fluorescent molecules in the inside of cells up to the depth of about 10 μm from the glass-medium interface. It is also useful for observation of living cell, which is sensitive to the illumination light, and for time-lapse observation over a long period.

(2) Single Molecule Imaging of Nucleocytoplasmic Transport in Cells and Quantitative Analysis of Interaction with Nuclear Pores

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

We examined behavior of green fluorescent protein (GFP)-tagged importin beta and GFP-tagged cargo in digitonin permeabilized cells, using the above new technique of thin layer illumination microscopy. Although recent years have seen considerable progress in researches on nucleocytoplasmic transport, molecular mechanisms of the transport and the interaction between nuclear pore complex (NPC) and molecules remain to be solved. Fluorescence images of importin beta or cargo molecules bound to the nuclear rim were obtained. Images were composed of many fluorescent spots, which represented single NPC's. Furthermore, imaging of single molecules of GFP-importin beta or GFP-cargo on the NPC has been also achieved.

Both visualization of single pores and single molecules enabled us to analyze images quantitatively at the molecular level and to obtain kinetic parameters in cells, i.e., retention time at the pore, number of molecules bound to pores, and binding constant. It was found that there were two kinds of binding site of importin beta on NPC in the absence of cargo and Ran-GTP, weak binding sites and strong ones. The former gathers many proteins on NPC, and the latter is related with the active site of transport. Thus, single molecule imaging has opened a new way to obtain quantitative information on kinetics of molecular interactions in cells.

(3) A novel protein p105: recruitment into mRNA-containing complex and actin- and microtubule-dependent transport

Nobuyuki SHIINA, Kazumi SHINKURA and Makio TOKUNAGA

p105 is a novel protein we identified originally as an antigen of a monoclonal antibody against *Xenopus* centrosomes. Here, we characterized the molecular constituent of p105-contaning complex and observed the behavior of the complex.

In p105-expressing cultured cells, p105 was localized in electron-dense structures, which contained ribosome-like particles and membranous structures. The p105-containing structures were immunostained with EF-1 alpha (a translation elongation factor) and beta-COP (a component of coated vesicles) antibodies, supported the existence of ribosomes and membranes in the structures. The p105-containing structures also contained mRNAs. Actin mRNA was not contained in the structures, indicating that some specific mRNAs were localized in the structures. Lines of biochemical evidence suggested that p105 was associated with

ribosomes via mRNA. These findings revealed that p105 forms a complex with specific mRNAs, ribosomes, translational machinery and beta-COP-containing membranes.

p105 was fused with GFP and its behavior was studied by time-laps observation. The p105-containing complex was transported close to the plasma membrane in a microtubule- and actin-dependent manner, and moved concomitantly with dynamic membranes, e. g. ruffling membranes and processes.

These characteristics of the p105-containing complex are similar to those of mRNA transporters identified in mammalian neurons and Drosophila oocytes. We are now characterizing the complex further in the point of view of cell polarity.

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

Michio HIROSHIMA and Makio TOKUNAGA

Intermolecular force microscopy (IMF) is able to measure forces of intermolecular and intramolecular interactions at single molecular level. This microscopy has achieved the force resolution of subpiconewton with the handmade cantilever which is over 100-fold more sensitive than commercial ones. And incorporating the feedback system which use laser radiation pressure to reduce thermal fluctuation of the cantilever, the cantilever position is controlled with a nanometre accuracy.

In conventional atomic force microscopy (AFM), optical lever method has been used for sensing the cantilever position, however, there were a few problems in applying this method to our IMF. We have devised new method in which the cantilever inclined at 45 degrees is illuminated and its position is detected as displacement of the image projected on the photosensor. In this refined system, the measurable range of forces is over 100pN which is comparable to that of the horizontal IMF developed in last year.

With the refined IFM, we have measured the binding forces between single bases of DNA and the unfolding process of single globular protein. Forces curves of single-molecule or single-bond interactions were obtained with the sub-nanometer and piconewton resolution, which were never obtained by conventional scanning probe microscopy.

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

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

We measured mechanical unfolding events of single staphylococcal nuclease (SNase) molecules with the intermolecular force microscope. Cysteinyl residues, which have an activity to chemisorb to gold, were introduced into both the N- and C-termini of SNase. One terminus of the protein was attached to the gold-coated glass surface and the other terminus was attached to the probe tip of the intermolecular force microscope. Then the protein was extended mechanically to obtain relations between the extension length and the force exerted on the protein. From the relations of force-extention length, a partially unfolded structure or an intermediate structure was found for the first time. The extension length of whole the protein was 45 nm, while partial unfolding yielded extension length of 15 nm. Supported with the result of the experiment in the presence of Ca ion, this partially unfolded structure was attributed to a structure in which alpha-helices in the C-terminal region are unfolded. Multi-pathways in the mechanical unfolding were also found. The results suggested two ways of the mechanical unfolding: unfolding by way of the partial unfolded structure, and direct unfolding through no intermediate structure.

(6) A Novel *in vitro* Assay System of Nucleocytoplasmic Transport

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

We have developed a novel *in vitro* assay system of nucleocytoplasmic transport. We aim at application of the method to new single-molecule experiments, imaging and nano- or force-measurement.

Nuclear envelope was formed on a planar surface of a small agarose plate. At first, agarose plates were modified with glutation. Then glutation-agarose plates were coated with GST-RanGDP fusion protein. Nuclear envelope was formed onto the RanGDP-coated surface using extracts from *Xenopus laevis* frog eggs.

Formation of Nuclear Pore Complexes was confirmed by observing import of fluorescently labeled proteins. This new cell-free system has marked advantages: 1) Solutions in both sides, pseudo-cytoplasmic and pseudo nucleoplasmic sides, can be replaced independently. 2) The shape of the nuclear envelope can be changed as one likes, for example, a vertical plane and a horizontal plane. 3) The system doesn't contain no organelle or cellular structures except the nuclear envelope. Using the *in vitro* system together with single molecule techniques or fluorescence resonance energy transfer should provide an innovative and powerful tool to investigate molecular mechanisms of transport.

Publications

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H-b. Molecular Biomechanism Laboratory

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

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Transcription initiation has been conventionally assumed to be a multi-step sequential process, although additional steps could exist. The in vitro initiation from T7A1 promoter in particular apparently behaves in a manner that can be fully explained by the sequential pathway. However, the initiation from λP_RAL promoter has been shown to follow the branched pathway, where a part of the enzyme-promoter complex is arrested at the promoter, raising the question as to which mechanism is general. We found that a moribund complex, which is generated in the arrested branch, is formed at the T7A1 promoter especially in low salt condition, indicating that the initiation mechanism for this promoter is also branched. The results of DNA footprinting suggested that holoenzyme in moribund complex is dislocated on DNA from its position of productive complex. However, only a small fraction of the binary complex becomes arrested at the promoter and the interconversion between subspecies of binary complex is apparently more reversible than at the λP_RAL promoter, explaining the reason why the reaction pathway looks sequential. These findings suggest a generality of the branched pathway mechanism, which would resolve contradictory observations that have been reported for various promoters.

We next addressed the question whether the branched pathway has physiological significance in E. *coli*, because the both promoters we have examined are bacteriophage promoters. The clue is the effect of GreA and GreB on promoter arrest, which was previously found in the initiation at the λP_RAL promoter. These two proteins relieve the arrest at the promoter by increasing the conversion of moribund subspecies of binary complex into productive one. By using genomic DNA array we selected candidate genes whose expressions are decreased in a strain with dusrupted *greA* and *greB*. By confirmation with Northerm blotting, I discovered that mRNA levels of at least 3 genes, *cspA*, *rpsA* and *atp*, are decreased by disruption of *greA* and *greB*. Their transcriptions were enhanced in a purified reconstitution system by adding GreA or GreB. Among them, *atp* operon was examined most detail. The formation of moribund complex at the *atp* promoter, the major promoter of *atp* operon, was confirmed by the most sensitive kinetic assay. The transcription showed littele pause and GreA and GreB were shown to enhance transcription through initiation efficiency by mitigating the promoter arrest. These findings suggest the pleiotropic existence of the branched pathway in *E.coli* cell. They also provide a new concept that the Gre factors are bonafide initiation factors, although they were discovered as elongation factors that mitigate elongation arrest in vitro.

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

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

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

(3) Does RNA polymerase track a groove of DNA during sliding?

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As the start of transcription of a gene, RNA polymerase first binds to a nonspecific site and then reaches a promoter by repeated nonspecific association and dissociation and by sliding along DNA. Sliding is one-dimensional random diffusion of proteins along DNA, the existence of which has been proved for *E. coli* RNA polymerase and several other DNA binding proteins by single-molecule dynamics and kinetics. There are two possible modes of movement during sliding. In the first mode, a protein molecule slides along the axis of DNA helix and thus contacts to one side of DNA. In the second, a protein molecule tracks a groove of DNA and makes relative rotational motion around the axis.

To determine the sliding mode for *E. coli* RNA polymerase, we devised an assay for detecting the tracking of a groove as rotation of DNA based on single-molecule manipulation. A nonspecific DNA is fixed to a 1 μm polystyrene bead coated with streptavidin by several biotin residues with no rotational freedom. A 0.1 μm fluorescent bead is covalently attached to the polystyrene bead to detect the rotational motion of the DNA-fixed beads under a fluorescent microscope. The polystyrene bead is held with optical tweezers at a distance from the surface of a glass slide on which RNA polymerase has been fixed. In this configulation, we moved RNA polymerase relative to the DNA by shifting the slide continuously in one direction. If RNA polymerase tracks a groove, it should rotate DNA and the rotation can be detected as the movement of the fluorescent bead.

We have observed rotational motions of the beads that could be caused by groove tracking of RNA polymerase in a small number of cases. Such motions were observed only when the distances between beads and the surface are smaller than the length of DNA. However, the number of such observation is not enough to conclude groove tracking. A short lifetime of sliding complex, about 1 sec in solution, complicates the estimation of the frequency of rotational motions. Further quantitative determination is undergoing to prove or disprove groove tracking.

