NATIONAL INSTITUTE FOR BASIC BIOLOGY

岡崎国立共同研究機構

基　礎　生　物　学　研　究　所

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ANNUAL REPORT

1996

The cover photograph indicates the enhanced tolerance to salt stress of seedlings of transgenic *Arapidopsis*, see p. 43.

CONTENTS

INTRODUCTION

We present here the 1996 Annual Report describing the research activities of the National Institute for Basic Biology (NIBB) during the past year. The NIBB is a government supported basic research institute established in 1977. It aims to stimulate and promote the biological sciences, by conducting first-rate research as a center of excellence, and by cooperating with other universities and research organizations. The Institute concentrates on the study of cellular functions, reproduction and development, neuronal and environmental control, and gene expression and regulation in eukaryotic organisms at the molecular level.

During the past year, a new division, Speciation Mechanisms II, was added to the Laboratory of Gene Expression and Regulation and Drs. Ken-ichirou Morohashi and M. Hasebe were appointed as Professor and Associate Professor of the new division, respectively. At the same time, the construction of a new building for the Laboratory of Gene Expression and Regulation has been made efficiently and was completed at the end of 1996. As a result, the Laboratory has been completely established and the number of divisions at the NIBB has now reached 17 in total.

Including the above-mentioned appointments, the turnover of personnel has been high as usual. In April, Drs. Shigeru Iida and Yoshinori Ohsumi were appointed as Professor of the Division of Gene Expression and Regulation I and Professor of the Division of Bioenergetics, respectively. Professor Yoshiki Hotta (University of Tokyo) together with Associate Professor H. Okamoto (Keio University) of the Adjunct Division of Cellular Communication completed their term of office at the end of March and were followed by Professor Ritsu Kamiya (University of Tokyo). Professor Goro Eguchi of the Division of Morphogenesis was appointed as the President of Kumamoto University and left the NIBB in November. Drs. T. Yoshimori, M. Mimuro, M. Maejima (Nagoya University), T. Takai (Osaka University), T. Hattori (Mie University) and H. Wada (Kyushu University) were appointed as Associate Professor of the Division of Bioenergetics, the Division of Gene Expression and Regulation I, the Adjunct Division of Cell Fusion, the Adjunct Division of Developmental Biology and the Adjunct Division of Behavior and Neurobiology, respectively. Associate Professor H. Iida moved to Tokyo Gakugei University. Furthermore, we newly appointed 9 research associates, 6 institute research fellows and 2 research assistants, while 4 research associates and 6 institute research fellows moved to other institutions or positions.

The NIBB plays important roles as a national and international center for biological research and is responsible for conducting research projects in cooperation with research groups of various universities and institutes. As a part of such cooperative activities, the NIBB carried out Special Programs which are currently directed to "Adaptation and Resistance to Environment" and "Trans-differentiation of Tissue Cells." Based on these programs, the NIBB held the 36th and 37th Conferences entitled as "Stress Signaling and Stress Responses in Plants" (organized by Professor Norio Murata) and "Approaches to Gene Function Controlling Developmental Processes" (organized by Professor G. Eguchi), respectively.

In addition, the Institute sponsors symposia and study meetings on current topics at the interdisciplinary level by inviting leading scientists in various related fields, both nationally and internationally. In 1996, an International Symposium "New Prospects of Photobiology and the Future Plan of the Okazaki Large Spectrograph" was held at the NIBB, sponsored by the Ministry of Education, Science, Sports and Culture. The NIBB also provided a training course in bioscience for young investigators and participated in the International Science School for high school students of Asian-Pacific countries.

At the beginning of October, the Director-General together with Professor N. Murata visited Canberra to sign a "Memorandum of Understanding between the NIBB and the Australian National University" in order to promote international cooperation between the two institutions. In turn, in October and November, Professor Howard A. Bern of the University of California, Berkeley, and Professor C. Barry Osmond of the Australian National University visited Okazaki to conduct a peer review of the activities and the future plan of the NIBB. We welcome any suggestion and

criticism concerning research activities of the NIBB from the outside.

Finally, I would like to congratulate Professor I. Takeuchi, the President of Okazaki National Research Institutes and the former Director-General of the NIBB, for being awarded the Minakata Kumagusu Prize for his pioneer work in developmental biology of the cellular slime mold. Professor (adjunct) M. Takeichi was awarded the Japan Academy Prize and the Uehara Prize for his excellent work on cadherins. In addition, Dr. H. Mohri was awarded a Purple Ribbon Medal for his contribution to reproductive biology.

Hideo Mohri, D. Sc.
Director-General

ORGANIZATION OF THE INSTITUTE

The National Institute for Basic Biology, NIBB is a part of the Okazaki National Research Institutes (ONRI) located on a hill overlooking the old town of Okazaki. The NIBB was established in 1977 and its activities are supported by Monbu-sho (the Ministry of Education, Science, Sports and Culture) of Japan. The ONRI are composed of three independent organizations, National Institute for Basic Biology (NIBB), National Institute for Physiological Sciences (NIPS) and Institute for Molecular Science (IMS).

Policy and Decision Making

The Director-General oversees the operation of the Institute assisted by two advisory bodies, the Advisory Council and Steering Council. The Advisory Council is made up of distinguished scholars representing various fields of science and culture, and advises the Director-General on the basic policy of the Institute. The Steering Council is made up of professors of the Institute and an equal number of leading biologists in Japan outside NIBB, and advises the Director-General on the scientific activities of the Institute. The Council advises on faculty appointments and on the Institute's annual budget as well as its future prospect.

Administration

Administration of the Institute is undertaken by the Administration Bureau of the Okazaki National Research Institutes under the direct auspices of the Ministry of Education, Science, Sports and Culture.

Research

The Institute conducts its research programs through three departments and one laboratory organized into 17 divisions.

Each division has its own research project and is staffed by a professor; an associate professor and two research associates. A division forms, in principle, an independent project team. Six of the divisions are for adjunct professorship and are under professors who hold joint appointments with other universities. The adjunct division has resident research associates. The arrangement aims to facilitate exchange in research activities in Japan. The Technical Department manages the activities of research techniques and helps to promote research activities of each division and also to maintain the research resources of the Institute. The Department also undertakes the technical education of its staff.

Research Support Facility

The research support facility of the NIBB consists of the Large Spectrograph Laboratory, Tissue and Cell Culture Laboratory, Computer Laboratory, Plant Culture Laboratory, Plant Cell Culture Laboratory, Experimental Farm, and Laboratory of Stress-Resistant Plants. In addition, seven facilities are operated jointly with the NIPS; they consist of the Radioisotope Facility, Electron Microscope Center, Center for Analytical Instruments, Machine Shop, Laboratory Glassware Facility, Animal Care Facility, and Low-Temperature Facility.

Campus

The Okazaki National Research Institutes covers an area of 150,000 m^2 with four principal buildings. The NIBB's main research building has a floor space of 10,930 m^2. Two-thirds of the space was completed in 1982 and the remaining third in June, 1983. The buildings which house the research support facility were also completed in June, 1983. A building for Laboratory of Gene Expression and Regulation (2,577 m^2) was newly built in December, 1996.

Department/Laboratory	Divisions
Department of Cell Biology	Cell Mechanisms Bioenergetics Cell Proliferation (adjunct) Cell Fusion (adjunct) Cellular Communication (adjunct)
Department of Developmental Biology	Reproductive Biology Cell Differentiation Morphogenesis Developmental Biology (adjunct)
Department of Regulation Biology	Molecular Neurobiology Cellular Regulation Biological Regulation (adjunct) Behavior and Neurobiology (adjunct)
Laboratory of Gene Expression and Regulation	Gene Expression and Regulation I Gene Expression and Regulation II Speciation Mechanisms I Speciation Mechanisms II

GRADUATE PROGRAMS

The NIBB carries out two graduate programs.

1. Graduate University for Advanced Studies

The NIBB constitutes the Department of Molecular Biomechanics in the School of Life Science of the Graduate University for Advanced Studies. The University provides a three year Ph.D. course. Those who have completed a master's course in a university or are qualified at an equivalent or higher level are eligible to apply.

The Department consists of the following Divisions and Fields:

Divisions	Fields
Molecular Cell Biology	Biomolecular Systems Cell Dynamics
Developmental Gene Expression and Regulation	Gene Expression Morphogenesis Transgenic Biology
Regulation Biology	Biological Regulation Biological Information

2. Graduate Student Training Program

Graduate students enrolled in other universities and institutions are trained to conduct research for fixed periods of time under the supervision of NIBB professors.

OFFICE OF DIRECTOR

Director-General: Hideo Mohri
Associate Professor: Shigeru Itoh
Research Associates: Mamoru Mimuro
Katsunori Aizawa (on leave)
Mika Tokumoto

Evolution of photoysnthesis and the mechanism of electron transfer

Shigeru Itoh

We study the evolution of photosyntheis focusing on the mechanism of solar energy conversion. Anoxygenic photosynthesis of bacteria seems to have evolved in the Precambrian Earth just after the evolution of life. Plant-type oxygen-evolving photosynthesis, then, was established in cyanobacteria 3500-2700 million years (Ma) ago and increased atmospheric oxygen, judging from the microfossils in Stromatolites and the oxidized iron sediments. Symbiosis of cyanobacteria inside larger cells seems to have produced first plant about 2000 Ma ago.

What can we study to explore the past? We take three approaches (1) Survey of electron transfer mechanisms in photosyntheic reaction center (RC) pigment-protein complexes. We replaced quinones and chlorophylls inside RC to see what happens. We use the ps-ns laser and ESR spectroscopies at 4-300 K in the quinone-replaced RCs and found that both the plant and bacterial RCs are highly optimized (Iwaki et al, Itoh et al. and Baba et al). The result that was the first evidence of the energy-gap-dependent ultra-rapid electron tunneling in plant, also teaches us the design of prototype RC. (2) Site-directed mutagenesis to construct a prototype of electron transfer protein. We modified a ferredoxin that functions in N2-fixation and found the conserved motif to be indispensable for the required extremely strong reducing power (Saeki et al). (3) Survey of new photosynthesis. We performed comparative studies of photosynthesis in purple, green sulfur, hellio-, cyano-bacteria and plants. Our topic in 1966 was the discovery of bacteria that undergo photosynthesis with Zn-bacteriochlorophyll (Wakao et al). The finding was a surprise by itself, and also questions for the reason of ubiquitous use of Mg-chlorophylls in ever known plant and bacterial photosynthesis. We are now studying another organism that undergoes oxygen-evolving photosynthesis with a completely new type of chlorophyll.

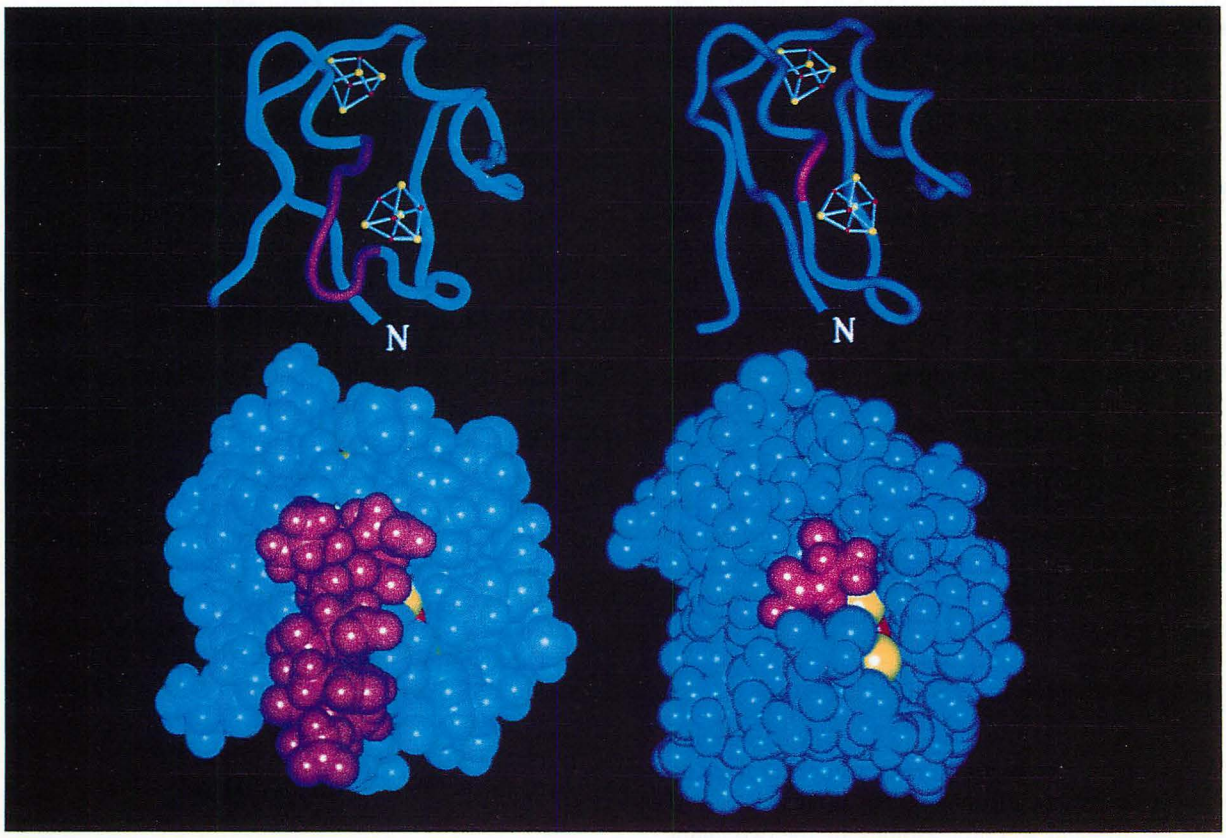

Fig. 1. Space-filling (lower) and wire-frame (upper) models of *R. capsulata* ferredoxin (FdxN). Pink-colored moiety in native FdxN (left) was altered (right) by site-directed mutagenesis. The mutation exposed the Fe-S cluster (yellow and red) and decreased its reducing power.