Publication

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H-c. Multicellular Organization Laboratory

(1) Analysis of Synthetic Dauer-constitutive Mutations in the Nematode *Caenorhabditis elegans*

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Eiko TSUCHIYA[1], Takeshi ISHIHARA and Isao KATSURA
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If newly hatched larvae of *C. elegans* are in a crowded state and given a limited food supply, they sense the environmental signal of pheromone and food with a head sensory organ called amphid, and deviate from the normal life cycle, ending in non-feeding larvae called dauer larvae. Since the assay of dauer formation is much less time-consuming than behavioral assays such as chemotaxis assays, we are analyzing the pathway of sensory signals in the amphid neural circuit by detecting dauer formation as the output. We found that mutations in more than 50 known genes show synthetic dauer-constitutive (Sdf-c) phenotypes, i.e., they develop to dauer larvae in a certain mutant background, regardless of the environmental conditions. The synthetic nature of the phenotype, we think, is based on the pathway of sensory signals. Namely, the signals are transmitted through parallel routes, and therefore two mutations are required to block them. We are determining the combinations of mutations for the Sdf-c phenotype and the pattern of suppression of the Sdf-c phenotype by various suppressor mutations. In this way we hope to elucidate the pathway of sensory signals for dauer regulation. Furthermore, to identify new genes required for the sensory signal transduction, we isolated and mapped 44 mutations that show the Sdf-c phenotype in the *unc-31(e169)* background, where *unc-31* gene encodes CAPS protein, which acts in secretion from dense core vesicles. Eight of the mutations mapped in 4 known genes, but most of the remaining 36 mutations, which map in at least 13 genes, seem to be located in novel genes, which we named *sdf* genes. Some of the mutants exhibit additional phenotypes after removal of the *unc-31(e169)* mutation. A mutant in *sdf-1* gene avoids volatile attractants sensed by AWC neurons and shows a thermophilic phenotype in thermotaxis. Mutants in *sdf-13* gene (2 alleles) show defects in the adaptation of chemotaxis to odorants sensed by AWC neurons, although they show normal chemotaxis to the same odorants. This gene encodes a homolog of mammalian Tbx2 and *Drosophila* Omb, and is expressed in AWB, AWC and ASJ sensory neurons and in many pharyngeal neurons. Mutants in *sdf-9* gene (5 alleles) form dauer larvae that recover spontaneously, if the promoter DNA fragment of *daf-7* gene, which encodes a TGF-β protein that inhibits dauer formation, is introduced into the mutants.

In the year 2001, the following results were obtained.

(a) The expression of a *daf-7*:: GFP fusion gene was investigated in various Sdf-c double mutants. In wild type animals this gene is expressed only when they adopt the normal life cycle, but not when they form dauer larvae. Hence, the results indicate whether the genes of the Sdf-c mutations act upstream of *daf-7* in the dauer regulatory pathway. The *daf-7*:: GFP fusion gene was not expressed in the double mutants *unc-31; osm-1*, *unc-31; unc-3*, and *egl-4; unc-3*, while it was expressed in the single mutants *unc-31*(CAPS), *unc-3* (Olf-1/ EBF), *egl-4*(PKG), and *osm-1* (required for sensory cilium formation). We speculate that the expression of *daf-7* is regulated by at least two parallel pathways: one for the activation of the UNC-3 transcription factor by an environmental signal that enters through sensory cilia, and the other(s) containing neurosecretion and PKG signal transduction.

(b) Using specific antibodies, we found that SDF-13 protein is localized mainly in cytoplasm. Although this localization does not change by the stimulus of odorants for adaptation, it is possible that SDF-13 may be regulated somehow by nuclear transport. We also found that the expression of *sdf-13* cDNA in AWC neurons alone can restore normal adaptation but not the synthetic dauer-constitutive phenotype. The results indicate that SDF-13 acts in AWC neurons for the adaptation to odorants sensed by the same neurons.

(c) We cloned *sdf-9* gene and found that it encodes a tyrosine phosphatase-like molecule. Since the consensus sequence HCxxGxxR is changed in this molecule, it may lack the enzyme activity. A functional *sdf-9*:: GFP fusion gene was expressed in a pair of cells anterior to the nerve ring.

(d) The dauer larvae formed by the double mutants *unc-31 (e169); sdf-14* (5 alleles) have two unique characteristics: (i) their percentage changes greatly by the volume of the *E. coli* liquid culture spread on the plates, and (ii) they recover easily to L4 larvae in spite of having the *unc-31 (e169)* mutation, which normally prevents such recovery. We narrowed down the position of *sdf-14* mutations within 3 cosmid clones by mapping and rescue experiments.

(2) Analysis of Mutants That Show Abnormality in the

Selection between Two Behaviors and in Behavioral Plasticity

Takeshi ISHIHARA, Yuichi IINO[1], Akiko MOHRI[2], Ikue MORI[2] and Isao KATSURA ([1]Molecular Genetics Laboratory, University of Tokyo, [2]Division of Biological Science, Nagoya University)

Animals receive environmental cues, select and integrate necessary information, and make proper responses, while all these steps can be modified by experience or memory. In *C. elegans*, many behavioral mutants in chemotaxis and thermotaxis, for instance, have been isolated and analyzed, and the molecular mechanisms of sensation have been elucidated. On this basis and as a next step, we are analyzing mutants that show abnormality in the learning and selection (evaluation) of sensory signals, to elucidate novel mechanisms of higher-order sensory signal processing.

C. elegans shows avoidance of copper ion and chemotaxis to odorants by receiving these stimuli with different sensory neurons in the head. We developed a behavioral assay method for the interaction between the two responses to learn a possible role of interneurons. Wild-type animals change their preference between the responses, depending on the relative concentration of copper ion and odorants. On the basis of the *C. elegans* neural circuitry, the result suggests that the two sensory signals interact with each other in a neural circuit consisting of about 10 neurons. While well-fed animals are usually used for this assay, we found that animals starved for 5 hours tend to prefer chemotaxis to odorants. The change is due to the desensitization of copper ion avoidance by starvation, and can be suppressed by serotonin, which mimics the effect of food. This desensitization is advantageous in natural environment, because starved animals can search for food over a wider area.

To elucidate the mechanism of behavioral interaction or selection, we are isolating and analyzing mutants that show abnormality in this assay. The mutant *ut236* has a tendency to choose avoidance of copper ion rather than chemotaxis to odorants, although it shows no abnormality in each behavior in case only one of the stimuli is given. The result shows that *ut236* is abnormal in the interaction between the two behaviors.

We identified the gene for *ut236* by positional cloning and named it *hen-1*. It encodes a novel secretory protein having an LDL receptor ligand-binding domain. Using antibodies against its recombinant protein, we found that the gene product is localized in the axon and cell body of each one pair of sensory and inter-neurons. The localization in the axon was abolished in *unc-104* (kinesin KIF1A homolog) mutants, which show defects in the transport of synaptic vesicles. Expression studies with various transcriptional promoters showed that this gene acts non-cell-autonomously in the nervous system. Studies using a heat-shock promoter revealed that the wild-type phenotype is recovered if *hen-1* gene is expressed after the mature neural circuitry is formed, but not during the development of the nervous system. These results indicate that the gene product is a novel neuromodulatory factor that acts in diverse functions such as the processing of sensory information and various types of learning (See below).

We isolated another mutant, *ut235*, which lacks the effect of starvation in this assay. This mutant behaved essentially normally in many other responses to starvation, such as the change of locomotion speed by starvation. Interestingly, the double-mutant *ut235; ut236* showed a preference to avoidance of copper ion, regardless of starvation. We are now trying to clone the gene for this mutation.

The *hen-1* mutant also shows abnormality in learning by paired presentation of starvation and NaCl (collaboration with Dr. Iino, University of Tokyo). While wild-type animals are normally attracted by NaCl, they avoid it after the conditioning. In contrast, *hen-1* mutant animals are only partially affected by the conditioning. Since the mutant shows normal behavioral responses to starvation alone, we think it has abnormality in the integration of starvation and other signals.

In 2001, we investigated the role of *hen-1* gene in learning by temperature and food/ starvation, in collaboration with Dr. Mori's Laboratory in Nagoya University. In thermotaxis, wild type animals prefer the temperature of their growth, but avoid it after they are starved at the same temperature. In contrast, *hen-1* mutant animals did not change their behavior by starvation, although they show normal thermotaxis without starvation. The results indicate that the *hen-1* mutant has abnormality in the behavioral plasticity after paired presentation of temperature and starvation, although it responds normally to each of the two stimuli.

(3) *C. elegans* Mutants in the Associative Learning of Odorants and Food

Ichiro TORAYAMA, Takeshi ISHIHARA and Isao KATSURA

The nematode *C. elegans* provides a good system for the study of associative learning. However, the mechanism of this learning looks different from that of classical conditioning, because (a) the unconditional stimulus is usually limited to food or starvation, and because (b) the learning is efficient, if the conditional stimulus is presented at the same time as but not before the unconditional stimulus. For instance, paired presentation of an attractive smell and starvation, but not the smell and food, leads to inefficient chemotaxis to the smell. However, this phenomenon may also be interpreted as the inhibition of olfactory adaptation by food. To elucidate the molecular mechanism of food and starvation signaling in such learning, we started the isolation and characterization of mutants that show abnormality in associative learning.

In the year 2001, we first examined the conditions for the associative learning of smell and food/ starvation and succeeded in efficient learning by using butanone as the odorant. Butanone is an attractant for naive worms, but chemotaxis to butanone becomes inefficient after conditioning with starvation and butanone, while it becomes more efficient after conditioning with food and butanone. Then, we isolated mutants that show abnormality in these responses. Some of them showed inefficient chemotaxis after conditioning with food and butanone (i. e., they adapted to butanone even in the presence of food), while others are attracted by butanone only after conditioning with food and butanone. We are mapping *ut305*, one of the mutations that belong to the former category. Interestingly, the mutant shows no adaptation to isoamyl alcohol and benzaldehyde, which are sensed by the same sensory neurons (AWC) as butanone.