Primary processes of photosynthesis were investigated from various points of view; phylogeny or systematics to ultrafast spectroscopy

Mamoru Mimuro

The origin of oxygenic photosynthetic organisms is now hypothesized to be symbiosis of two different types of photosynthetic bacteria. However, it is very hard to reproduce process(es) of symbiosis in a laboratory. We started to study this process by using marine phytoplankton, dinoflagellates. We think that the first step of symbiosis is recognition of counterpart for symbiosis, thus we tried to find chemical compound(s) responsible for recognition. We found that some dinoflagellates contain lectin and some showed the lectin-receptor activity (Hori et al.). These compounds might be included in the recognition process.

Chl dimer structure is found both in reaction centers and in antenna. We have already shown that this dimerization induced a systematic decrease in an intensity of magnetic circular dichroism (MCD). We extensively analyzed this phenomenon on bacterial reaction center, green plant reaction center and also bacterial antenna (Kobayashi et al.). Furthermore, we applied this method to artificially formed chlorophyll *a* epimer (so called Chl *a*'). The MCD intensity was also half of that of monomer, indicating that decrease in the MCD intensity is universal phenomenon (Oba et al.). Spectroscopic properties of Chl *a*' dimer indicated a stacked face-to-face dimer structure.

Excited state dynamics of carotenoids were analyzed by the femto-second up-conversion method. Relaxation processes from the second excited singlet state (S_2 state) were shown for the first time to follow the energy gap law of internal conversion (Mimuro et al., J. Am. Chem. Soc. in press). We applied this method to the peridinin-chlorophyll-complex (PCP) isolated from a dinoflagellate, and determined the energy transfer pathway from peridinin to Chl *a*; from the S_1 state of peridinin to the S_1 state of Chl *a* (Akimoto et al.). This is unique in the transfer pathway in algal photosynthesis. We proposed that this transfer pathway is realized by a unique chemical structure of peridinin.

Molecular structure of antenna system in a green photosynthetic bacterium, *Chloroflexus aurantiacus* was proposed; an array of dimer units which are arranged in a spoke-like manner in a rod structure of chlorosomes (Mimuro et al.).

Role of proteasome in meiotic cell division

Mika Tokumoto

All eukaryotic cells, from yeast to human, contain large protease complexes called proteasomes, of which the 20S and 26S proteasomes are the two major types. Recent evidence indicates that proteolysis may play an important role in the regulation of the meiotic and mitotic cell cycles. Inhibitor studies suggest that proteasomes are involved in meiotic maturation of animal oocytes. To investigate the roles of proteasomes in meiotic maturation polyclonal antisera against 20S proteasomes was used to examine changes of components of proteasome during oocyte maturation and early development of *Xenopus laevis*. Although no significant changes in the proteins common to 20S and 26S proteasomes were observed during oocyte maturation and early development, the amount of a unique 48 kDa protein (p48) component of the 26S proteasome decreased during oocyte maturation to the low levels seen in unfertilized eggs. p48 levels remained low in fertilized eggs until after the midblastula transition. These results show that at least one component of 26S proteasomes changes during oocyte maturation and early development, and suggest that alterations in proteasome function may be important for the regulation of developmental events, such as the rapid cell cycles, of the early embryo. Further molecular characteristics of p48 will be required to determine the function of p48.

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DEPARTMENT OF CELL BIOLOGY

Chairman: Mikio Nishimura

The department consists of two regular divisions and three adjunct divisions. One regular division and one adjunct division were started in 1996. The department conducts studies on molecular dynamics of the cell in higher plants and animals such as organelle differentiation, autophagy, cell motility and expression of cell cycle genes and odrant receptor genes.

DIVISION OF CELL MECHANISMS

Professor: Mikio Nishimura
Associate Professor (adjunct): Masayoshi Maeshima
Research Associates: Makoto Hayashi
Ikuko Hara-Nishimura
Tomoo Shimada
JSPS Postdoctoral Fellow: Tetsu Kinoshita
NIBB Fellow: Akira Kato
Graduate Students: Nagako Hiraiwa
Shoji Mano
Hiroshi Hayashi
Kenji Yamada
Daigo Takemoto [1)]
Yuki Tachibe [2)]
Technical Staffs: Maki Kondo
Katsushi Yamaguchi
Monbusho Foreign Scientist: Luz Marina Melgarejo [3)]
Visiting Scientists: Yasuko Koumoto
Miwa Kuroyanagi

[1)] *from Nagoya University*
[2)] *from Hiroshima University*
[3)] *from Colombia National University*

Higher plant cells contain several distinct organelles that play vital roles in cellular physiology. During proliferation and differentiation of the cells, the organelles often undergo dynamic changes. The biogenesis of new organelles may occur, existing organelles may undergo a transformation of function, while other organelles may degenerate. Because the dynamic transformation of organellar function (differentiation of organelles) is responsible for the flexibility of differentiation events in higher plant cells, the elucidation of regulatory mechanisms underlying organelle transformation are currently being studied in this division.

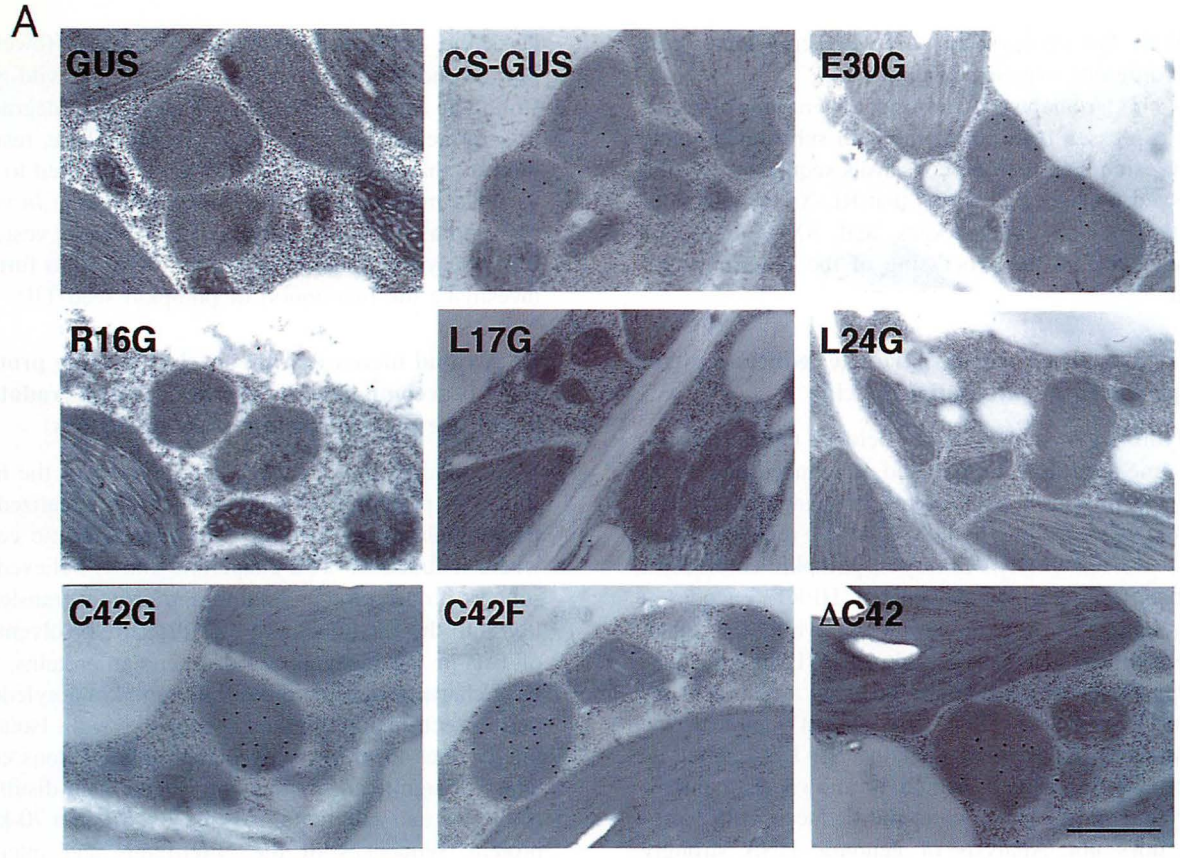
I. Regulation at the level of protein transport to microbodies during the microbody transition

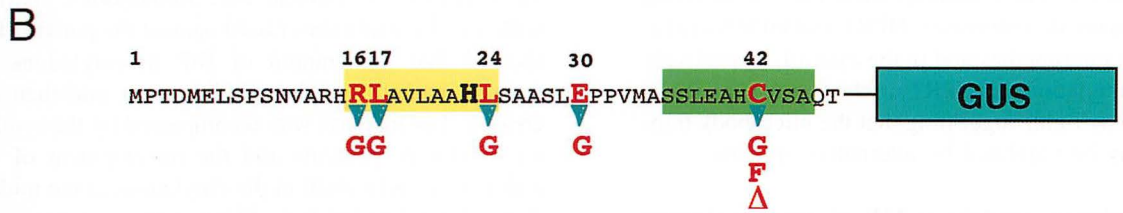
Dramatic metabolic changes which underlie the shift from heterotrophic to autotrophic growth occur in greening associated with seed germination. Accompanying these metabolic changes, many constitutive organelles are functionally transformed. For example, etioplasts differentiate into chloroplasts and mitochondria acquire the ability to oxidize glycine. Glyoxysomes which are microbodies engaged in the degradation of reserve oil via β-oxidation and the glyoxylate cycle, are transformed into leaf peroxisomes that function in several crucial steps of photorespiration. After the functional transition of glyoxysomes to leaf peroxisomes during the greening of pumpkin cotyledons, the reverse microbody transition of leaf peroxisomes to glyoxysomes occurs during senescence. Microbody enzymes function after their transport to microbodies. Since the enzyme compositions and functions of glyoxysomes and leaf peroxisomes differ from each other, it was likely that the two types of microbodies possess different machineries for protein import.

Microbody proteins are synthesized in the cytosol on free polysomes and are transported post-translationally into microbodies. Two types of targeting signals to microbodies have been reported. One type of the targeting signal is part of the mature protein. One such signal, the tripeptide Ser-Lys-Leu at the C-terminal end, has been identified as a targeting signal. Ser-Lys-Leu and related amino acid sequences commonly function in mammals, insects, fungi, and plants. Glyoxysomal enzymes, such as malate synthase and isocitrate lyase, and leaf peroxisomal enzymes, such as glycolate oxidase and hydroxypyruvate reductase, contain the targeting signal at their C-terminal ends.

To characterize the targeting signal, transgenic *Arabidopsis* plants that expressed a fusion protein composed of the C-terminal five amino acids of pumpkin malate synthase and a bacterial protein, β-glucuronidase (GUS) were generated. Immunocytochemical analysis of the transgenic plants revealed that the carboxy-terminal five amino acids of pumpkin malate synthase were sufficient for the transport of the fusion protein into glyoxysomes in etiolated cotyledons, into leaf peroxisomes in green cotyledons and in mature leaves, and into unspecialized microbodies in roots, although the fusion protein was no longer transported into microbodies when SRL at the carboxyl terminus was deleted. Transport of proteins into glyoxysomes and leaf peroxisomes was also observed when the carboxy-terminal amino acids of the fusion protein were changed from SRL to SKL, SRM, ARL or PRL.

A second type of targeting signal involved a cleavable N-terminal sequence. A small group of microbody proteins, such as 3-ketoacyl-coenzyme A (CoA) thiolase, malate dehydrogenase and glyoxysomal citrate synthase, (gCS) are synthesized as precursor proteins with larger molecular masses than those of the mature proteins. Each of these proteins has a cleavable presequence at its N-terminal end. Swinkels and colleagues showed that the N-terminal presequence of 3-ketoacyl-CoA thiolase from rat liver functions as a targeting signal. The N-terminal region of gCS is highly homologous to those of other microbody proteins that are synthesized as larger precursors. There are two conserved sequences in their N-terminal regions. One sequence is RL-X_5-HL, first recognized by de Hoop and Ab. The other sequence, SXLXXAXCXA, is located at the cleavage site of the presequence. Transgenic *Arabidopsis* plants that expressed a fusion protein composed of the N-terminal region of gCS and GUS, were generated and their localization and processing were characterized by immunological and immunocytochemical analyses (Fig. 1). The fusion protein was transported into functionally different microbodies, such as glyoxysomes, leaf peroxisomes and unspecialized microbodies, and was subsequently processed. These observations indicated that the transport of gCS is mediated by its amino-terminal presequence and that the transport system is functional in all plant microbodies. Therefore, it is





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Construct	CS-GUS	R16G	L17G	L24G	E30G	C42G	C42F	ΔC42
Localization	Mb	Cyt	Cyt	Cyt	Mb	Mb	Mb	Mb
Processing	++	−	−	−	+	+	+	−

Mb, microbody; Cyt, cytosol.

Fig. 1. Targeting and processing of the chimeric proteins between N-terminal sequence of glyoxysomal citrate synthase (gCS) and β-glucuronidase (GUS). (A) Localization of the chimeric proteins in transgenic *Arabidopsis* plants analyzed by immunoelectron microscopy. 13-day-old cotyledons were fixed and embedded in LR-white resin. The thin sections were stained with anti-GUS antibodies. Gold particles indicated the localization of the GUS chimeric proteins in microbodies. Bar in panel ΔC42 indicates 1 μm. (B) Construction of the chimeric proteins. The yellow and green boxes show the consensus sequences which were found in the N-terminal region of the microbody proteins that contained the cleavable N-terminal presequences. Mutated amino acids are indicated by red letters. (C) Summary of the localization and processing of the chimeric proteins. Arg-16, Leu-17 and Leu-24 in gCS, which are located in the yellow box, function in the targeting to microbodies, whereas Cys-42 located in the green box functions in the processing of the presequence.

unlikely that glyoxysomes and leaf peroxisomes possess different targeting machineries.