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

Akane OISHI, Minoru KAWAKAMI[1], Akiko KAMAMOTO, Takeshi ISHIHARA and Isao KATSURA ([1]Gothenburg University)

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

In the year 2001, we worked on the positional cloning of *flr-5* gene and the expression of *flr-5* gene. We mapped a *flr-5* mutation precisely by classical methods and limited the position of *flr-5* gene within several genomic clones by the activity of rescuing the Flr-5 phenotype (suppression of the slow growth phenotype of *flr-3*) in a *flr-3; flr-5* double mutant.

In the past study we found that a *flr-3*:: *lacZ* gene is expressed in the intestine. However, this expression may not reflect the expression of the intrinsic *flr-3* gene, because we later discovered that *flr-3* gene is

transcribed together with a neighboring gene as an operon. Hence, we isolated a genomic clone covering the operon, inserted GFP cDNA at the end of the *flr-3* coding region, and introduced the GFP fusion gene into the wild type. The transformant expressed GFP also in the intestine. Since the transformant grows very slowly, we are investigating whether the *flr-3*:: GFP fusion gene acts as a dominant negative allele.

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H-d. Biomolecular Structure Laboratory

(1) Crystallographic Study of F1-ATPase

Yasuo SHIRAKIHARA, Aya SHIRATORI and Chikako SHIRAKIHARA

F1-ATPase is the catalytic sector in ATP synthase that is responsible for ATP production in living cells. F1 has molecular mass of 380,000 dalton and a subunit composition of $\alpha 3\beta 3\gamma\delta\varepsilon$. The unique rotational catalysis mechanism of F1 includes rotation of the rod-like γ subunit, which is thought to control the conformations of the three catalytic β subunits in a cyclic manner by its rotation. We have recently concentrated on the structural study of the $\alpha 3\beta 3\gamma\varepsilon$ sub-assembly of thermophilic F1, after solution of the structure of the $\alpha 3\beta 3$ sub-assembly and committed attempts to crystallize the $\alpha 3\beta 3\gamma$-sub-assembly. Employing the $\alpha 3\beta 3\gamma\varepsilon$ sub-assembly that show catalytic properties very similar to those of F1, we aim to detect structural changes caused by different nucleotide occupancy, which should provide with structural basis for understanding the rotational catalysis mechanism.

The Mg ion is absolutely required for catalysis, but its absence forces the sub-assembly into only slightly inhibited state. The $\alpha 3\beta 3\gamma\varepsilon$ sub-assembly crystals were grown in broad conditions that vary in content of both nucleotide and magnesium ion. However, the Mg-free conditions generally gave better-diffracting crystals than the Mg-included conditions. When experimented with Spring8 beam, crystals grown in absence of Mg diffracted to 4 Å at 100K. The diffraction capability of these crystals did not depend much on nucleotide contents in the mother liquor. The better resolution of the data, compared with that reported last year, are due to the improved flash-cooling method that included the use of oil paratone. The diffraction data have been collected at Spring8 from the crystals that were grown in the presence of varying concentrations of ADP and ATP but in absence of Mg.

Following the analysis of the data from the crystals grown in presence of 0.5mM ADP at 4.5 Å resolution reported last year, we analyzed the data from a crystal grown in absence of nucleotide to 4.0 Å resolution. Unexpectedly the sub-assembly molecules in the crystal represented Mg-free one-ADP bound form, probably because the sub-complex molecules that bound single ADP tightly and occupied 10-20% population in our partially nucleotide-depleted preparations, appear to have exclusively crystallized. In contrast to the analysis of 0.5mM ADP form (representing two ADP bound structure), the current analysis of one ADP bound form allowed to identify the structures of the γ-and ε-subunits.

The structure of the sub-complex with Mg-free ADP bound to only one β-subunit has the features distinct from those in all the previous F1 crystal structures with two or three nucleotide-bound β-subunits. The β-subunit with the Mg-ADP-bound catalytic site in the previous F1-structures is replaced by the nucleotide-free β-subunit in this structure. The structural change appears to be responsible for substantial structural changes in the stalk, 30-degree twist in major part of the γ-subunit and penetration of the C-terminal helices of the ε-subunit into the $\alpha 3\beta 3$ cavity. The new conformation of the C-terminal helices of the ε-subunit explains well the ε-inhibition.

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

(2) Crystallographic Study of the Transcription Activator, PhoB

Kazuyasu SHINDOH, Katsumi MAENAKA and Yasuo SHIRAKIHARA

PhoB Protein is a positive transcriptional activator for the genes in the phosphate (*pho*) regulon of *E. coli*, such as *phoA* and *pstS* that are induced by phosphate starvation. PhoB acts by binding to the *pho* box in the promoter region, which is the consensus sequence shared by the regulatory regions of the genes in the regulon. The activity of PhoB is regulated by phosphorylation by PhoR. The N-terminal domain of PhoB is responsible for this regulatory role, whereas the C-terminal domain (PhoBC: spanning from a residue 125 to the C-terminus) has a DNA binding ability. In order to resolve the detailed interaction between PhoB and *pho* box DNA, we have made the crystallographic study of PhoBC and of its complex with the DNA.

Using the 3.0 Å resolution MAD (Multi-wavelength Anomalous Dispersion) data from the selenomethionyl derivative of PhoBC, a good density map has been obtained which allows identification of the fold of PhoBC. The observed fold, classified as a variant of the winged-helix-turn-helix, is consistent with the previous NMR structure but includes the DNA-binding loop that have been unclear so far. Refinement of this model using the 2.0 Å resolution native data has come to the final stage.

We have searched for the crystallization conditions of the complex of PhoBC and DNA and got the crystals of the complex in the conditions using the PEG as a precipitant. Diffraction data to 3.0 Å resolution have been collected at PF. The data analysis has been in progress.

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

(3) Structural and functional studies of immunoglobulin(Ig)-like receptors

Katsumi MAENAKA, Kimie AMANO and Yasuo SHIRAKIHARA

Since the immunoglobulin (Ig) superfamily domains are very common among the cell surface receptors in immune system, it is important to clarify the molecular recognition of Ig-like cell surface receptors towards various ligands. We are studying two Ig-like receptors, human killer cell Ig-like receptors (KIRs) and Fcγ receptors (FcγR).

Fcγ receptors (FcγRs) are expressed on all immunologically active cells and bind the Fc portion of IgG to trigger a range of immunological functions. By using surface plasmon resonance (SPR), we have studied the kinetic and thermodynamic properties of Fc binding of human low-affinity Fcγ receptors (FcγRs (IIa, IIb and IIIb), whose structure have unique topology (intermediate between I set and C2 set) revealed by several structural studies of KIRs and FcγRs including ours. All three receptors bind Fc or IgG with low affinities (K_D ($\sim 10^{-6}$M) and fast kinetics, which is similar to other receptors involved in cell-cell interactions. It suggests that FcγR recognition of IgG-coated cells is mechanistically similar to cell-cell recognition. On the other hand, the Fc receptors show distinct thermodynamic properties. While the binding of the FcγRIIa and FcγRIIb to Fc is driven by favourable entropic and enthalpic changes, the binding of FcγRIII is characterised by highly unfavourable entropic changes, suggesting that the molecular events coupled to the binding differ amongst the low-affinity FcγRs.

We got very small crystals for KIR2DL3 complexed with MHC class I (HLA-Cw7) in the conditions including PEG20000 as a precipitant, but further refinement of crystallization conditions is needed for determination of the complex structure.

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

(4) Structural study of Na^+-translocating ATPase

Yasuo SHIRAKIHARA

Na^+-translocating ATPase from Enterococcus hirae, classified as a Vacuolar-type ATPase (V-type), is expected to have a structure similar to F1-ATPase (F-type ATPase). Na^+-translocating ATPase was highly purified and was subjected to a crystallization experiment. However, the crystals obtained under the previously identified conditions (27% PEG4000, 0.2 M $LiSO_4$, 10 mM Tris-HCl, pH 7.5, 10% glycerol) were

found to be from enolase that was co-purified with ATPase, in spite of its very low content (a few percent). Enolase was purified and again was subjected to a crystallization experiment in order to search for different crystal forms, because the original crystal form contained as much as four molecules in the asymmetric unit. The new crystal form (I4, a=b=153.5 Å, c= 90.6 Å) was found to be suited for the molecular replacement analysis. Refinement, after finishing the molecular replacement calculations, has been in progress.

The crystallization experiment with Na^+-translocating ATPase was carried out again for its authentic crystals. Several crystal forms, with plate-like and needle-like shapes, were obtained. The most hopeful crystals diffract to 4.5 Å resolution and belong to space group $P2_12_12_1$with cell dimensions of a=122.0 Å, b=122.0 Å, c=240 Å. Structural analysis by molecular replacement using the F1 structure is in progress.

This work has been done in collaboration with Toshiaki Hosaka, Toshiyuki Meguro and Ichiro Yamato, Science University of Tokyo.

(5) Structural analysis of glutaminase from *Micrococcus luteus K-3*

Yasuo SHIRAKIHARA and Aya SHIRATORI

Glutaminase (EC 3.5.1.2) catalyzes the hydrolytic degradation of L-glutamine to L-glutamic acid. The salt-tolerant glutaminase from marine *Micrococcus luteus* K-3 has an unusual property that its maximun activity is observed at about 2M salt, and also has industrial relevance that this enzyme may be more efficient than the conventional ones in the soy sauce fermentation that is carried out in the high-salt environments.