A similar analysis of transgenic *Arabidopsis* plants that expressed fusion proteins with substituted amino acid residues in the two consensus sequences by site-directed mutagenesis shows that RL-X_5-HL functions in targeting to microbodies and SXLXXAXCXA plays a role in the processing of the N-terminal region.

II. Two forms of hydroxypyruvate reductase might be produced by alternative splicing

Hydroxypyruvate reductase belongs to the leaf-peroxisome-specific enzymes and is induced and accumulated in microbodies during greening. Two different cDNAs encoding hydroxypyruvate reductase were isolated from a cDNA library of pumpkin cotyledons. One of the cDNAs, designated HPR1, encodes a polypeptide of 386 amino acids, while the other, HPR2, encodes a polypeptide of 381 amino acids. Although the nucleotide and deduced amino acid sequences of these cDNAs are almost identical, the deduced HPR1 protein contains Ser-Lys-Leu at its carboxy-terminal end, which is known as a microbody-targeting signal, while the deduced HPR2 protein does not. Analysis of genomic DNA strongly suggests that HPR1 and HPR2 are produced by alternative splicing. These findings show that two different hydroxypyruvate reductases, HPR1 and HPR2, are localized in microbodies and in the cytosol, respectively and accumulation of HPR1 in leaf peroxisomes is increased by light, suggesting that the microbody transition may be regulated by alternative splicing.

III. Membrane protein α-TIP of protein-storage vacuoles

The vacuole in the cells of plant seeds shows dramatic changes in its morphology and function during seed maturation through seed germination. To investigate this transition, we prepared protein-body membranes from dry seeds of pumpkin (*Cucurbita* sp.) and characterized their protein components. Five major membrane proteins, designated MP23, MP27, MP28, MP32 and MP73, are located in the protein body membrane of pumpkin seeds. Both MP28 and MP23 belong to the seed TIP (tonoplast intrinsic protein) subfamily. TIP is an integral membrane protein that is found in plant seeds and belongs to the MIP (major intrinsic protein) family. Both MP28 and the 29-kDa precursor to MP23 accumulate on vacuolar membranes before the deposition of storage proteins, and then the precursor is converted to the mature MP23 at the late stage. The two TIPs of pumpkin seeds, pMP23 and MP28, were expressed in yeast cells under control of the *GAL1* promoter, and the subcellular localization of the proteins was analyzed. The pMP23 and MP28 proteins stably accumulated in the yeast vacuolar membrane when the proteins were expressed in the proteinase A-deficient strain (*pep4*), which lacks the activities of vacuolar proteases. However, pMP23 and MP28 did not accumulate in the wild-type strain; the expressed pMP23 and MP28 were degraded in a proteinase A-dependent manner. These results indicate that pMP23 and MP28 are transported to the vacuolar membrane when expressed in yeast. *In vitro* transport assays using the vacuolar membrane vesicles from the yeast transformants will allow us to further investigate the function(s) of pumpkin seed TIP.

IV. A rapid increase in the level of binding protein (BiP) is accompanied by synthesis and degradation of storage proteins in pumpkin cotyledons

The binding protein (BiP) is a member of the heat shock 70 protein (Hsp70) family that is localized to the endoplasmic reticulum (ER) of eukaryotic cells, where it functions as a chaperone and is believed to support proper protein folding and protein translocation into the ER lumen. To elucidate the involvement of BiP in the biosynthesis of vacuolar proteins, we have characterized the protein in pumpkin cotyledons during seed maturation and seedling growth. Isolated microsomes from maturing pumpkin cotyledons contained a significant amount of BiP, protein-disulfide isomerase and calreticulin. We have purified a 70-kDa protein; sequences of the N-terminus and internal fragments of this protein exhibited a high identity to the sequence of soybean BiP. Immunoblot analysis with specific antibodies raised against the purified BiP showed that the amount of BiP in cotyledons increased markedly at the middle stages and then decreased. The increase was accompanied by the synthesis of storage proteins and the development of the endoplasmic reticulum in the cotyledons at the middle stage of seed maturation. Most of these storage proteins degraded dramatically between 2 and 5 days after seed germination, and the degradation was also accompanied by a rapid increase in the level of BiP. Subcellular fractionation of the 4-day-old cotyledons showed a high accumulation of BiP in the endoplasmic reticulum. It is possible that BiP might be involved in the synthesis of seed storage proteins during maturation and in the synthesis of hydrolytic enzymes responsible for the degradation of the storage proteins during seed germination.

V. A vacuolar processing enzyme responsible for conversion of proprotein precursors into their mature forms

Processing enzymes responsible for the maturation of seed proteins belong to a novel group of cysteine proteinases with molecular masses of 37 to 39 kDa. However, the processing enzyme activity can be found not only in seeds but also in vegetative tissues such as hypocotyls, roots and mature leaves. Thus, we designated the enzyme as a vacuolar processing enzyme (VPE). Members of the VPE family can be separated into two subfamilies, one that is specific to seeds and another that is specific to vegetative organs.

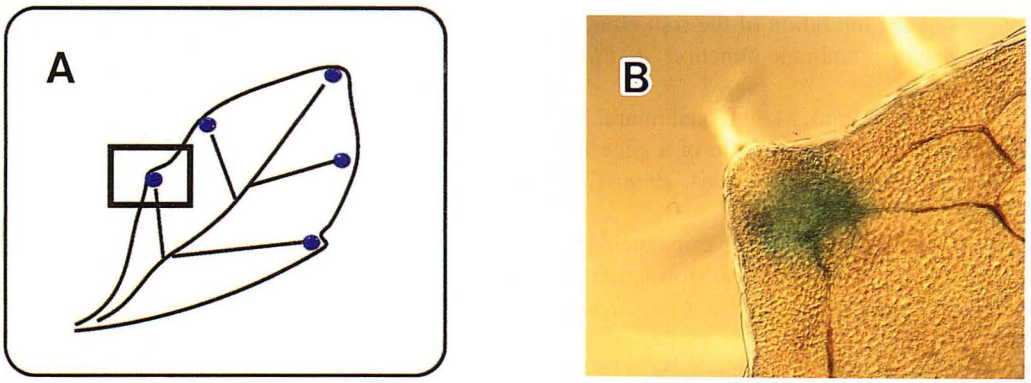

Fig. 2. Histochemical localization of GUS activity in the hydathode of *Arabidopsis* transformed with the γ-VPE promoter-*gus* fusion gene. *Arabidopsis* was transformed with a reporter gene composed of the promoter of the γ-VPE gene and the coding region of β-glucuronidase (GUS). GUS activity was observed in the hydathodes at the ends of veins along with the leaf margins, as shown in (A). Hydathodes are structures for that discharge water from the interior of the leaf to its surface. A photograph of the hydathode with DIC optic of the enclosed region in A is shown in (B).

The molecular characterization of all the members of the VPE family in *Arabidopsis* is required if we are to elucidate the mechanisms of regulation of genes for VPE homologues and the physiological functions of these proteins in protein-storage vacuoles and vegetative vacuoles. We isolated the three genes of VPEs (α-VPE, β-VPE and γ-VPE) from a genomic library of *Arabidopsis*. To demonstrate temporal and spatial expression of the promoters of the VPE genes, we transformed *Arabidopsis* plants with a reporter gene containing the promoter of the VPE genes and the coding region of β-glucuronidase (GUS). The β-VPE gene was expressed in seeds, but α-VPE and γ-VPE were not. The GUS activity for γ-VPE gene was predominatly expressed in the hydathode tissues of leaves (Fig. 2).

VI. Role of molecular chaperones in organelle differentiation

Molecular chaperones are cellular proteins that function in the folding and assembly of certain other polypeptides into oligomeric structures but that are not themselves, components of the final oligomeric structure. To clarify the roles of molecular chaperones on organelle differentiation, we have purified and characterized chaperonin and Hsp70s and analyzed their role in the translocation of proteins into chloroplasts. We isolated a cDNA for the chaperonin 10 homologues from *Arabidopsis thaliana* by functional complementation of the *E. coli groES* mutant. The cDNA was 647 bp long and encoded a polypeptide of 98 amino acids. The deduced amino acid sequence showed approximately 50% identity to mammalian mitochondrial Cpn10s and 30% identity to GroES. A. Northern blot analysis revealed that the mRNA for the Cpn10 homologue was expressed uniformly in various organs and was markedly induced by heat-shock treatment. The Cpn10 homologue was constitutively expressed in transgenic tobaccos. Immunogold and immunoblot analyses following the subcellular fractionation of leaves from transgenic tobaccos revealed that the Cpn10 homologue was localized in mitochondria and accumulated at a high level in transgenic tobaccos.

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DIVISION OF BIOENERGETICS

Professor: Yoshinori Ohsumi
Associate Professor: Tamotsu Yoshimori
Research Associates: Takeshi Noda
Yoshiaki Kamada
Graduate Students: Kanae Shirahama (Univ. of Tokyo)
Tomoko Funakoshi (Univ. of Tokyo)
Satoshi Kametaka (Univ. of Tokyo)
Chikara Tokunaga (Univ. of Tokyo)
Takayoshi Kirisako (Univ. of Tokyo)
Technical Staff: Yukiko Kabeya
Visiting Professor: Michael Filosa

This division aims to elucidate the mechanism and regulation of intracellular protein degradation in a lytic compartment and its physiological function. Recently it was realized that degradation process plays essential role for cellular regulation. In the cytoplasm selective protein degradation takes place by ubiquitin/proteasome system. Short-lived or abnormal proteins are selectively eliminated by this pathway. While vacuole/lysosome contributes to the bulk turnover of cytosolic and organelles proteins. However, little is known about the mechanism of protein degradation in contrast with biosynthesis. Bulk protein degradation is induced by various nutrient starvation condition, which is obligatory to cell differentiation and maintenance of cell viability. Autophagy is a major route for sequestration of proteins to the lytic compartment. Vacuole/lysosome is also the destination for endocytic pathway. Since in 1952 du Duve identified cellular lytic compartment, lysosome, enzymatic characterization and biogenesis of lysosomal enzymes have been studied thoroughly. However, the mechanisms of delivery of proteins to the lysosomes are not known at a molecular level. Dynamism of lysosomal system has been studied mostly with electron microscope. Autophagy is one of the most important problems in cell biology remained to be solved.

Yeast Induces Autophagy as Mammalian Cells

Recently we discovered yeast, *Saccharomyces cerevisiae*, induces bulk protein turnover in the vacuoles under various starvation conditions. This whole process corresponds to the process of macroautophagy in higher eukaryotic cells. By electron microscopic analyses we succeeded to detect double membrane structure enclosing a portion of cytosol in the cytoplasm. These yeast autophagosomes immediately fuse with vacuolar membrane, resulting to deliver a single membrane bound vesicles, autophagic bodies in the vacuoles. When vacuolar proteinase activities are blocked genetically or by specific inhibitor such as PMSF autophagic bodies are accumulated in the vacu-

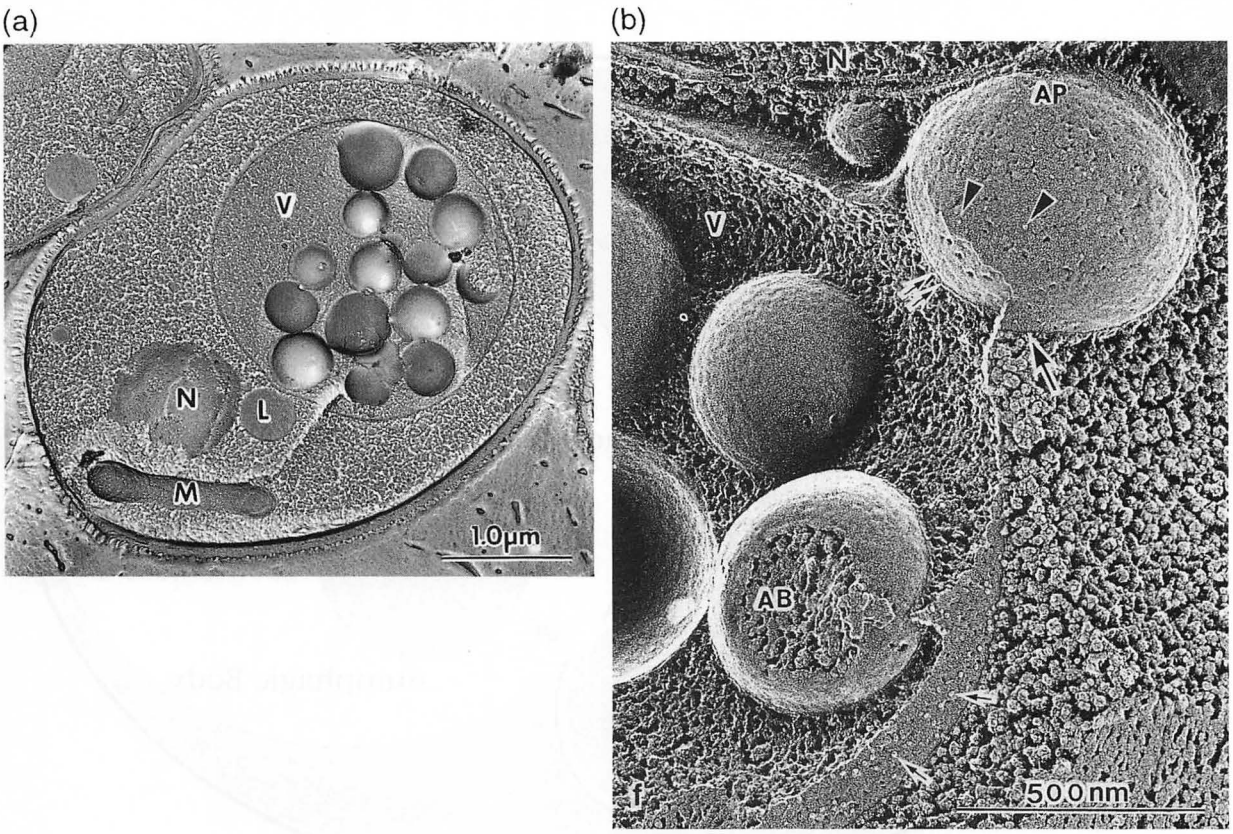

Fig. 1. (a) Freeze-Fracture Image of Yeast under Nitrogen Starvation.
When vacuolar proteinases are blocked, spherical membrane structure, autophagic bodies accumulate in the vacuole.
(b) Fusion of Autophagosome to the Vacuolar Membrane.
Double membrane structure, autophagosome, encloses a portion of the cytosol, then fuses with the vacuole to deliver an autophagic body in the vacuole. These membrane structures show a few intramembrane particles.

oles. They moves around vigorously in the vacuoles by Brownian motion, and are easily observed by light microscope. Thus we can follow the progression of autophagy by the accumulation of autophagic bodies in real time. Biochemical and immunoelectron microscopic analyses of vacuoles containing autophagic bodies showed that starvation-induced sequestration is non-selective, that is, any kind of cytosolic enzymes and organelles are sequestered to the vacuoles to be degraded. Freeze-fracture electron microscopy showed that autophagosomal membrane has characteristic feature and density of intramembrane particles is quite few. The origin of the autophagosomal membrane and mechanism of formation are key problems remained to be solved.

Genetical dissection of autophagic process

Yeast, *S. cerevisiae*, has made great contribution to solve many fundamental problems in cell biology because of tractability of genetic and molecular biological techniques. In order to dissect the complex process of autophagy to its elementary steps we started to isolate mutants in the process of autophagy, taking advantage of morphological selection under light microscope. Total 14 *agp* mutants, defective in the induction of autophagy, were isolated. They cannot induce protein degradation upon shift from growth medium to the starvation medium. But they grow normally in a rich medium, but start to die after 2 days in the starvation medium. This suggests that the autophagy is necessary for long time maintenance of cell viability. Homozygous diploid of each *apg* mutants is sporulation defective as expected.

Analyses of *APG* genes

Now we are focusing on characterization of these *APG* genes, essential for autophagy. This year we have finished cloning of twelve *APG* genes. So far ten *APG* genes have been identified. *APG1* codes a novel Ser/Thr kinase essential for the induction of autophagy. This provides the first direct evidence for involvement of protein phosphorylation in the process of autophagy. So far all genes analyzed are novel and non-essential for vegetative growth. Using the assay system of autophagy we developed, we are now analyzing genetic interaction among *APG* genes. Further molecular biological and biochemical studies on these *APG* gene products might provide important clues to understand the mechanism of autophagy at a molecular level. Now we obtained antibodies against to these *APG* gene products. Intracellular localization of them may give us clues to elucidate the function of these proteins.

Diverse pathways of autophagy

Autophagy is one of the fundamental function of all eukaryotic cells. We found yeast cells induce macro-

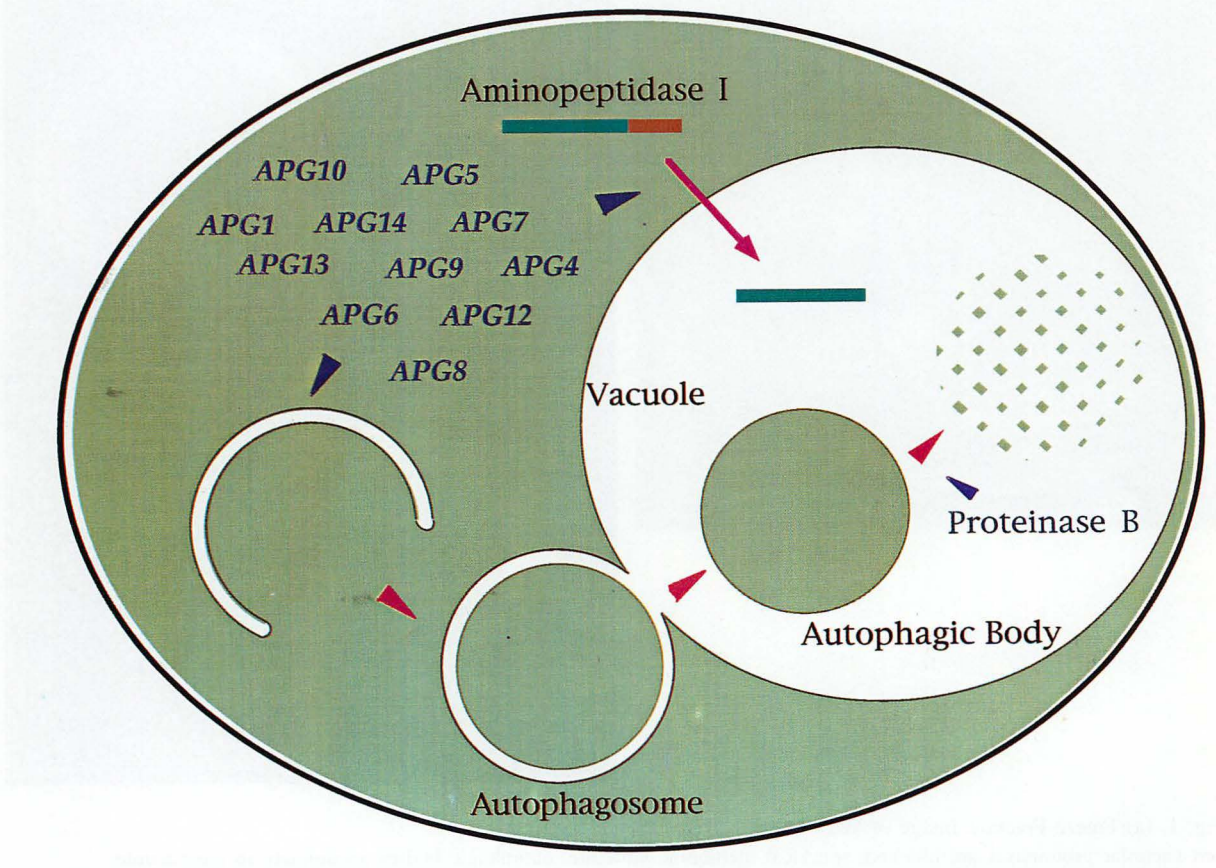

Fig. 2. Scheme of Autophagy in Yeast
All *APG* genes identified are novel and required at or before the formation of autophagosome.

autophagy under starvation, which is a main route for bulk and non-selective protein degradation. However, it is also reported that some enzymes are selectively taken up to vacuoles according to the physiological demands. In methylotrophic yeast, *Pichia pastoris* degrades peroxisomes by invagination of vacuolar membrane, microautophagic process. Further studies will uncover more sophisticated pathways for the degradation in the lysome/vacuoles. Recently it was found that all *APG* genes are reuired for targeting of API from cytosol to the vacuole. It is interest problem how this selective and constitutive process shares machinery with the autophagy.

Perspective

Autophagy is essential for maintenance of cell viability during starvation. Degradation products may provide essential nutrients necessary for minimal protein synthesis, or reduction of certain critical activities in the cytosol may be essential for the maintenance of viability. Basic molecular devices for the membrane dynamics are conserved from yeast to higher eukaryotes. We realized some *APG* genes in yeast show homologues in mammals or higher plants. We are now developing systems of autophagy in mammalian cultured cell.

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DIVISION OF CELL PROLIFERATION (ADJUNCT)

Professor: Masayuki Yamamoto
Research Associate: Masuo Goto
Institute Research Fellow: Takashi Kuromori
Visiting Scientist: Satsuki Okamoto
Graduate Students: Chikako Kitayama (from The University of Tokyo)
Masahiro Fujita (from The University of Tokyo)

The major goal of this Division is to elucidate regulatory mechanisms of meiosis. Meiosis is a crucial step in gamete formation and is essential for sexual reproduction. Meiotic steps are highly conserved among eukaryotic species. We have been screening for genes that may be relevant to the regulation of meiosis in animals and plants, by using transcomplementation methods. In the fission yeast *Schizosaccharomyces pombe*, which is a unicellular eukaryotic microorganism, genes controlling meiosis have been cloned and characterized extensively, and mutants defective in these genes are available. We have isolated animal and plant genes that can functionally complement such *S. pombe* mutants. Analysis of the cloned genes is in progress. In addition, efforts are also paid to in-depth analysis of regulatory mechanisms of meiosis in the fission yeast, to facilitate the above strategy.

I. Genes encoding farnesyl cysteine carboxyl methyltransferase in fission yeast and *Xenopus*

The mating pheromone signaling is essential for the induction of meiosis in *Schizosaccharomyces pombe*. The *S. pombe mam4* mutation causes mating deficiency in cells of mating-type *M* but not in *P. M* cells defective in *mam4* do not secrete active mating pheromone M-factor. We cloned *mam4* by complementation. The *mam4* gene encodes a protein of 236 amino acids with several potential membrane-spanning domains, which is 44% identical with farnesyl cysteine carboxyl methyltransferase encoded by *STE14* and required for the modification of **a**-factor in *Saccharomyces cerevisiae*. Analysis of membrane fractions revealed that *mam4* is responsible for the methyltransferase activity in *S. pombe*. Cells defective in *mam4* produced farnesylated but unmethylated cysteine and small peptides, but no intact M-factor. These observations strongly suggest that the *mam4* gene product is farnesyl cysteine carboxyl methyltransferase that modifies M-factor. Furthermore, transcomplementation of *S. pombe mam4* allowed us to isolate an apparent homologue of *mam4* from *Xenopus laevis* (*Xmam4*). In addition to its sequence similarity to *S. pombe mam4*, the product of *Xmam4* was shown to give a farnesyl cysteine carboxyl methyltransferase activity in *S. pombe* cells. These results will open the way to intensive studies of the role for methylation in a large body of proteins including *ras*-superfamily proteins and its relevance to meiosis. This work will be published shortly (Y. Imai et al. (1997) Mol. Cell. Biol., in press).

II. Phosphorylation of RNA-binding protein controls cell cycle switch from mitotic to meiotic in fission yeast

Little is known about the molecular controls over the switch from mitotic to meiotic cell cycles. In *S. pombe*, the switch from the mitotic to the meiotic cell cycle is controlled by the antagonistic effects of the

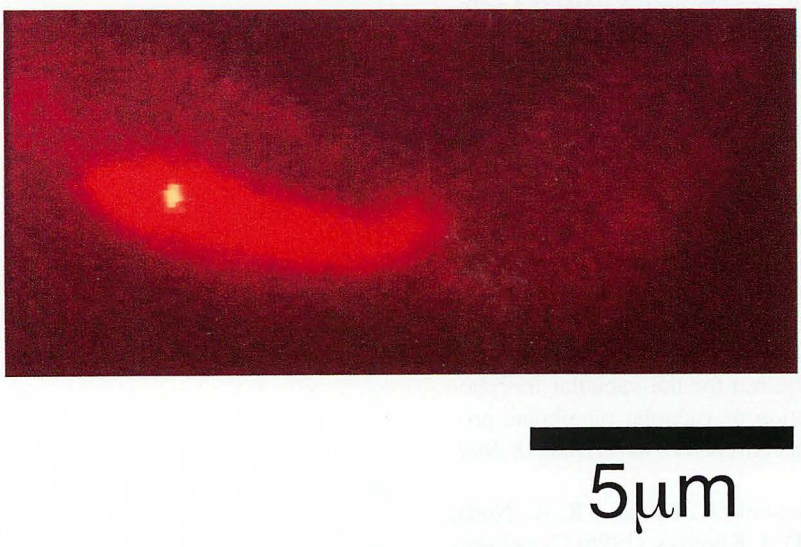

Fig. 1. A "dot" formed by the RNA-binding protein Mei2 in a meiotic prophase nuclei. Mei2 protein fused to GFP, which emits green fluorescence, was live-recorded. The yellow spot represents fluorescence of Mei2-GFP, whereas the red region represents nuclear DNA. The whole cell is faintly visible in red. Prophase nuclei of fission yeast move back and forth, assuming morphology like a horse tail. Mei2 appears to occupy a fixed position in the meiotic nucleus. See Watanabe et al. (Nature 386, 187-190, 1997) for more details. We were assisted by Y. Chikashige and Y. Hiraoka (Kansai Advanced Research Center, Communications Research Laboratory) in taking this photograph.

Pat1 protein kinase and the Mei2 RNA-binding protein. We have shown that Mei2 is phosphorylated on Ser438 and Thr527 by Pat1 kinase, whose inactivation is necessary to induce meiosis. Mutant Mei2 carrying alanine in these two positions induces meiosis without inactivation of Pat1 kinase, establishing that Mei2 is the critical target of Pat1. Mei2 localizes mainly in the cytoplasm of proliferating cells, but is found in a single 'dot', closely opposed to the spindle pole body, in prophase nuclei during meiosis I (Fig. 1). Our results emphasize the crucial role of RNA-binding proteins in the initiation and execution of meiosis. This work has been published lately (Y. Watanabe et al. (1997) Nature 386, 187-190).