The enzyme was found to form good crystals that diffracted better than 3 Å, as reported last year. Crystals of Se-Met derivative have been obtained in the conditions similar to those for the native enzyme. The MAD data for this intact enzyme was collected to 2.6 Å resolution However, the truncated enzyme, loosing the C-terminal 80 residues out of 480 residues in the intact enzyme and being identified in the latest preparation, formed much better crystals for native and Se-Met derivative forms than the intact molecule. The 2.4 Å resolution MAD data from the Se-Met derivative of the truncated enzyme produced good maps, allowing us to finish the tracing the main chain. The model building is in progress. We plan to utilize the model in determining the structure of the intact enzyme by molecular replacement.

The structural study was done in collaboration with Panuwan Chantawannakul, Kazuaki Yoshimune and Mitsuaki Moriguchi at Oita University, and Mamoru Wakayama at Ritsumeikan University.

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H-e. Gene Network Laboratory

Nucleocytoplasmic exchange of macromolecules

Naoko IMAMOTO

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

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

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

(1) Analysis of translocation process through nuclear pores using single molecule imaging techniques

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

In contrast to our growing knowledge of receptor molecules for nucleocytoplasmic transport, function of nuclear pore complex (NPC), as well as mechanism of translocation step of transport is still very poorly understood. This is due to the complexity of NPC structure, and difficulty in biochemical handling of NPC as a functional unit. As one approach to investigate the function of NPC, we are trying to visualize behavior of transport factors and transport substrates at NPC, using modified objective-type total internal reflection fluorescence microscopy (TIRFM). We examined behavior of green fluorescent protein (GFP)-tagged importin β and GFP-tagged NLS-cargo in digitonin permeabilized cells. Fluorescence images of importin β or cargo molecules bound to the nuclear rim were obtained. Images were composed of many fluorescent spots, which represented single NPC's. Furthermore, imaging of single molecules of GFP-importin β on the NPC has been also achieved. Both imaging of single pores and single molecules enabled us to analyze images quantitatively at the molecular level and to obtain kinetic parameters of translocation events, i.e. retention time at the pore, number of molecules bound to pores, and binding constant. Based on quantitative analysis of images, we are currently working to propose a model to explain the molecular interactions of translocation events.

(2) Analysis of nuclear migration of importin β through the nuclear pore complex

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

Importin β is a nuclear transport factor, which mediates the nuclear import of various nuclear proteins. We have found that importin β shuttles rapidly between the nucleus and the cytoplasm by direct interaction with components of the nuclear pore complex (NPC). However, it remains unclear how translocation directionality is determined.

The crystal structure of uncomplexed form of importin β has been solved. Its global feature is a right-handed superhelical structure formed by consecutively stacked HEAT repeats. To understand the mechanism of nucleocytoplasmic migration of importin β, we made various importin β mutants. These mutants were mutated in amino acids between HEAT repeat 4B and 6A, which we have shown to be necessary and sufficient for the translocation activity of importin β through NPC. By transient transfection and microinjection experiments, we observed that one of the mutants showed inefficient accumulation to the nucleoplasm, while the other localized within the nucleus more efficiently than wild type.

To investigate the rate of nuclear accumulation of these mutants, we measured nuclear accumulation of GFP-fusion importin β mutants in permeabilized cells in real time. This assay revealed that the rate of nuclear import of one mutant (inefficiently accumulating mutant to the nucleus), but not the other (efficiently accumulating mutant), is faster than that of wild type, while the rate of nuclear export of inefficiently accumulating mutant, but not the efficiently accumulating mutant, is faster than that of wild type. These differences in the activity of translocation through NPC between importin β mutants may be due to the change of affinities between importin β mutants and components of NPC. The interaction between importin β mutants and various nucleoporins are now under investigation.

(3) Analysis of mechanism of nuclear import and export of β-catenin

Makiko KOIKE and Naoko IMAMOTO

β-catenin plays a key role in Wnt signaling pathway. Upon activation, β-catenin accumulates and enters the nucleus, where it modulates the activation

of the target gene expression. Therefore, it is important to understand the mechanism of nucleocytoplasmic transport of β-catenin.

Our previous studies indicate that β-catenin can enter the nucleus on its own, and shuttles between the cytoplasm and nucleus. The export of β-catenin examined in living cells showed that this protein exit the nucleus in CRM1-independent and Ran-independent manner.

In order to determine the region of β-catenin necessary and sufficient for its import and export, we made series of deletion constructs of β-catenin, expressed in bacteria, and purified as recombinant proteins. Using digitonin-permeabilized cell free import assay, and recently developed our in vitro export assay, we examined the ability of β-catenin fragments to exit and enter the nucleus. We confirmed that a β-catenin fragment containing both Arm repeats 10-12 and the C-terminus possesses most strong nuclear import and export activity. The import and export activities of these fragments were also confirmed in living cells by microinjection experiments. Further dissection of this fragment is now under taken, in order to know whether import and export activity of β-catenin can be separated.

(4) Nuclear import under stress condition and normal condition

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

Heat-shock induces a strong stress response and alters gene expression drastically in cells. Investigation of nuclear transport activity of certain substrates under heat-shock condition and normal condition may provide us an information concerning whether specific transport pathway or transport factors function under different cellular conditions.

We first examined the nuclear transport of 70kDa heat-shock protein (hsp70/hsc70) under normal condition and heat-shock condition. When injected into the cell cytoplasm, only little amount of hsc70 migrated into the nucleus. When injected into the cell nucleus, only little amount of hsc70 migrated into the cytoplasm. These results show that hsc70 possess very poor nuclear import and export activity under normal condition. On the other hand, when hsc70 is microinjected into the cytoplasm of heat shock cells, we found that hsc70 efficiently migrates into the nucleus. From our results, we concluded that nuclear import of hsc70 is strongly and specifically stimulated under heat shock condition.

We next examined importin α/β mediated nuclear import of SV40 T-antigen NLS under normal condition and heat shock condition. When microinjected into the cell cytoplasm, we found that nuclear import efficiency of SV40T-antigen NLS is clearly down regulated under heat-shock condition.

These results strongly indicate that different transport pathways or transport factors function under different cellular conditions. In order to elucidate these phenomena at molecular level, we are now developing an in vitro transport assay that mimics in vivo transport of heat shock condition and normal condition.

(5) Molecular cloning of a novel importin α homologue from rice, by which constitutive photomorphogenic 1 (COP1) nuclear localization signal (NLS)-protein is preferentially nuclear imported

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Nuclear import of proteins that contain classical nuclear localization signals (NLS) is initiated by importin α, a protein that recognizes and binds to the NLSs in the cytoplasm. We have cloned a cDNA for a novel importin α homologue from rice which is in addition to our previously isolated rice importin α1a and α2, and we have named it rice importin α1b. In vitro binding and nuclear import assays using recombinant importin α1b protein demonstrated that rice importin α1b functions as a component of the NLS-receptor in plant cells. Analysis of the transcript levels for all three rice importin α genes revealed that genes were not only differentially expressed but that they also responded to dark-adaptation in green leaves. Furthermore, we also show that the COP1 protein bears a bipartite-type NLS and its nuclear import is mediated

preferentially by the rice importin α1b. These data suggest that each of the different rice importin α proteins carry distinct groups of nuclear proteins, such that multiple isoforms of importin α contribute to the regulation of plant nuclear protein transport (for detail, see Ref1).

(6) Transforming growth factor-β induces nuclear import of Smad3 in an importin-β1 and Ran-dependent manner

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Smad proteins are cytoplasmic signaling effectors of transforming growth factor-β (TGF-β) family cytokines and regulate gene transcription in the nucleus. Receptor-activated Smads (R-Smads) become phosphorylated by the TGF-β type I receptor. Rapid and precise transport of R-Smads to the nucleus is of crucial importance for signal transduction. By focusing on the R-Smad Smad3 we demonstrate that 1) only activated Smad3 efficiently enters the nucleus of permeabilized cells in an energy- and cytosol-dependent manner. 2) Smad3, via its N-terminal domain, interacts specifically with importin-β1 and only after activation by receptor. In contrast, the unique insert of exon3 in the N-terminal domain of Smad2 prevents its association with importin-β1. 3) Nuclear import of Smad3 in vivo requires the action of the Ran GTPase, which mediates release of Smad3 from the complex with importin-β1. 4) Importin-β1, Ran, and p10/NTF2 are sufficient to mediate import of activated Smad3. The data describe a pathway whereby Smad3 phosphorylation by the TGF-β receptor leads to enhanced interaction with importin-β1 and Ran-dependent import and release into the nucleus. The import mechanism of Smad3 shows distinct features from that of the related Smad2 and the structural basis for this difference maps to the divergent sequences of their N-terminal domains (for detail, see Ref2).

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I. CENTER FOR INFORMATION BIOLOGY AND DNA DATA BANK OF JAPAN

I-a. Laboratory for DNA Data Analysis

(1) Expression profile of normal developing mouse eye and brain

Chi Chiu WANG, Kazuho IKEO, Takashi GOJOBORI, Michael Scott ROGERS[1] ([1]Department of Obstetrics and Gyraecology, The ChineseUniversity of Hong Kong)

This is only the first phase to the project of "Developmental damage, embryonic molecular regulation in early embryogenesis in both normal and diabetic environment". The application of comprehensive gene expression profiling technologies by DNA microarray to compare normal and diseased tissues/cells or to assess molecular alterations resulting from various experimental interventions has the potential to provide highly detailed qualitative and quantitative information. We have received a copy of 15K mouse cDNA clone set from NIA/NIH in which there are more than 15k unique sequence verifies clones amongst 52,374 ESTs from pre- and peri-implantation embryos. It has been successfully adopted to employ to our high density in-house cDNA chip systems for array experiments, including sufficient number of internal, quantitative, RNA amplification probes and negative controls. Initially normal brain and eye tissues from different gestational stage of development have been speculated whilst E8.5 of C57BL/6J strain was taken as reference for competitive hybridization. Whereas the variances of transcript regulations in forebrain, midbrain and hindbrain were evaluated by using lasar captured microdissection technology. In additional we occupied to use a new neural network system to analyse the expression profiles. The findings have strong implications for our current understanding of the processes of brain and eye development and differentiation.