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DIVISION OF CELLULAR COMMUNICATION (ADJUNCT)

Professor: Ritsu Kamiya
Associate Professor: to be appointed
Research Associate: to be appointed

The research in this laboratory, started in November, 1996, is aimed at an understanding of the molecular mechanisms that regulate the assembly and function of cytoskeletal proteins. Current research effort is centered on the function of axonemal dyneins, microtubule-based motor proteins that produce force for flagellar beating. The organism employed is *Chlamydomonas*, a biflagellate green alga particularly suited for genetic and molecular biological studies.

A single flagellar axoneme contains at least eleven kinds of dynein heavy chains in inner and outer arms. To understand the specific function of each heavy chain, we have been isolating and characterizing mutants that lack different kinds of axonemal dyneins. Their motility phenotypes have indicated that different dynein species differ in function in a fundamental manner. For example, the outer arm heavy chains are important for flagellar beating at high frequency, whereas most of the inner arm heavy chains are important for producing proper waveforms. In addition, indirect evidence has suggested that the force generation properties differ greatly among different heavy chains. We are currently trying to measure the force production by different dyneins using micro-physiological techniques.

The inner dynein arms are known to contain actin as a subunit. Thus the two independent motility systems of eukaryotes – the actin-based and microtubule-based motility systems – should somehow cooperate in the inner arm, although the function of actin in dynein arms is unknown at present. The mutant *ida5* we isolated as a mutant that lacks four subspecies of inner-arm dynein was recently found to have a mutation in the actin-encoding gene. This mutant as well as another independently isolated mutant (*ida5-t*) has a deletion in the actin gene and express no conventional actin. The cytoplasm and axonemes of these mutants lack the conventional actin entirely but contain two novel proteins which are immunologically distinct from, but related to, actin. Since *Chlamydomonas* has been shown to have only a single copy of actin-encoding gene, it is likely that the two novel proteins in these mutants originate from other gene(s) that codes for an actin-like protein which has hitherto been undetected in wild-type cells. The net growth rate of the mutants did not differ from that of wild type, but the mating efficiency was greatly reduced because of their deficient fertilization tubule growth. These results raise the possibility that *Chlamydomonas* can live without conventional actin.

The discovery of the mutants that lack the conventional actin entirely should open the way to studying the function of actin in the cell as well as in the dynein arm. We have succeeded in transforming the mutant *Chlamydomonas* with cloned actin gene and found that inner dynein arms become restored upon transformation. Studies with artificially mutated actin gene will enable us to determine whether actin is really essential for cytokinesis or other fundamental processes in *Chlamydomonas*, and what functions are carried out by actin in the axoneme and cytoplasm. A most important immediate project will be to characterize the novel proteins found only in the mutants that lack actin.

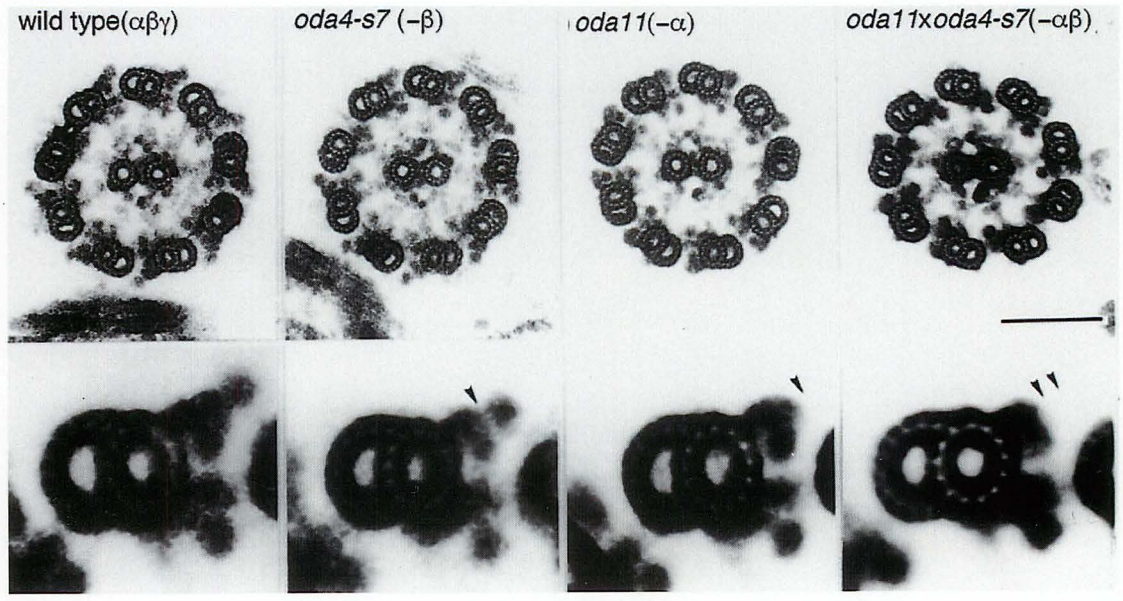

Fig. 1. Wild-type and mutant axonemes of *Chlamydomonas* that lack different heavy chains of outer arm dynein. Wild-type outer arm contains three heavy chains, α, β and γ, of which the mutant *oda4-s7* lacks β and *oda11* lacks α. Lower photographs are the averaged images of the outer doublet microtubules, showing the structural defect in each mutant (arrowhead).

DIVISION OF CELL FUSION (ADJUNCT)

Professor: Hitoshi Sakano
Research Associates: Akio Tsuboi (until November)
Kanae Muraiso (on leave)
Institute Research Fellow: Hiroaki Kasai (until March)
Graduate Student: Nika Yamazaki (from Tokyo Institute of Technology)

Recently, hundreds of odorant receptor (OR) genes have been reported in the olfactory system, although how the expression of this gene system is regulated has yet to be studied. It is assumed that only a limited number of the OR genes (possibly one) are activated in each olfactory neuron. In addition, neurons expressing a given OR gene are known to project their axons to a limited number of glomeruli at the fixed sites on the olfactory bulb.

We have been studying how individual neurons express a limited number of OR genes while keeping the rest of the genes silent, and how neurons expressing a given OR gene project their axons to a few subsets among the 2000 glomeruli within the olfactory bulb. In order to study the selective expression of the OR genes, we have been characterizing a P1 clone containing two highly related genes, MOR10 and MOR28. These genes are 92% homologous within their coding regions and linked in tandem on mouse chromosome 14. *In situ* hybridization has revealed that both genes are expressed in the same spatial zone within the olfactory epithelium, but never expressed simultaneously in the same neuron.

Developmental analyses have revealed that MOR10-expressing neurons appear earlier during embryogenesis than those MOR28-expressing. However, the number of MOR28 neurons is greater than that of MOR10 neurons after birth and continues to increase to a ratio of 2.5:1 by adulthood. These results suggest that individual olfactory neurons may activate OR genes through a stochastic mechanism even between two closely related OR genes; yet this selection appears to be biased in both the onset and level of expression. Interestingly, olfactory neurons expressing either MOR10 or MOR28 project their axons to two distinct, but adjacent subsets of glomeruli on the olfactory bulb. We conclude that two highly homologous and tandemly linked OR genes are expressed in a mutually exclusive manner in individual olfactory neurons, and that neurons expressing either of these genes project their axons to two discrete, but neighboring subsets of glomeruli on the olfactory bulb.

For the study of the mutually exclusive expression of OR genes, we have generated transgenic mice which are devised to express the MOR28 gene in every olfactory neuron under the control of the olfactory marker protein (OMP) promoter. Since the OMP gene is expressed in the mature neurons within the olfactory epithelium, the transgene is expected to be activated in all olfactory neurons. Recently, a transgenic line is found to express the OMP-MOR28 transgene in most of the mature neurons within the olfactory epithelium as well as within the vomeronasal organ. We believe that the study of such transgenic mice will give us new insight into the molecular mechanisms for OR gene expression, as well as for neuronal projection to the olfactory bulb.

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DEPARTMENT OF DEVELOPMENTAL BIOLOGY

Chairman: Yoshiaki Suzuki

Three regular divisions and one adjunct division construct the Department. The department members conduct molecular analysis on various aspects of developmental phenomena; differentiation and maturation of the germ cells, molecular basis of the body plans, and gene regulation in different phases of the cell cycle, in the differentiating tissues, and during changes of the differentiated states.

DIVISION OF REPRODUCTIVE BIOLOGY

Professor: Yoshitaka Nagahama
Associate Professor: Michiyasu Yoshikuni
Research Associates: Minoru Tanaka
Tohru Kobayashi
Institute Research Fellow: Akihiko Yamaguchi
JSPS Postdoctoral Fellows: Yoshinao Katsu
Zuxu Yao
NIBB Postdoctoral Fellows: Kunimasa Suzuki
Won-Kyo Lee
Jain-Quiao Jiang
Xiao-Tian Chang
JSPS Research Associates: Mika Takahashi
Takashi Todo
Daisuke Kobayashi
Graduate Students: Yuichi Ohba (Graduate University for Advanced Studies)
Jun Ding (Graduate University for Advanced Studies)
Masatada Watanabe (Graduate University for Advanced Studies)
Guijun Guan (Graduate University for Advanced Studies)
Monbusho Foreign Scientist: Graham Young (University of Otago)
JSPS Visiting Scientist: Allen Schuetz (University of Maryland)
Visiting Scientist: Craig Morrey (University of Hawaii)

The division of reproductive biology conducts research on the endocrine regulation of differentiation, growth and maturation of germ cells in multicellular animals, using fish as a primary study model.

I. Endocrine regulation of oocyte differentiation, growth and maturation

Our research effort in previous years concentrated on the identification and characterization of the molecules (gonadotropins, gonadal steroid hormones, and cell cycle-regulated molecules) that stimulate and control germ cell growth and maturation. We identified, for the first time in any vertebrate, 17α,20β-dihydroxy-4-pregnen-3-one (17α,20β-DP) as the maturation-inducing hormone of amago salmon (*Oncorhynchus rhodurus*). Along with estradiol-17β, which was identified as the major mediator of oocyte growth, we now have two known biologically important mediators of oocyte growth and maturation in female salmonid fishes. It is established that the granulosa cells are the site of production of these two mediators, but their production by the ovarian follicle depends on the provision of precursor steroids by the thecal cell (two-cell type model). A dramatic switch in the steroidogenic pathway from estradiol-17β to 17α,20β-DP occurs in ovarian follicle cells immediately prior to oocyte maturation. This switch is a prerequisite step for the growing oocyte to enter the maturation phase, and requires a complex and integrated network of gene regulation involving cell-specificity, hormonal regulation, and developmental patterning.

We have isolated and characterized the cDNA encoding several ovarian steroidogenic enzymes of several fish species which are responsible for estradiol-17β and 17α,20β-DP biosynthesis: cholesterol side-chain cleavage cytochrome P450 (P450scc), 3β-hydroxysteroid dehydrogenase (3β-HSD), 17α-hydroxylase/C17,20-lyase cytochrome P450 (P450c17) and P450 aromatase (P450arom). More recently, cDNA clones encoding 20β-hydroxysteroid dehydrogenase (20β-HSD; a critical enzyme which converts 17α-hydroxyprogesterone to 17α,20β-DP) were isolated from cDNA libraries of ayu (*Plecoglossus altivelis*). The amino acid sequence deduced from the isolated cDNA had 276 amino acid residues and shared approximately 60% homology with mammalian carbonyl reductase. The clear lysate, which was prepared from *E. coli* harboring the cDNA, catalyzed the production of 17α,20β-DP. The identification of 17α,20β-DP was confirmed by two dimensional thin layer chromatography, followed by recrystallization. Purification of the *E. coli*-expressed cDNA product revealed that it possessed carbonyl reductase activity and 17α-hydroxyprogesterone, the endogenous immediate precursor of 17α,20β-DP, was a good substrate.

Northern and Western blots revealed a single P450arom mRNA and a single protein in tilapia ovarian tissue respectively. These analyses also revealed that the levels of both P450arom mRNA and protein were low in early vitellogenic follicles, increased in midvitellogenic follicles, and declined to non-detectable levels in postvitellogenic follicles. Changes in the ability of follicles to convert exogenous testosterone to estrogens (aromatase activity) were similar to those of P450arom mRNA and protein. These observations indicated that the capacity of tilapia ovarian follicles to synthesize estradiol-17β is closely related to the contents of P450arom mRNA and protein within them. The mRNA levels of P450scc, 3β-HSD, and P450c17 are barely detected in ovarian follicles during the midvitellogenic stage, and are abundant in follicles during the postvitellogenic stage and oocyte maturation. Our preliminary results indicate that forskolin-induced 17α,20β-DP production is accompanied by a dramatic decrease in P450arom mRNA levels in granulosa cells isolated from postvitellogenic follicles. A 2- to 3-fold increase in P450scc and 3β-HSD mRNAs and a slight decrease in P450c17 mRNA are also observed during forskolin-induced 17α,20β-DP production. Northern hybridization analysis has revealed that 20β-HSD mRNA transcripts are present in fully vitellogenic follicles and increased as oocyte maturation proceeded. Time course studies further suggest that *de novo* synthesis of 20β-HSD *in vitro* in response to gonadotropin and cAMP occurs, and consists of gene transcriptional events within the first 6 hr of exposure to gonadotropin and cAMP and translational events 6-9 hr after the exposure to gonadotropin and cAMP. Thus, these results suggest that gonadotropin causes the *de novo* synthesis of 20β-HSD in the granulosa cell through a mechanism dependent on RNA synthesis.

17α,20β-DP acts via a receptor on the plasma mem-

brane of oocytes. We have identified and characterized a specific 17α,20β-DP receptor from defolliculated oocytes of several fish species. Scatchard analysis revealed two different receptors: a high affinity with a Kd of 18 nM and a Bmax of 0.2 pmoles/mg protein and a low affinity receptor with a Kd of 0.5 μM and a Bmax of 1 pmole/mg protein. 17α,20β-DP receptor concentrations increase during oocyte maturation. The interaction between 17α,20β-DP receptors and G-proteins was examined. Pertussis toxin (PT) catalyzed the ADP ribosylation of a 40 kDa protein in crude membranes from rainbow trout oocytes. The 40 kDa protein was recognized by an antibody against the α subunit of inhibitory G-protein. Treating the membrane fraction with 17α,20β-DP decreased the PT-catalyzed ADP ribosylation of the 40 kDa protein. The specific binding of 17α,20β-DP was decreased by PT. We conclude that the PT-sensitive Gi is involved in the signal transduction pathway of 17α,20β-DP in fish oocytes. We have cloned two Giα cDNAs from a medaka oocyte cDNA library. Specific polyclonal antibodies against Giα subunits were generated. These antibodies were used to examine changes in Giα content during 17α,20β-DP-induced oocyte maturation and to immunoprecipitate solubilized Giα. Western blot analysis showed that 17α,20β-DP receptor concentrations and Giα content decreased concomitantly in membrane preparations during oocyte maturation. We also found that significant amounts of 17α,20β-DP receptors in the immunoprecipitates, indicating that the 17α,20β-DP membrane receptors are directly coupled with Gi. This is the first demonstration of direct coupling of the maturation-inducing hormone (steroid) receptor and heteromeric G-proteins (Fig. 1).

The early steps following 17α,20β-DP action involve the formation of the major mediator of this steroid, maturation-promoting factor or metaphase-promoting factor (MPF). MPF activity cycles during 17α,20β-DP-induced oocyte maturation with the highest activity occurring at the first and second meiotic metaphase. Studies from our laboratory and others have shown that MPF activity is not species-specific and can be detected in both meiotic and mitotic cells of various organisms, from yeast to mammals.

Fish MPF, like that of amphibians, consists of two components, catalytic cdc2 kinase (34-kDa) and regulatory cyclin B (46- to 48-kDa). Goldfish immature oocytes contain 35-kDa inactive cdc2 kinase. Although immature oocytes contain mRNA for cyclin B, they do not contain cyclin B protein. 17α,20β-DP induces oocytes to synthesize cyclin B. The preexisting 35-kDa inactive cdc2 kinase binds to *de novo* synthesized cyclin B at first, then is rapidly converted into the 34-kDa active form. Introduction of a bacterially produced goldfish cyclin B into immature goldfish oocyte extracts induces cdc2 kinase activation. Phosphoaminoacid analysis shows that threonine (Thr) phosphorylation of the 34-kDa cdc2 kinase is associated with the activation. The sites of Thr phosphorylation on cdc2 kinase was mapped to reside Thr-161. Since goldfish cyclin B mRNA contains four copies of the usual cytoplasmic polyadenylation element in the 3'UTR, the initiation of its synthesis during oocyte maturation may be controlled by the elongation of poly (A) tail. We examined the polyadenylation state of cyclin B mRNA during goldfish oocyte maturation by means of a PCR poly (A)

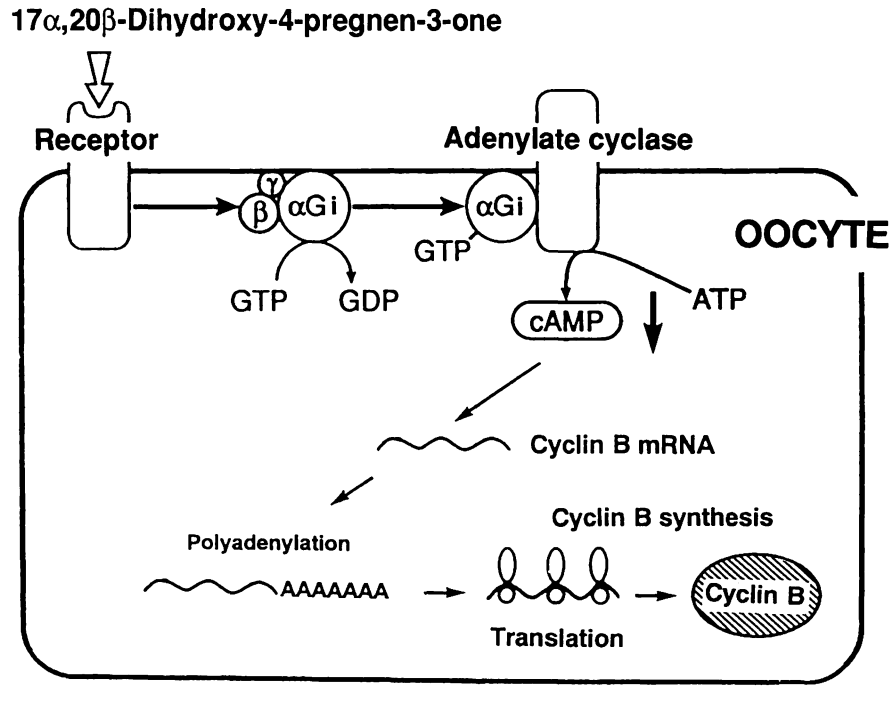

Fig. 1. Mechanisms of 17α,20β-dihydroxy-4-pregnen-3-one (17α,20β-DP)-induced cyclin B synthesis by fish oocytes. Inhibitory G-proteins (Gi) is involved in the signal transduction pathway of 17α,20β-DP.

test, and found that cyclin B mRNA is polyadenylated during oocyte matuation. Furthermore, cordycepin which inhibits poly (A) addition of mRNA, prevented 17α,20β-DP-induced oocyte maturation in goldfish. It is concluded that elongation of poly (A) tail of cyclin B mRNA is required for the initiation of its translation (Fig. 1).

Immediately prior to the transition from metaphase to anaphase, MPF is inactivated by degradation of cyclin B. We investigated the role of proteasomes (a nonlysosomal large protease) in cyclin degradation, using *E. coli*-produced goldfish cyclin B and purified goldfish proteasomes (20S and 26S). The purified 26S proteasome, but not 20S proteasome, cleaved both monomeric and cdc2-bound cyclin B at lysine 57 (K57) restrictively *in vitro*, and produced a 42 kDa N-terminal truncated cyclin B, which was transiently detected at the initial phase of the normal egg activation. The 42 kDa cyclin B, as well as full-length one, was degraded in *Xenopus* egg extracts, but a mutation on K57 (K57R) inhibited both the digestion by 26S proteasome and the degradation in *Xenopus* egg extracts. These findings strongly suggest that the involvement of 26S proteasome in cyclin degradation through the first cleave on its N-terminus.

II. Endocrine regulation of male germ cell development and maturation

We have identified two steroidal mediators of male germ cell development in salmonid fishes (11-ketotestosterone for spermatogenesis and 17α,20β-DP for sperm maturation). A steroidogenic switch, from 11-ketotestosterone to 17α,20β-DP, occurs in salmonid testes around the onset of final maturation. *In vitro* incubation studies using different testicular preparations have revealed that the site of 17α,20β-DP production is in the sperm, but its production depends on the provision of precursor steroids by somatic cells. The site of 11-ketotestosterone production is in the testicular somatic cells.

In the cultivated male Japanese eel (*Auguilla japonica*), spermatogonia are the only germ cells present in the testis. A serum-free, chemically defined organ culture system developed for eel testes was used to investigate the effect of various steroid hormones on the induction of spermatogenesis *in vitro*. We obtained evidence that 11-ketotestosterone can induce the entire process of spermatogenesis *in vitro* from premitotic spermatogonia to spermatozoa within 21 days.

We have used subtractive hybridization to identify genes that are expressed differentially in eel testes in the first 24 hr after HCG treatment *in vivo*, which ultimately induces spermatogenesis. One up-regulated cDNA was isolated from subtractive cDNA libraries derived from mRNA extracted from control testes and testes one day after a single injection of HCG. From its deduced amino acid sequence, this clone was identified as coding for the activin βB subunit. Using Northern blot analysis and *in situ* hybridization techniques, we examined sequential changes in transcripts of testicular activin βB during HCG-induced spermatogenesis. No transcripts for activin βB were found in testes prior to HCG injection. In contrast, 3.3 kb mRNA transcripts were prominent in testes one day after the injection. The transcript concentration began to decrease three days after the injection and there was a further sharp decrease by nine days. The HCG-dependent activin βB mRNA expression in the testes was confirmed by *in situ* hybridization using a digoxigenin-labelled RNA probe: the signal was restricted to Sertoli cells in testes treated with HCG for one to three days. A marked stimulation of activin B production, but not either activin A or activin AB, was observed in testes after HCG and 11-ketotestosterone treatment. Addition of recombinant human and eel activin B to the culture medium induced proliferation of spermatogonia, producing mitotic spermatogonia, within 15 days in the same manner as did 11-ketotestosterone. Taken together, these findings suggest the following sequence of the hormonal induction of spermatogenesis in the eel. Gonadotropin stimulates the Leydig cells to produce 11-ketotestosterone, which, in turn, activates the Sertoli cells to produce activin B. Activin B then acts on spermatogonia to induce mitosis leading to the formation of spermatocytes (Fig. 2).

To characterize the pathway of activin signal transduction involved in the process of spermatogenesis, we recently initiated studies on the cloning and expression of activin receptors of eel testes. Three kinds of cDNA clones encoding eel activin type I receptors were isolated from an eel testis cDNA library. The predicted proteins of these receptors consist of 510, 493, and 505 amino acids with their deduced amino acid sequences having a putative signal peptide, a cystein-rich extracellular domain containing a potential N-glycosylation site, a putative transmembrane domain with a well conserved GSGSG motif, and a serine/threonine kinase domain. Northern blot hybridization was performed in testes from eels injected with hCG for 1-18 days. The results were that mRNA transcripts of the three eel activin type I receptors were expressed in the testes of eels prior to hCG injection, with marked increased in the testes after hCG injection for 9 days. *In situ* hybridization revealed that these activin type I receptors are localized in germ cells (spermatogonia and spermatocytes) and Sertoli cells. To further understand the molecular mechanisms of activin B-induced initiation of mitosis in spermatogonia, we recently isolated cDNA clones encoding eel homologs of cdc2 kinase, cdk2 kinase, cdk4 kinase, cyclin A1 and A2, cyclin B1 and B2, cyclin D1, and cyclin E and E' from cDNA libraries of eel testes. Specific antibodies against some of these proteins were also raised.

III. Endocrine regulation of gonadal sex differentiation

Recently, we, in collaboration with Drs. K. Ozato

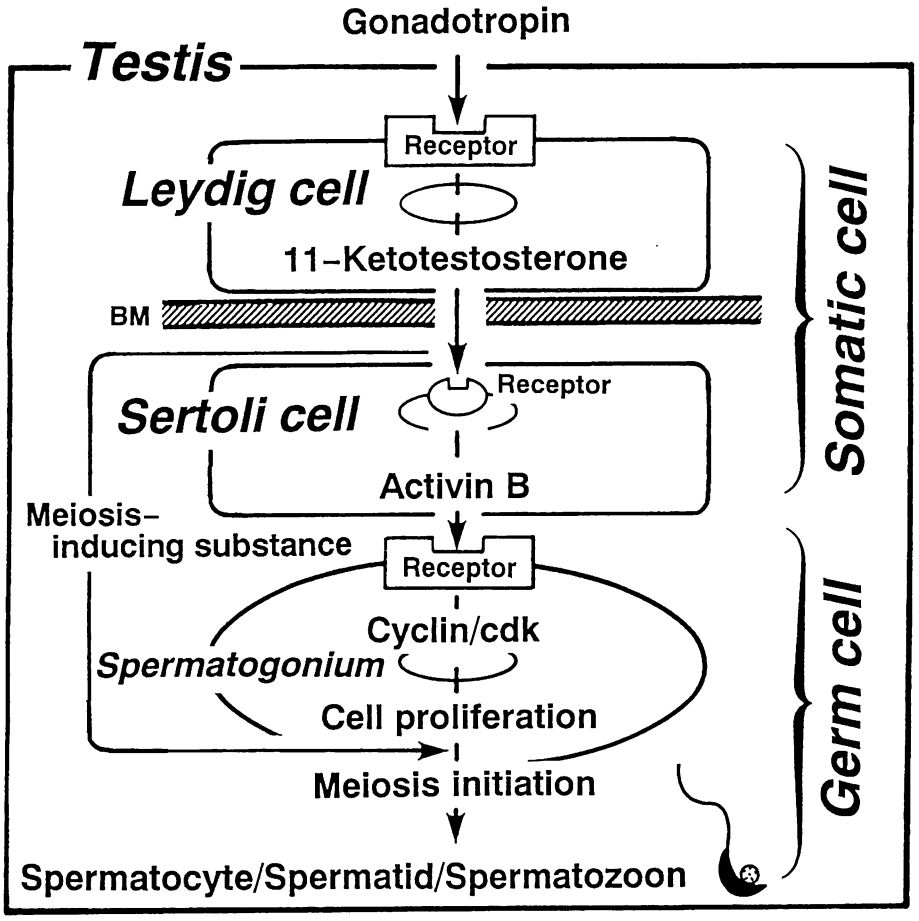

Fig. 2. Hormonal regulation of spermatogenesis in the eel testis.