(2) The study of the genetic program for planarian brain using cDNA chip technology

Masumi NAKAZAWA and Takashi GOJOBORI

The origin of the brain remains a challenging problem in evolutionary studies. To understand the brain on the viewpoint of the evolution, we analyzed the central nervous system (CNS) of a lower invertebrate, planarian. We conducted a large-scale screening of the head part-specific genes in the planarian by constructing a cDNA microarray. We created a microarray of 3,555 non-redundant genes, randomly chosen from 9,000 EST sequences. Competitive hybridization of cDNAs between a head portion and the other body portion of planarians revealed 205 genes with head part-specific spikes, including homologues of *synapsin*, *synaptotagmin* and *arrestin* genes essential in the vertebrate nervous system. The expression patterns of the top 30 genes showing the strongest spikes implied that the planarian brain has undergone functional regionalization. These data indicate that a brain character to be highly organized had already emerged by the time of planarian divergence. Moreover with analysis of their function by RNA interference (RNAi) method, we identified a gene that restricts brain formation in the head region, since brain-like structures expand into the entire region of the body in the gene-knockout planarian. We are also using the microarray in order to find out the downstream genes of one of homeobox containing gene, *otx* which is essential for head formation in higher organisms. Our results show that planarian *otx* (*DjotxA*) network contains the homologous genes related to the eye development in higher organisms. These results should give good insights to understand a complex gene network of mammalian CNS.

(3) Comparative study for the CNS evolution using neural-related genes found in the planarian EST project

Katsuhiko MINETA, Masumi NAKAZAWA, Francesc CEBRIÁ, Kazuho IKEO, Kiyokazu AGATA and Takashi GOJOBORI

In the bilateral animals, the centralized nervous system was found in both clades of deuterostome and protostome. It is thought that central nervous systems (CNSs) were derived from a common ancestor between deuterostome and protostome. It is an essential question to know what kinds of genes had existed in

the CNS of the common ancestor. To answer this question, we took a comparative approach using different species, focusing on one of the lower bilateral animals, planarian (Platyhelminthes, Tricladida) which is known to possess the CNS. We determined the nucleotide sequence of expressed sequence tags (ESTs) from planarians, obtaining 3,101 non-redundant EST clones. As a result of homology search, we found that 116 clones had significant similarity to the genes related to the nervous system. Here, we conducted a comparative analysis using these planarian 116 EST clones with all ORFs of the complete genome sequences of human, *D. melanogaster* and *C. elegans*, showing 100%, 97% and 95% of the evolutionary conservation of these nervous system-related genes, respectively. Moreover, we found that about 30% of planarian neural-related genes had homologous sequences in *A. thaliana* and *S. cerevisiae*, implying that the origin of neural-related genes was much older than the emergence of the nervous system. Our results provide the first evidence of an extreme extent of the conservation of the CNS at the molecular level during a long-term evolution.

(4) Identification of Neurotransmitter Receptor Genes under Significantly Relaxed Selective Constraint by Orthologous Gene Comparisons between Humans and Rodents

Hisakazu IWAMA and Takashi GOJOBORI

Neurotransmitter receptors (neuroreceptors) are classified into two types, G-protein coupled receptors (GPCRs) and ligand-gated ion channels (LGICs). The former occupies a small part of the large GPCR superfamily, whereas the latter consists of three superfamilies. In these superfamilies, humans and rodents share almost the same set of neuroreceptor genes. This neuroreceptor gene set is good material to examine the degree of selective constraint exerted on each member gene of a given superfamily. If there are any neuroreceptor genes under the degree of selective constraint that is very different from that of the other member genes, they may have influenced the functional features characteristic of human neural activities. With the aim of identifying such neuroreceptor genes, we collected sequence data of orthologous neuroreceptor genes for humans, mice and rats by database searches. This data set included orthologue pairs for 141 kinds of neuroreceptor genes, which covered almost the whole set of neuroreceptor genes known to be expressed in the human brain. The degree of selective constraint was estimated by computing the ratio (d_N/d_S) of the number of nonsynonymous substitutions to that of synonymous substitutions. We found that the d_N/d_S ratio ranged widely and its distribution fitted a gamma distribution. In particular, we found that four neuroreceptor genes are under the significantly relaxed selective constraint. They are an ionotropic glutamate receptor subunit NMDA-2C, two $GABA_A$ receptor subunits, *i.e.* $GABA_A$-ε and $GABA_A$-θ, and a dopamine receptor D_4. Interestingly, these neuroreceptors have been reported to be associated with cognitive abilities such as memory and learning, and responsiveness to novel stimuli. These cognitive abilities can influence the behavioral features of an individual. Thus, it suggests that the relaxed-constraint neuroreceptor genes have evolved, assuring that the nervous system responds to a variety of stimuli with proper flexibility.

(5) Simulating interaction among multiple gene expression with GASS (Genetic Algorithm for S-System) model

Takaho ENDO and Takashi GOJOBORI

DNA microarray technology and EST profiling provide us a great amount of information about gene expression and some of them trail expression change along time. However, it is hardly possible to predict expression network from the data because of the amount of data themselves. We adopted S-System to describe gene network from observed data. S-System is one of the model to simulate chemical reaction and requires optimization of $2n(n+1)$ parameters for n molecules. The number of n should be more than 100 for expressed genes and it should be hard to solve the because of the number of parameters. Therefore we programmed parallel processing program running on supercomputer Supernig with 128CPUs (the program uses 64 of them) and the program optimizes parameters by GA(Genetic Algorithm) to save the calculation time within realistic period. This S-System simulating program with GA is named GASS model. We used ascidian larval expression data to evaluate how well GASS model makes the profile of the genes and the result was sufficient one. We would adopt GASS to other public DNA microarray data and would like to

predict unknown interaction pathway among genes.

(6) Identification of apoptotic machinery in lower metazoan phylum, platyhelminth

Jung-Shan HWANG, Kazuho IKEO and Takashi GOJOBORI

The freshwater flatworm, planarian belongs to the phylum platyhelminth and it is phylogenetically placed at the base of the lineage of protostomes and deuterostomes as according to the 18S ribosomal sequences analysis. Planarian is also well known by its regenerative ability in which piece of its body part can be regrown into an entire body. Regeneration bears some similarities to embryonic development in that most basic processes such as cell proliferation and differentiation, axial polarity, and patterning of structures occur in both phenomena. Our study concerns the identification of programmed cell-death in the lower metazoan such as planarian and the conservation of this process in the evolutionary development. It has been reported that two apoptosis-related genes, caspase and Bcl-2 homologues, were identified in two lower metazoan organisms, hydra and sponge, respectively. Nevertheless, the involvement of apoptosis during morphogenesis has not yet been documented in both cases and here we carried out an approach to study how apoptosis taking part during planarian regeneration and the conservation of this machinery. In order to show apoptosis occurring in planarian, planarian was cultured in the presence of colchicine and then dissociated cells were examined by TUNEL assay. Signals due to DNA fragmentation were clearly observed under the fluorescent microscopy. Four full-length clones were isolated from a planarian cDNA library, three are caspase homologues (Dj-cas1, Dj-cas2 and Dj-cas3) and one is Bcl-2 homologue (Dj-blg). Sequence alignment showed that Dj-cas1, 2 and 3 contain conserved amino acids residues in the CASc domain (large subunit) and phylogenetic analysis indicated that all three clones are clustered with caspase 3, 6 and 7. Similarly, two conserved domains (BH2 and 3) were identified in Dj-blg and they were shown phylogenetically closer to the pro-apoptotic Bcl-2 family member. Whole mount *in situ* hybridization was carried out to the 2-hour, 1-day, 3-day, -5-day, 7-day amputated heads, tails and intact body. Expressions of Dj-cas2 and 3 (expression of Dj-cas1 is low) were observed at the blastema-forming region of head piece from three to seven days after cutting, while their expressions in tail piece were not specific. Surprisingly, Dj-cas 2 and 3 were also expressed in the brain region and these results were further confirmed by the *in situ* hybridization on paraffin section. Whole mount *in situ* hybridization detected the expression of Dj-blg in the stump region, not in the blastema of the head piece at 5-7 days of regeneration, while its expression in the tail piece is specifically found at the region nearby intestine duct at 3 days of regeneration. The present studies provide evidence that the process of cell death plays a critical role during the regeneration of planarian and in fact the whole apoptotic pathway may have well developed and conserved back to the earliest multicellular animals.

(7) *In Silico* Chromosome Staining: Reconstruction of Giemsa Bands from the Whole Human Genome Sequence

Yoshihito NIIMURA and Takashi GOJOBORI

Giemsa staining has been used for identifying individual human chromosomes. Giemsa-dark (G) and -light (R) bands are generally thought to correspond to GC-poor and GC-rich regions; however, several experiments showed that the correspondence is quite poor. To elucidate the precise relationship between GC content and Giemsa banding patterns, we developed an "*in silico* chromosome staining" method for reconstructing Giemsa bands computationally from the whole human genome sequence. Here we show, 850-level Giemsa bands are best correlated with the difference in GC content between a local window of 2.5 Mb and a regional window of 9.3 Mb along a chromosome. The correlations are of strong statistical significance for almost all of 43 chromosomal arms. Our results clearly show that G bands are *locally* GC-poor regions compared with the flanking regions. These findings are consistent with the model that matrix-associated regions (MARs), which are known to be AT-rich, are present more densely in G bands than in R bands.

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I-b. Laboratory for Gene-Product Informatics

(1) GTOP: a database of protein structures predicted from genome sequences

Takeshi KAWABATA, Satoshi FUKUCHI, Keiichi HOMMA, Motonori OTA, Jiro ARAKI[1], Takehiko ITO[1], Nobuyuki ICHIYOSHI[1] and Ken NISHIKAWA ([1]Mitsubishi Res. Ins.)

Large-scale genome projects generate an unprecedented number of protein sequences, most of them are experimentally uncharacterized. Predicting the 3D structures of sequences provides important clues as to their functions. We constructed the Genomes TO Protein structures and functions (GTOP) database, containing protein fold predictions of a huge number of sequences. Predictions are mainly carried out with the homology search program PSI-BLAST, currently the most popular among high-sensitivity profile search methods. GTOP also includes the results of other analyses, e.g. homology and motif search, detection of transmembrane helices and repetitive sequences. We have completed analyzing the sequences of 41 organisms, with the number of proteins exceeding 120000 in total. GTOP uses a graphical viewer to present the analytical results of each ORF in one page in a "color-bar" format. The assigned 3D structures are presented by Chime plug-in or RasMol. The binding sites of ligands are also included, providing functional information. The GTOP server is available at:
http://spock.genes.nig.ac.jp/~genome/gtop.html.
See Ref. 8 for details.

(2) Comparison of energy components of proteins from thermophilic and mesophilic organisms

Akira R. KINJO and Ken NISHIKAWA

In order to infer the energetic determinants of thermophilic proteins, molecular mechanics calculations were applied to five proteins from thermophilic eubacteria and their mesophilic homologs. The energy function includes a hydration term as well as the electrostatic contribution from the solvent in addition to the usual conformational energy terms. We calculated energy values for three different states of each protein: the native, near-native, and unfolded structures. The energy difference and its components between pairs of these states were compared. The hypothetical near-native structures have almost the same backbone conformation as the native structure but with largely distorted side-chain packing, thus enabling us to extract the energy components important for stabilizing the native backbone topology itself, irrespective of structural details. It was found that the sum of the electrostatic and hydration energies, although of large positive values, were consistently lower for the thermophilic proteins than for their mesophilic counterparts. This trend was observed in the energy difference not only between the native and unfolded structures, but also between the near-native and unfolded structures. In contrast, the energy components regarding side-chain packing did not show any clear tendency. These results suggest that the thermophilic proteins are stabilized so that the precise packing of the native structure does not significantly affect the stability. Implications of this conclusion are also discussed. See Ref. 2 for details.

(3) Protein Surface Amino Acid Compositions Distinctively Differ Between Thermophilic and Mesophilic Bacteria

Satoshi FUKUCHI and Ken NISHIKAWA

One of the well-known observations of proteins from thermophilic bacteria is the bias of the amino acid composition in which charged residues are present in large numbers, and polar residues are scarce. On the other hand, it has been reported that the molecular surfaces of proteins are adapted to their subcellular locations, in terms of the amino acid composition. Thus, it would be reasonable to expect that the differences in the amino acid compositions between proteins of thermophilic and mesophilic bacteria would be much greater on the protein surface than in the interior. We performed systematic comparisons between proteins from thermophilic bacteria and mesophilic bacteria, in terms of the amino acid composition of the protein surface and the interior, as well as the entire amino acid chains, by using sequence information from the genome projects. The biased amino acid composition of thermophilic proteins was confirmed, and the differences from those of mesophilic proteins were most obvious in the compositions of the protein surface. In contrast to the surface composition, the interior composition was not distinctive between the thermophilic and mesophilic proteins. The frequency of the amino acid pairs that are closely located in the space was also analyzed to show the same trend of the single amino acid compositions. Interestingly, extracellular proteins from mesophilic bacteria showed an inverse trend against thermophilic proteins (i.e. a reduced number of charged residues and rich in polar residues). Nuclear proteins from eukaryotes, which are known to be abundant in positive charges, showed different compositions as a whole from the thermophiles. These results suggest that the bias of the amino acid composition of thermophilic proteins is due to the residues on the protein surfaces, which may be constrained by the extreme environment. See Ref. 3 for details.

(4) Knowledge-based potential defined for a rotamer library to design protein sequences

Motonori OTA, Yasuhiro ISOGAI[1] and Ken NISHIKAWA
([1]RIKEN at Wako)

A knowledge-based potential for a rotamer library was developed to design protein sequences. Protein side-chain conformations are represented by 56 templates. Each of their fitness to a given structural site-environment is evaluated by a combined function of the three knowledge-based terms, i.e. two-body side-chain packing, one-body hydration and local conformation. The number of matches between the native sequence and the structural site-environment in the database and that of the virtually settled mismatches, counted in advance, were transformed into the energy scores. In the best-14 test (assessment for the reproduction ability of the native rotamer on its structural site within a quarter of 56 fitness rank positions), the structural stability analysis on mutants of human and T4 lysozymes and the inverse-folding search by a structure profile against the sequence database, this function performs better than the

function deduced with the conventional normalization and our previously developed function. Targeting various structural motifs, *de novo* sequence design was conducted with the function. The sequences thus obtained exhibit reasonable molecular masses and hydrophobic/ hydrophilic patterns similar to the native sequences of the target and act as if they were the homologs to the target proteins in BLASTP search. This significant improvement is discussed in terms of the reference state for normalization and the crucial role of short-range repulsion to prohibit residue bumps. See Ref. 4 for details.

(5) X-Ray Crystalline Structures of Pyrrolidone Carboxyl Peptidase from a Hyperthermophile, *Pyrococcus furiosus*, and Its Cys-Free Mutant

Hideaki TANAKA[1], Masanobu CHINAMI[2], Tsunehiro MIZUSHIMA[1], Kyoko OGASAHARA[1], Motonori OTA, Tomitake TSUKIHARA[1] and Katsuhide YUTANI[1] ([1]Ins. Protein Res., Osaka Univ., [2]Kyushu Women's Univ.)

In order to elucidate the mechanism of the thermostability of proteins from hyperthermophiles, X-ray crystalline structures of pyrrolidone carboxyl peptidase from a hyperthermophile, *Pyrococcus furiosus* (*Pf*PCP), and its mutant protein with Ser substituted at Cys142 and Cys188 were determined at 2.2 and 2.7 A resolution, respectively. The obtained structures were compared with those previously reported for pyrrolidone carboxyl peptidases from a hyperthermophilie, *Thermococcus litoralis* (*Tl*PCP), and from a mesophile, *Bacillus amyloliquefaciens* (*Ba*PCP). The *Pf*PCP structure is a tetramer of four identical subunits similar to that of the *Tl*PCP and *Ba*PCP. The largest structural changes among the three PCPs were detected in the C-terminal protrusion, which interacts with that of another subunit. A comparison of the three structures indicated that the high stability of *Pf*PCP is caused by increases in hydrophobic interactions and hydrogen bonds, the formation of an intersubunit ion-pair network, and improvement to an ideal conformation. On the basis of the structures of the three proteins, it can be concluded that *Pf*PCP does not have any special factors responsible for its extremely high stability and that the conformational structure of *Pf*PCP is superior in its combination of positive and negative stabilizing factors compared with *Ba* PCP. See Ref. 5 for details.

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2. Kinjo, A. and Nishikawa, K.: Comparison of energy components of proteins from thermophilic and mesophilic organisms. *Eur. Biophys. J.*, **30**, 378-384, 2001.

3. Fukuchi, S. and Nishikawa, K.: Protein surface amino-acid compositions distinctively differ between thermophilic and mesophilic bacteria. *J. Mol. Biol.*, **309**, 835-843, 2001.

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8. Kawabata, T., Fukuchi, S., Homma, K., Ota, M., Araki, J., Ito, T., Ichiyoshi, N. and Nishikawa, K.: GTOP: a database of protein structures predicted from genome sequences. *Nucl. Acids Res*, **30**, 294-298, 2002.

I-c. Laboratory for Gene Function Research

(1) Evolution of the MHC class I genome region in man and chimpanzee

Y. TATENO, K. FUKAMI-KOBAYASHI, H. INOKO[1] and M.

YAMAZAKI[2] ([1]Tokai University, [2]Fujiya Bioscience Institute)

MHC class I genome region contains not only MHC genes such as A, B, C and others but also MHC related genes such as MICA and MICB and repetitive sequences such as LINEs and SINEs. As well known, this region is remarkably dynamic in view of evolution. We can observe almost every evolutionary event in this region; mutation, selection, transposition, duplication, deletion and insertion. We have paid special attention to evolutionary aspects of the B and C loci and MICA and MICB loci. Our first attempt is to elucidate the evolutionary origin of the B and C loci and that of MICA and MICB. The two pairs were each produced by genome fragment duplication. To implement our attempt we first sequenced the MHC class I regions of man and chimpanzee. We have then confirmed that chimpanzee has similar genome structures of the B and C loci to those of man. Interestingly, however, chimpanzee lacks the pair of MICA and MICB. Instead, it has fused one that was reformed by deletion of a region between the MICA and MICB loci. We are now analyzing the two MHC class I genome sequences paying attention to LINEs included together in the duplicate genome fragments. Those repetitive sequences are fragmentary and thus considered to have evolved by neutral mutation.

(2) Parallel evolution of ligand specificity between LacI/GalR family repressors and periplasmic sugar-binding proteins

K. FUKAMI-KOBAYASHI, Y. TATENO and K. NISHIKAWA

The bacterial LacI/GalR family repressors such as lactose operon repressor (LacI), purine nucleotide synthesis repressor (PurR) and trehalose operon repressor (TreR) consist of not only the N-terminal helix-turn-helix DNA-binding domain but also the C-terminal ligand binding domain that is structurally homologous to periplasmic sugar-binding proteins. These structural features imply that the repressor family evolved by acquiring the DNA-binding domain in the N-terminal of an ancestral periplasmic binding protein (PBP). Phylogenetic analysis of the LacI/GalR family repressors and their PBP homologues revealed that the acquirement of the DNA-binding domain occurred first in the family, and then ligand specificity evolved. The phylogenetic tree also indicates that the acquirement has occurred only once before the divergence of the major lineages of eubacteria, and that the LacI/GalR and the PBP families have undergone extensive gene duplication/loss along the evolutionary lineages. Multiple alignment of the repressors and PBPs furthermore revealed that repressors and PBPs with the same ligand specificity have the same or similar residues in their binding sites. The result, together with the phylogenetic relationship, elucidated that the repressors and the PBPs independently acquired the same ligand specificity by homoplasious replacement, even though their genes are encoded in the same operon.