(Nagoya University), M. Nakamura (Teikyo University) and E.G. Grau (University of Hawaii), have initiated studies on the roles of sex steroid hormones in sex determination and differentiation using medaka, tilapia (*Oreochromis niloticus*), and a sex change fish, *Thalassoma duperrey*. In tilapia, a pair of gonadal primordia formed on both sides of the intestine at 15 days after hatching. The gonadal anlage consists of several roundish germ cells surrounded by a few stromal cells. At 20 days after hatching, prior to the period of sex differentiation, positive immunostaining for P450scc, 3β-HSD, P450c17, and P450arom antibodies becomes evident for the first time in gonads. Immunostained stromal cells are observed in the vicinity of blood vessels. From 23-26 days after hatching, morphological gonadal sex differentiation begins to be recognized. Initial ovarian differentiation is marked by the appearance of a narrow space in the stromal tissue, representing the formation of the ovarian cavity. On the other hand, initial testicular differentiation is characterized by the appearance of a narrow space in the stromal tissue, representing the efferent duct construction. At this stage, germ cells in testes and ovaries remain in the gonial stage. In ovaries, positive immunostaining for four kinds of steroidogenic enzymes is recognized in large stromal cells located in the vicinity of blood vessels. In contrast, no immunoreaction is evident in testes during sex differentiation. This situation continues until testes initiate spermatogenesis. Thus, tilapia ovaries express the steroidogenic enzymes required for estradiol-17β biosynthesis from cholesterol before sexual differentiation, which is consistent with the concept that estrogen biosynthesis is essential for sexual differentiation of female phenotype during early development.

In *T. duperrey*, steroid-producing cells have identified at different stages of sex change using immunocytochemical methods. Immunoreactive P450scc, the first enzyme in the steroidogenic pathway, initially localizes in thecal cells. As sex change progresses towards testis formation, P450scc becomes localized in interstitial cells. It appears that thecal cells migrate towards the interstices and become very active androgen-producing cells.

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Division of Cell Differentiation

Professor: Yoshiaki Suzuki
Associate Professor: Kohji Ueno
Research Associates: Kaoru Ohno
Hiroki Kokubo
Graduate Students: Katsuyoshi Matsunami (Graduate University for Advanced Studies)
Yoshinori Ueno (Graduate University for Advanced Studies)
Technical Staff: Chikako Inoue

We conduct two well-associated projects. One is to understand how a special tissue like the silk gland of *Bombyx mori* differentiates along the developmental programs and results in transcribing a specific set of genes like the silk fibroin and sericin-1 genes. The other concerns with what the specificity of the *Bombyx* body plan is and how the developmental regulatory genes dictate a set of target genes in specifying the identities of various regions of the embryos.

I. Genes and factors that control the silk gland development and the silk gene transcription

We have been trying to understand the networks of regulation hierarchy that function in the processes of silk gland development and differentiation. As a bottom-up type approach for this project, analyses on the molecular mechanisms that control the differential transcription of the fibroin and sericin-1 genes in the silk gland should provide an information about a part of the networks. In complementing this approach, a top-down type approach should also help understanding the networks; analyses of regulation hierarchy of the homeobox and other regulatory genes, and identification of their target genes expressed in the labial segment, where the silk glands originate.

During the past year we have succeeded in purifying the SGF-2, a key transcription factor for the fibroin gene, which binds to FC, FD, and FE elements in the enhancer I and enhances transcription specifically in the posterior silk gland (K. Ohno et al., unpublished). The purification steps include SP-Sepharose FF, Source 30Q, Dyna beads-FE element multimers, BioSilect 250, Mini S, and Mini Q. The SGF-2 is a huge complex revealing an apparent molecular mass of about 1.1 MDa and accommodating 12 components; groups of p30/p32/p33, p36/p47B/p50B/p55, and p45/p46/p47G/p48/p50G. Through a partial peptide sequencing and PCR analysis, p36 was identified as a Lim homeodomain family protein, and p47B/p50B/p55 were as nuclear Lim interactor homologues. p30/p32/p33 were identified as variants of p25 which was known as an associating protein to the fibroin heavy chain-light chain complex. This finding raises the possibility that the p25 variants might be a chaperone to the transcription complex. By the use of antibodies against p45, p47B, p47G, and p50G, it was found that these components were not or barely detectable in the extract from the middle silk gland where the fibroin gene is not expressed. Functional reconstitution experiments as well as developmental expression pattern analysis on these components are being planned.

Previously we reported cloning and labial segment-

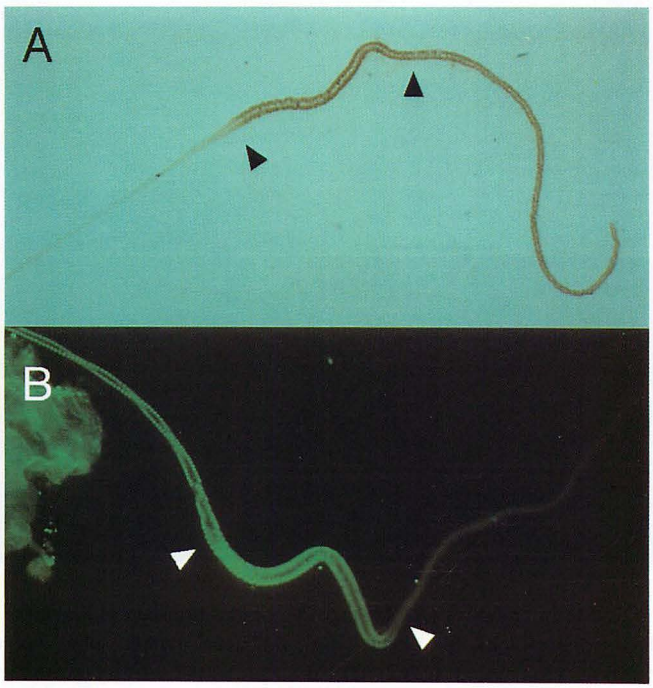

Fig. 1. Expression of SGF-1/Bm Fkh (A) and SGF-3/POU-M1 (B) in embryonic silk gland. (A) At stage 23, signals are seen in the middle and posterior silk gland. Revised from Kokubo et al. (1996). (B) At stage 21, signals are weakened in the posterior region. Revised from Kokubo et al. (1997) Dev. Genes Evol. **206**, in press. Arrowheads indicate the borders between the anterior, middle, and posterior silk gland. All scale bars represent 100 μm.

specific expression of the *Bombyx Scr*. Silk gland specific transcription factor-1 (SGF-1) is known to interact with the SA site of the sericin-1 gene and FA and FB sites of the fibroin gene. Also previously, we reported the SGF-1 to be a new member of the *fork head/HNF-3β* family. Expression patterns of *SGF-1/fkh* mRNA and protein in developing embryos were also described. At stage 20, the transcripts and protein were detected in the invaginating silk glands. Interestingly, preceding the appearance of the *Bombyx* Fkh protein in the invaginating silk glands, *Bombyx* Scr disappeared from the spots. This observation suggests the possibility that the *Bombyx* Scr is necessary to determine the nature of the labial segment and induce the silk gland invagination accompanied by the *Bombyx fkh* expression but the Scr protein is probably not necessary for the direct induction of *Bombyx fkh* expression in the invagination spots (Kokubo et al., Dev. Biol., in press).

The following observations supported above suggestions. In the *Nc/Nc* embryos described by Itikawa in 1944 that lack the *Bombyx Antp* gene (Nagata et al., 1996), we observed ectopic expression of *Bombyx* Scr in the thoracic and abdominal segments. These ectopic expressions resulted in inducing ectopic formation of invaginating silk glands in the prothoracic, mesothoracic, and metathoracic segments all of which revealed ectopic expression of *Bombyx* Fkh (Kokubo et al., Dev. Biol., in press).

By the time when the blastokinesis finishes (stage 25) and the silk gland fully develops, the *Bombyx fkh* transcripts and protein were restricted to the middle and posterior regions of the silk gland (Fig. 1A). These results suggest that besides the role of transcription factor for the silk genes the *Bombyx fkh/SGF-1* may play important roles during the silk gland development.

We reported that the POU-M1 which binds to the

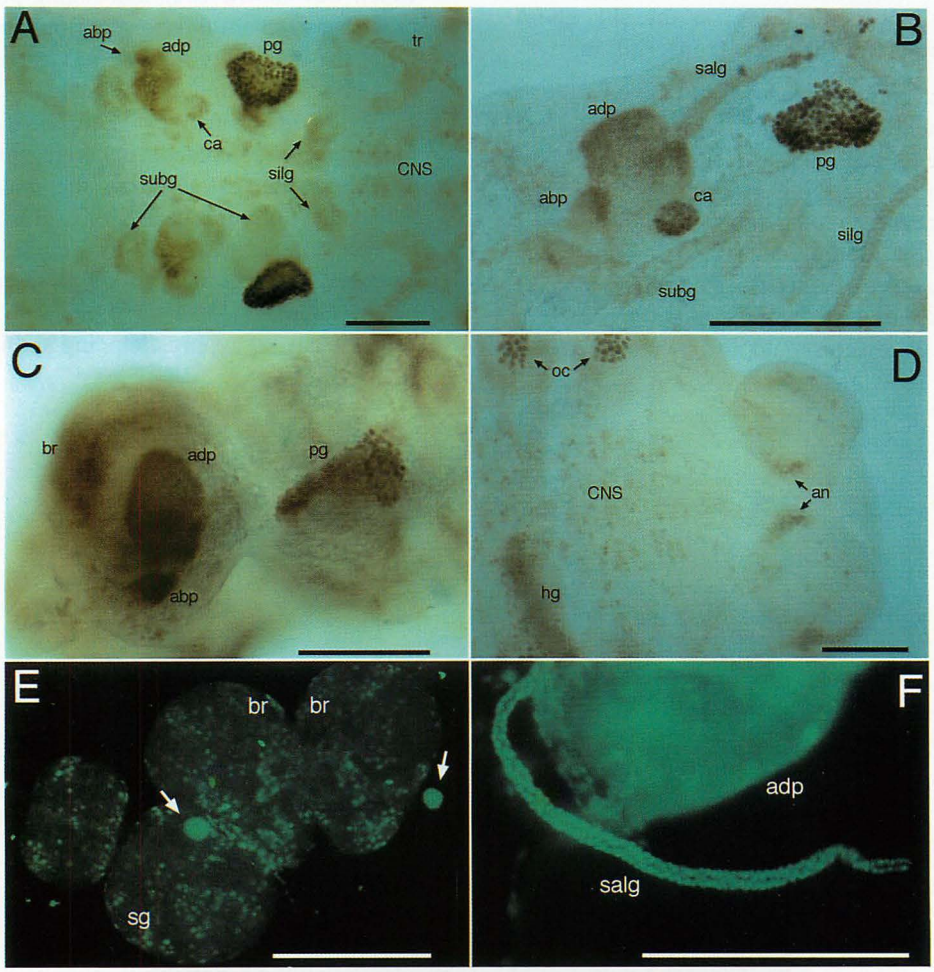

Fig. 2. Expression of SGF-3/POU-M1 in embryos. (A) At stage 20, signals are seen in precursor cells of the prothoracic glands (pg), adductor plates (adp), abductor plates (abp), silk glands (silg), subbuccal glands (subg), corpora allata (ca), tracheal system (tr), and in some cells of the central nervous system (CNS). (B) At stage 21, the salivary glands are elongated from the posterior part of the adp. Both invaginated cells in the anterior mandibular and posterior maxillary segments are fused to form the subg. (C) At stage 23, signals are also seen in the adp, abp, and the pg that reveal mature form. (D) Magnified in posterior region of an embryo at stage 21. Signals are seen in parts of the anus. (E) At stage 25, signals are seen in some cells of the brain (br) and the suboesophageal ganglion (sg), and in the ca (arrows). (F) At stage 25, signals are seen in the salivary glands (salg) and the adp. Anterior side is always to the left and dorsal side is up. All scale bars represent 100 μm. Revised from Kokubo et al. (1997) Dev. Genes Evol. **206**, in press.

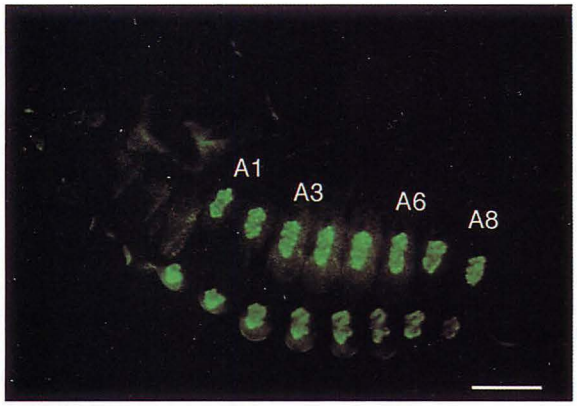

Fig. 3. Expression of p260/p270 in the embryonic abdominal regions at stage 21A. The scale bar represents 100 μm. Revised from Ueno and Suzuki (1997), J. Biol. Chem. **272**, 13519-13526.

SC site of the sericin-1 gene accommodates a POU-domain identical to *Drosophila* Cf1-a. The expression of the *POU-M1* gene has been analyzed in *Bombyx* embryos by *in situ* hybridization and immunohistochemistry (Fig. 2). The gene was expressed specifically for the first time at stage 18-19 in a pair of restricted sites of the labial segment where a pair of prothoracic glands is going to be formed by invagination. After the silk gland invagination, the *POU-M1* expression was detected in the developing silk gland and confined to the anterior and middle portions of the silk gland by late embryonic stages (Fig. 1B).

A *Bombyx* homologue of *trachealess* has been cloned, identified by sequence comparison, and named *Bm trh* (K. Matsunami et al., unpublished). The *Bm trh* is expressed first at the invagination sites of trachea, and continued to be expressed along the trachea development. Later, it is also expressed in the invagination sites of the silk glands. The expression in the silk gland continues along the silk gland development but disappears first in the posterior silk gland and then in the middle silk gland leaving the expression only in the anterior silk gland. This expression pattern differs from that of *trh* in the *Drosophila* salivary gland.