Publications

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4. Fukami-Kobayashi, K. and Nishikawa, K.: Parallel evolution at molecular level: Phylogenetic analysis of the LacI/GalR family repressors and their PBP homologues. *Genes and Genetic Systems* **76**, 439, 2001.

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I-d. Laboratory for Research and Development of Biological Databases

(1) Research and Development for DDBJ

1) Introduction of XML and enhancement of processing massive data

Satoru MIYAZAKI and Hideaki SUGAWARA

It is necessary for bioinformatics to integrate multiple information resources such as databases and analytical tools. Thus the interoperability is one of key issues in bioinformatics. We have applied XML technology to DDBJ entries and find that the technology is useful to improve the interoperability. We name the XML DDBJ-XML. Each DDBJ entry and the whole DDBJ entries are now available in the XML format

Last year, we could process human draft sequences by use of the mass submission system (MSS) and TSUNAMI system. Even after the completion of human genome draft sequencing, the size of the International Nucleotide Sequence Database (INSD) expanded at the rate of 1.7 times a year. It exceeded 13 millions entries and 14 Giga base-pairs in October, 2001. Thus we keep upgrading the system for capturing, reviewing and distributing massive data.

2) Expansion of Genome Information Broker (GIB)

Satoru MIYAZAKI, Masaki HIRAHATA and Hideaki SUGAWARA

GIB was originally created for *E. coli* genome for the retrieval and analysis of genomic information in a set. We implemented microbial genome data into GIB whenever genome sequencing was completed and the data is made open to the public. At the GIB Web page (http://www.ddbj.nig.ac.jp/), key word search, homology search, links to DBGET, KEGG and GTOP and visualization of the data are available for 70 organisms as of February 2002. We have utilized XML and CORBA to integrate distributed computational resources in order to cope with the explosion of microbial genome information.

3) Survey on a backbone system for genomics

Hideaki SUGAWARA

DDBJ is a member of the International Nucleotide Sequence Database. Therefore, it is a mandatory task for DDBJ to archive all the nucleotide sequence data that are in public domain. However, it is expected that DDBJ will contribute more to genomics. Thus we carried out survey on a backbone system for genomics and drew up a schema of the system. This task was sponsored by Japan Science and Technology Corporation.

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

1) WDCM

Yumi FUJISAWA, Satoru MIYAZAKI and Hideaki SUGAWARA

WFCC and MIRCEN stand for World Federation for Culture Collections and Microbial Resource Centers network respectively. The laboratory is a host of WDCM that is the data center of WFCC and MIRCEN. We maintain the world directory of 500 culture collections in 60 countries in databases of "CCINFO" and "STRAIN" that are accessible at http://wdcm.nig.ac.jp/. We introduced XML technology into the database and the WDCM Web pages. The Web site now includes on-line system for registration and updating of the database. We also published the World Directory of Collections of Cultures of Microorganisms, and Technical Information Sheet and Guidelines for culture collections in CD-ROM. This activity was sponsored by UNESCO.

2) Development of an e-Workbench for Biological Classification and Identification (InforBIO)

Naoto TANAKA, Satoru MIYAZAKI and Hideaki SUGAWARA

We have developed an e-Workbench named InforBIO. In InforBIO, users are able to integrate databases and analytical tools that are distributed in the Internet including their own resources. We aim at an open system by use of JAVA, XML, and tools of CORBA and a relational database management system in the public domain. We now distribute InforBIO in CD-ROM to get feedback from microbiologists and staffs in culture collections.

(3) Others

1) Biological Resources Centers (BRC)

Hideaki SUGAWARA

The Working Party for Biotechnology (WPB) of OECD set up a task force on BRC in February1999 to

develop a policy guidance to support BRCs. Professor Hideaki Sugawara is the chair of the task force and compiled a report entitled "Biological Resource Centres: Underpinning the future of life sciences and biotechnology". The report calls for actions in OECD countries and beyond. The report and a follow-up task force were approved by WPB on 16 February 2001. He joins the new task force and now chairs a focus group on international linkage.

2) Global Biodiversity Information Facility (GBIF)

Hideaki SUGAWARA

GBIF (http://www.gbif.org/) is a scheme that was created by a Biological Informatics working group in Mega-science Forum of OECD and the GBIF secretariat was established in Copenhagen in 2001. GBIF will improve the accessibility of all kinds of data of all kinds of organisms on the globe and we will make scientific and technical contribution to GBIF.

Publications

1. Goto, K., Miyazaki, S. and Sugawara, H.: Genome Information Broker for Data Retrieval and Comparative Analysis of Microbial Genomes. *Journal of Japan Society of Information and Knowledge* **10**, 4-13, 2001.

2. Miyazaki, S. and Sugawara, H.: Visualization of features in the Flat File by use of DDBJ-XML. *Currents in computational molecular biology*, 249-250, 2001.

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J. RADIOISOTOPE CENTER

(1) Radioisotope Center

Yoshiharu YAMAICHI, Katsunori YATA and Hironori NIKI

Mechanisms regulating the partitioning of the pokaryotic genome. We are studying the proteins and the DNA sites responsible for the regulation of pokaryotic DNA segregation using a combination of genetic, molecular, biochemical, cell-biological, and genomic approaches in *Escherichia coli*. Prokaryotes are not known to have a eukaryotic-like mitotic apparatus, and little is known about the mechanisms controlling chromosome partitioning. We visualized bacterial chromosome DNA and plasmid DNA in cells using fluorescence in situ hybridization (FISH) during the cell-division cycle. We have revealed the dynamic migration patterns of replication origin and terminus on the chromosome during active partitioning of daughter chromosomes.

Current work focuses on identifying the chromosomal segments involved in positioning and migration of the chromosomal domains. To identify functional region for bipolar migration of the Ori domain, we constructed a series of new mutants including a circular chromosome was split into two circular chromosomes. The chromosome-split mutants in which 220 kb of the chromosomal region [84.7-89.4 min] in the Ori domain is separated from the original chromosome showed irregular localization of nuceloids and were defective in bipolar migration of *oriC* segments. The results suggest that a *cis*-acting DNA site for chromosome positioning/ migration is located on this chromosomal region.

Furthermore, we are investigating the segregation of plasmid with the ParABC partitioning system. We have revealed that altered membrane structure affects the localization or activity of a putative plasmid partitioning apparatus located at positions equivalent to 1/4 and 3/4 of the cell length. We hope elucidating the mechanism of position recognition by the Par proteins provide key insight into how to determine middle site in bacterial cells.

Publication

1. Inagawa, T., Kato, J., Niki, H., Karata, K., Ogura, T.: Defective plasmid partition in *ftsH* mutants of *Escherichia coli*. *Mol Genet Genomics*. **265**, 755-762, 2001.

K. EXPERIMENTAL FARM

(1) Development of new experimental lines and reevaluation of the genetic stocks of rice

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

Production, evaluation and maintenance of rice genetic stocks and research works employing such resources carried out here are cooperative works between the experimental farm and the plant genetics laboratory. For details, see the reports of plant genetics lab.

In brief, we conducted reproduction and distribution of rice genetic stocks of over 5,000 wild and cultivated strains. Information about these strains can be accessed at the web site of "Oryzabase", which is a comprehensive rice genome resources database at http://www.shigen.nig.ac.jp/rice/oryzabase/index.html. From 1998, Yukihiro ITO (Plant Genetics Lab.) and we have been developing a new rice genetic stock of enhancer trap lines aiming to generate over ten thousands lines, then to characterize, propagate and distribute them as rice resources for functional genomics.

Addition of new information and revision of the "Oryzabase" will be performed occasionally to include more useful information for rice basic research under the guidance of the rice genetic resources committee in Japan (chair :N. Kurata).

Other works are more or less depending on the experiments using plant resources; e.g. many numbers of transgenic rice, chemically induced mutant strains, strains of insertional mutagenesis and so on. These materials grown in the greenhouses and the exprimental farm are indispensable for plant genetics and genomics.

Publications

1. Nonomura, K-I. and Kurata, N.: The centromere composition of multiple repetitive sequences on rice chromosome 5. *Chromosoma*, **110**, 284-291, 2001.

2. Ito, Y., Eiguchi, M. and Kurata, N.: KNOX homeobox genes are sufficient in maintaining cultured cells in an undifferentiated state in rice. *Genesis*, **30**, 231-238, 2001.

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6. Nonomura, K-I., Miyoshi, K., Eiguchi, M., Miyao, A., Hirochika, H. and Kurata, N.: *acd1* mutant aberrant for archesporial cell division in the anther and ovule of rice. *Rice Genet. Newsl.* **18** (in press).

7. Miyoshi, K., Kurata, N. and Nonomura, K.I.: Detection of the pre-meiotic DNA synthesis in pollen mother cells by immunofluorescence technique in rice. *Rice Genet. Newsl.* **18** (in press).

8. Kurata, N., Nonomura, K-I. and Harushima, Y.: Rice genome organization focusing on centromere and genome interaction studies. *Annals of Botany* (in press).

9. Kurata, N. and Fukui, K.: Chromosome research in genus *Oryza*. in Monograph in genus Oryza. *Nanda JS. Ed, Springer* (in press).

10. Ito, Y., Hirochika, H. and Kurata, N.: Organ specific alternative transcripts of KNOX family class 2 homeobox genes of rice. *Gene* (in press).

ABSTRACTS OF DIARY FOR 2001

Biological Symposium

Jan. 19 Using threading in protein classification and genome analysis (Anna R. PANCHENKO)

Jan. 25 Signal transduction and developmental strategies in Drosophila hematopoiesis and neural development (Utpal BANERJEE)

Jan. 29 The evolution of gene structure (Walter GILBERT)

Feb. 1 DNA damage checkpoints and replication controls in budding yeast (Marco FOIANI)

Feb. 9 Gene silencing by RNA in Drosophila: mechanism and applications (Richard CARTHEW)

Feb. 13 Epigenetics and the plant genome (Robert MARTIENSSEN)

Feb. 21 Machine learning with Splines (Young TRUONG)

Feb. 27 Recent advance in DNA polymerases for translesion synthesis. (Haruo OHMORI)

Mar. 5 Mechanisms and consequences of genomic imprinting (Wolf REIK)

Mar. 8 DNA supercoiling and transcription in Escherichia coli-the FIS connection (Andrew TRAVERS)

Mar. 8 Molecular polymorphism of O alleles in five populations of different ethnic origins (Antoine BLANCHER)

Mar. 9 Evolution and dynamics of centromeric histones (Steven HENIKOFF)

Mar. 12 Formation of new genes and new species (Chung-I WU)

Mar. 12 How was the metazoa threshold crossed: the urmetazoa (Werner E. G. MULER)

Mar. 26 The three-dimensional structure of bacteriorhodopsin (light-driven proton pump) and aquaporin-1 (membrane water channel): mechanism of proton transprot. (Kaoru MITSUOKA)

Mar. 26 Knowledge discovery by use of transcriptome analysis (Kousaku OKUBO)

Apr. 5 Using bioinformatics to find restriction enzymes (Richard ROBERTS)

Apr. 10 Regulation of DNA replication in budding yeast (Seiji TANAKA)

Apr. 13 Site specific proteolysis and its regulation in cell death and Alzheimers disease: insights and approaches using Drosophila (Ming GUO)

Apr. 25 Molecular genetic analysis using Arabidopsis thaliana and its related species–pollen tube guidance, reproductive isolation, and morphological evolution–(Kentaro SHIMIZU)

May. 14 Development of genome ontologies and information extraction from biological literature (Toshihisa TAKAGI)

May. 17 Glycobiology in the post-genome era (Tatsuro IRIMURA)

May. 28 Dynamics of core histones in living human cells: relationship of nucleosome stability with chromatin activity (Hiroshi KIMURA)

May. 29 Signaling pathways that generate olfactory diversity and odor discrimination in C. elegans (Cori BARGMANN)

June. 6 Using yeast to analyze interactions of proteins, nucleic acids and small molecules (Stanley FIELDS)

June. 8 Gene expression measurement using DNA arrays: present results, future trends (Bertrand R. JORDAN)

June. 11 The hippocampal serotonin Type 1A receptor and its interaction with the membrane (Amitabha CHATTOPADYAY)

June. 14 SOCS-1, a negative regulator of the JAK/STAT pathway, inhibits cell growth and is silenced in hepatocellular carcinoma by DNA methylation (Hirohide YOSHIKAWA)

June. 14 DNA distortion mechanism for transcription activation by a Zinc-responsive gene-regulatory protein ZntR in E.coli (Thomas V. O'ALLORAN)

June. 19 Mechanisms underlying vertebrate endoderm development: analyses with zebrafish mutants. (Yutaka KIKUCHI)

June. 27 The moving landscape of comparative genomics for mammals (Stephen J. O'BRIEN)

July. 2 WNT signals control FGF-dependent limb initiation and AER formation in the chick embryo

(Yasuhiko KAWAKAMI)

July. 3 Regulatory mechanism of the heat shock response: search for the the thermosensors (Takashi YURA)

July. 4 Flies and drugs: studying drug abuse in Drosophila (Ulrike HEBERLEIN)

July. 5 Molecular signaling mechanisms guiding cell migration (Yi RAO)

July. 5 Comparison of the morphological segmentation along the embryonic anterposterior axis and leg proximodistal axis of Drosophila melanogaster (Vincent STEPHANE)

July. 6 The evolution of segmentation and axis formation (Nipam PATEL)

July. 13 A perfect vulva every time: changes in signaling and gene function during nemetode evolution (Ralf SOMMER)

July. 16 Associative information access using DualNAVI--Is it applicable to the navigation of biological information?- (Akihiko TAKANO)

July. 17 Zebrafish mutants reveal a link between pronephric cyst formation and epithelial cell polarity (Tomoko OBARA-ISHIHARA)

July. 24 Computation and annotation of human genome sequence variations (Stephen T. SHERRY)

July. 26 Analysis of genomic regulation of the Dlx3-Dlx7 cluster of mouse (Kenta SUMIYAMA)

July. 30 Chromosome versus genic mechanisms of hybrid sterility in mammals (Pavel M. BORODIN)

Aug. 31 Disease centric rat gene initiative (Peter J. TONELLATO)

Aug. 31 Molecular genetic and evolution of color vision in vertebrates (Shozo YOKOYAMA)

Sep. 3 A molecular mechanism for cells to find their own positions (Hisao HONDA)

Sep. 26 Spectral imaging for single cell biochemistry (Tokuko HARAGUCHI)

Oct. 11 Signaling from the endoplasmic reticulum-toward understanding of the molecular mechanism of the quality control of proteins (Kazutoshi MORI)

Oct. 11 Membrane traffic as a bearer of cell structure and function: Molecular machinery of endosomal system and autophagy (Tamotsu YOSHIMORI)

Oct. 18 Cloning of the human telomerase gene: complete genomic sequence and analysis of tandem repeat polymorphism in intronic regions (Leem SUN-HEE)

Oct. 24 Vertebrate segmentation: the mechanisms underlying boundary formation and intercellular signaling (Yoshiko TAKAHASHI)

Oct. 29 Ran tells cells where their chromosomes are (Iain MATTAJ)

Oct. 30 Epigenetic regulation of redundant genes in polyploid genomes (Jeffrey Z. CHEN)

Nov. 1 Initiation mechanism of DNA replication in hyperthermophilic archae, Pyrococcus (Fujihiko MATSUNAGA)

Nov. 8 Information extraction from molecular biology journal articles (Nigel Collier)

Nov. 12 Genetic complexity (Sydney BRENNER)

Nov. 12 Evolutionary relationships among the yeasts from multigene phylogenetic analyses (Cletus P. KURTZMAN)

Nov. 15 A novel action of axon guidance molecules: semaphorin induces axoplasmic transport and its intracellular signal transduction mechanisms in neuronal cells (Yoshio GOSHIMA)

Nov. 15 Coupling gene expression to flagellar assembly by secretion of anti-sigma factor (Kelly HUGHES)

Nov. 16 Roles of DNA dynamics in adaptation and variation: Changes in DNA supercoiling and DNA double-strand breaks induced by environmental stress (Yasuyuki OGATA)

Nov. 19 Arabidopsis quanitative genomics (Thomas MITCHELL-OLDS)

Nov. 22 Optical and genetic approaches toward understanding spinal circuits in zebrafish (Shin-ichi HIGASHIJIMA)

Nov. 26 Development and its application of a Drosophila gene search system (Toshiro AIGAKI)

Nov. 26 Towards original and exciting imagings (Atsushi MIYAWAKI)

Nov. 26 Evolution of genome size and diversity in microorganisms (Charles G. KURLAND)

Nov. 28 Construction of a genome-wide mutant bank of Drosophila using induced type of RNAi (Ryu UEDA)

Nov. 28 Molecular determinants for birth order identity of nerve cells-a study using Drosophila (Takako

ISSHIKI)

Dec. 3 Genome-wide screening for mutations affecting morphogenesis in small fishes (Makoto SEIKI)

Dec. 5 Molecular basis of cold adaptation-the role of membrane lipids (S. SHIVAJI)

Dec. 7 Regulation of cell migration in Drosophila (Penille RORTH)

Dec. 7 Boundary formation in Drosophila wing development (Stephen COHEN)

Dec. 14 Genetic pathway of eye primordium formation and optic stalk specification in zebrafish (Masaya TAKE-UCHI)

Dec. 17 Role of a cGMP-dependent protein kinase and sensory inputs in the regulation of body size and exploratory behavior of the nematode *C. elegans* (Manabi FUJIWARA)

Dec. 25 Towards understanding of the regulatory mechanisms of perception and processing of environmental signals (Makoto KOBAYASHI)

Dec. 25 Toward understanding transcriptional gene silencing in Arabidopsis (Yoshiki HABU)

Dec. 26 Introduction of a medaka transposon system into zebrafish: development of a new gene transfer/ insertional mutagenesis technology (Koichi KAWAKAMI)

Dec. 26 Fish as a model system to study early development, cell differentiation, morphogenesis and formation of fine brain structure of vertebrates (Kohei HATTA)

FOREIGN VISITORS IN 2001

Jan. 19 Anna R. Panchenko, National Center for Biotechnology Information, NIH.
Jan. 25 Utpal Banerjee, Department of Molecular Cell & Developmental Biology, University of California, Los Angeles
Jan. 29 Walter Gilbert, Dept. of Molecular and Cellular Biology, Harvard University
Feb. 1 Marco Foiani, F.I.R.C. Institute of Molecular Oncology, Milan
Feb. 7 Masazumi Tada, University College London
Feb. 9 Richard Carthew, Department of Biological Sciences, University of Pittsburgh
Feb. 13 Robert Martienssen, Cold Spring Harbor Laboratory
Feb. 21 Young K. Truong, Department of Statistics and Applied Probability, National University of Singapore
Mar. 5 Wolf Reik, Laboratory of Developmental Genetics and Imprinting, The Babraham Institute, Cambridge
Mar. 8 Andrew Travers, MRC Laboratory of Molecular Biology
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Mar. 9 Steven Henikoff, Division of Basic Sciences, Fred Hutchinson Cancer Research Center
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