II. Genes associated with abdominal leg development

Through the studies on the mechanisms of abodominal leg development, we found that a high molecular weight protein (p260/270) was expressed specifically in abdominal leg cells (Fig. 3) during early embryonic stages and disappeared by a late stage. p260/270 consists of two polypeptides with molecular weights of 260 and 270 kDa. We have established a purification procedure for p260/270 and have raised an antibody against p260/270. Immunoblot analysis of the E^{Ca}/E^{Ca} (additional crescent) and E^{N}/E^{N} (new additional crescent) mutants (Itikawa, 1943), which lack the *Bombyx abdominal-A* gene (Ueno et al., 1992) and therefore do not express abdominal legs, demonstrated that both mutants lacked p260/270. Therefore we speculate the expression of p260/270 may be regulated by the *Bombyx abdominal-A* gene. cDNA cloning and sequencing demonstrated that p260 and 270 have structures similar to rat fatty acid synthase, which synthesizes palmitate. Most of the enzymatic domains for palmitate synthesis were well conserved in the amino acid sequences of p260 and p270, while the thioesterase domains of p260 and p270 were less conserved to that of rat fatty acid synthase (Ueno and Suzuki (1997), J. Biol. Chem. **272**, 13519-13526). Purified p260/270 can transfer palmitate to cysteine residues of synthetic peptides *in vitro*. We propose that p260/270 may be involved in protein palmitoylation and may function in abdominal leg development.

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DIVISION OF MORPHOGENESIS

Professor: Goro Eguchi
Associate Professor: Ryuji Kodama
Research Associates: Makoto Mochii
Mitsuko Kosaka
Visiting Scientists: Takamasa S. Yamamoto
Akio Iio (from Chubu National Hospital)
Shin-ichi Higashijima (from Japan Science and Technology Corporation)
Panagiotis A. Tsonis (from University of Dayton)
Katia Del Rio-Tsonis (from University of Dayton)
Thomas Reh (from University of Washington)
Graduate Student: Harutoshi Hayashi (from School of Agriculture, University of Tokyo)
Technical Staffs: Chiyo Takagi
Sanae Oka

In the newt and some other limited animal species, the lens and neural retina can be completely regenerated through transdifferentiation of pigmented epithelial cells (PECs). Such a phenomenon, transdifferentiation, as observed in regeneration of ocular tissues seems to be a highly powerful model for studying stability and instability in differentiation of tissue cells. From this view point, lens transdifferentiation of PECs of vertebrate has been studied using *in vivo* and *in vitro* systems, and our *in vitro* studies have revealed that dormant potential of PECs to transdifferentiate into lens cells is widely conserved throughout vertebrate species and that such potential becomes evident when PECs are cultured *in vitro*. Such transdifferentiation can be accelerated in the presence of phenylthiourea and testicular hyaluronidase and cells passes through a dedifferentiated status, where expression of PEC-specific genes, such as *mmp115*, *tyrosinase*, *TRP-1*, and *pP344* are suppressed. On the other hand, *c-myc* and *pax6* expressions are elevated in the dedifferentiated PECs. We have extended our study to the regulatory mechanisms controlling gene expression during transdifferentiation and following results are obtained.

I. *In vitro* culture system of iris pigmented epithelial cells for molecular analysis of lens regeneration

We have succeeded in purely isolating and cultivating the iris pigmented epithelial cells of 2 day-old chick using chemical treatment by dispase and EDTA. The iris PE cells continued to proliferate very stably in the control medium and transdifferentiate into lens cells by the addition of only two defined growth factors to the culture medium. The process of transdifferentiation in this system is very reproducible and this culture system of the iris PE has made it possible to prepare homogeneous cell population at various stages during the transdifferentiation process, thus providing the way for biochemical and molecular biological analyses. The Northern blot analysis suggests that the transcription level of *pax6* gene closely correlate with the progression of the transdifferentiation process of the iris PE cells to the lens.

II. Role of the *mi* gene in differentiation and transdifferentiation of the pigmented epithelial cells

The *mi* gene was first isolated as a mouse gene in the *microphthalmia* (*mi*) locus and shown to encode a basic-helix-loop-helix-leucine zipper (bHLHzip) transcription factor, which is a possible regulatory gene functioning in PEC differentiation. We have revealed that *mi* is specifically expressed in PECs and precursors of PECs in the embryo, and that *mi* starts to be expressed at the optic vesicle stage when no other marker genes are expressed yet. Expression of *mi* is also correlated with PEC differentiation in the culture condition, where *mi* expression is down-regulated under the condition for dedifferentiation but up-regulated under the condition for redifferentiation.

In order to elucidate the role of *mi* in PEC differentiation, cultured PECs were infected with a retrovirus construction including *mi* sequence. The *mi* overexpression did not cause significant changes in morphology but caused a dramatic change in the pattern of gene expression. PECs infected with the retrovirus encoding *mi* continued to express *mmp115*, *tyrosinase* and *TRP-1* but not *pP344* even under a culture condition which promote dedifferentiation. Transfection and CAT assay revealed that *mi* transactivates *mmp115* promoter. These results suggest that *mi* has a critical role in PEC differentiation by activating *mmp115*, *tyrosinase* and *TRP-1*. Moreover, the result that *pP344* was not transactivated by *mi* expression suggested the presence of another regulatory factor in PEC differentiation which is required to promote *pP344* expression. Our findings revealed that at least a part of the changes in gene expression pattern accompanying dedifferentiation is regulated by *mi* inactivation which is induced by dedifferentiation signals.

III. Analysis of *silver* quail mutant

In the homozygous *silver* mutant of Japanese quail (*Coturnix coturnix japonica*), a part of the outer layer of retina, which normally differentiates into the pigmented epithelium of the retina, forms an ectopic neural retina tissue (Fig. 1). The ectopic neural retina was suggested to be formed through transdifferentiation from pigmented epithelium according to morphological observations, but there have been no direct evidence. We found that the outer layer of retina in *silver* homozygote once differentiates to pigmented epithelium judging from the expression of *mmp115* in 4-day embryo. After 5th day of incubation, however, the outer layer starts to express neural marker genes which indicates the beginning of the transdifferentiation into the neural retina. The process to form the ectopic neural retina in *silver* embryo is, therefore, a

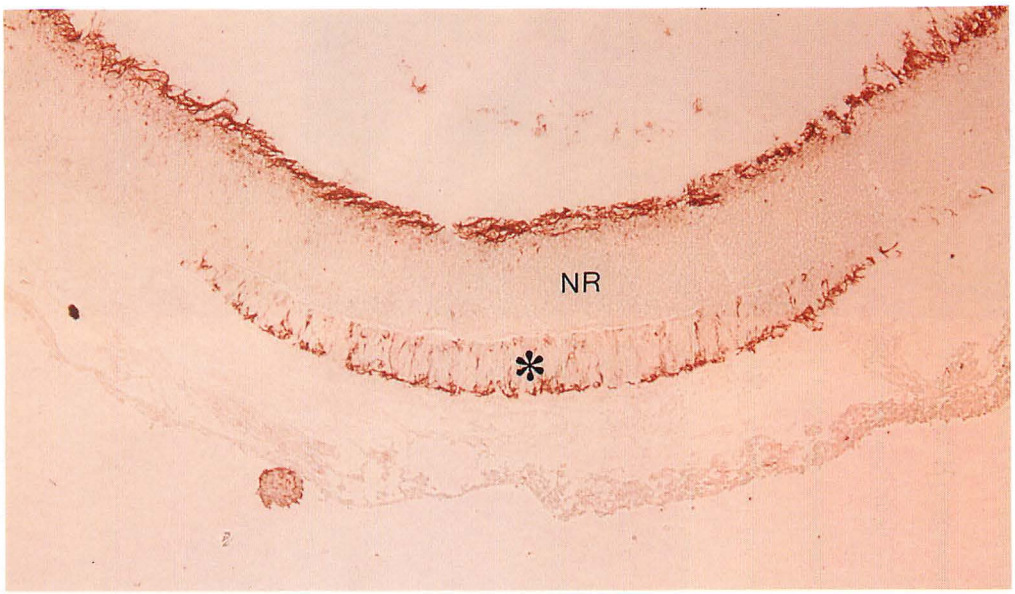

Fig. 1. Ectopic neural retina in *silver* mutant quail.
Retinal section of 7-day-old embryo from *silver* homozygote was stained with anti-neurofilament antibody. Ectopic neural retina formed via transdifferentiation of pigmented epithelium is positively stained (*).
NR: original neural retina

transdifferentiation process. Furthermore, we analysed the sequence of *mi* gene in the *silver* homozygote and found that there is a two-base deletion which causes a production of truncated *mi* protein. We have confirmed using an *in vitro* assay that the truncated *mi* protein has less activity in activating the *mmp115* promoter. These results suggest that the mutation in the *mi* gene induces transdifferentiation of PECs to neural retina cells in *silver* homozygote.

IV. Isolation of a novel chick homologue of *Serrate* and its coexpression with *C-Notch-1* in chick development

Intercellular signaling mediated by the transmembrane proteins, Notch as receptor and its ligands, Delta and Serrate, plays essential roles in the developmental fate decision of many cell types in *Drosophila*. The *Notch* genes are highly conserved both in invertebrates and in vertebrates, suggesting that Notch pathway regulates cell fate decisions during vertebrates development.

As a starting point of our study on the role of the Notch pathway in the control of the differentiation of ocular tissues, we cloned homologues of *Notch*, *Delta* and *Serrate* in the chicken. Among them, there was a novel chick homologue of *Drosophila Serrate*, named as *C-Serrate-2*. We examined the expression patterns of *C-Serrate-2* and other homologs during the early chick development using whole-mount *in situ* hybridization. Tissues with conspicuous expression of Notch receptors and ligands included the forebrain, the myotome and the apical ectodermal ridge (AER) of the limb bud of a 4-day-old chick embryo.

In the early development of ocular tissues, *C-Serrate-1* expression was observed in the thickened ectoderm of the lens primordium at stage 13 (Fig. 2). At stage 14, *C-Notch-1* transcripts were coexpressed with *C-Serrate-1* in the lens placode. When the lens placode invaginates to form a lens vesicle in 3-day-embryos (stage 18-21), *C-Serrate-1* expression was restricted to the posterior region of the lens containing differentiating lens fiber cells. On the other hand, *C-Notch-1* was restricted to the anterior region of the lens containing proliferating cells.

These combination of expression patterns during developmental processes is useful in predicting possible roles and mode of functions of intercellular signalling through the Notch pathway.

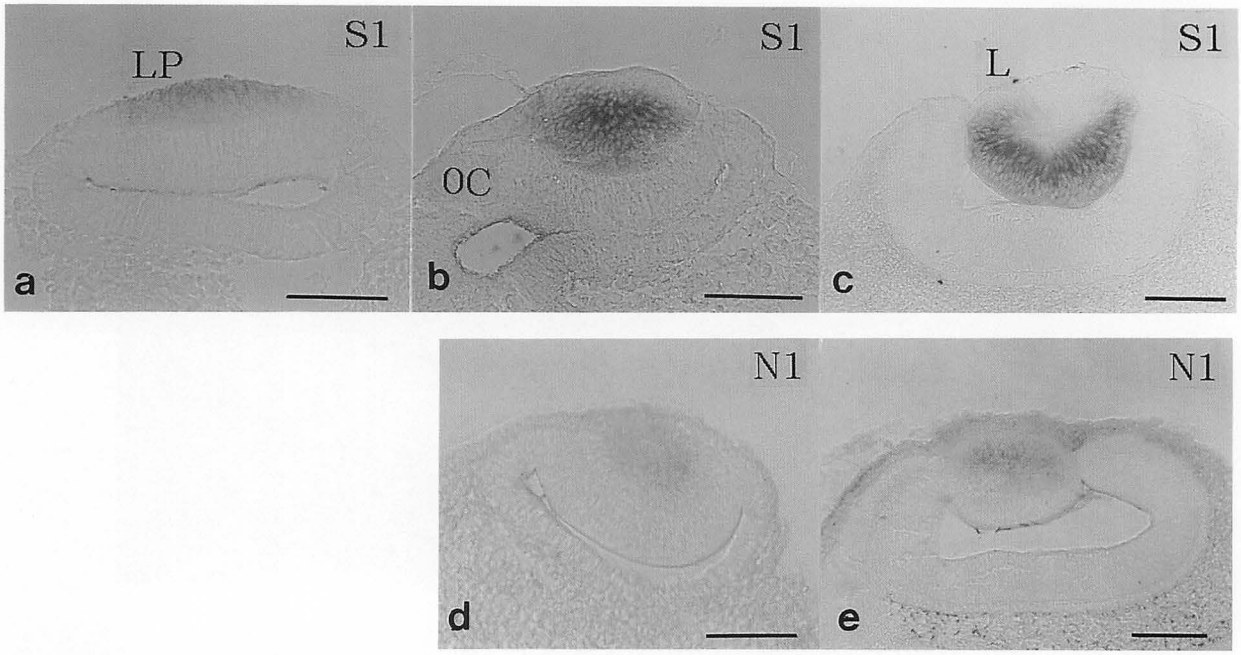

Fig. 2. Expression of *C-Serrate-1* and *C-Notch-1* in the eye primordium of the chick embryo.
Embryos at stage 13 (a), stage 14 (b, d) and stage 18-21 (c, e) were hybridized with *C-Serrate-1* (S1) or *C-Notch-1* (N1) probes. LP: lens placode, L: lens, OC: optic cup, the scale bar: 100 μm

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DIVISION OF DEVELOPMENTAL BIOLOGY (ADJUNCT)

Professor: Kenzo Nakamura (Nagoya University)
Associate Professor: Tsukaho Hattori (Mie University)
Research Associate: Masa-aki Ohto
Graduate Students: Yasuhiro Koide (Nagoya University)
Hideyuki Hirano (Nagoya University)
Masamitsu Kuriyama (Nagoya University)
Yukiko Iwata (Nagoya University)

Our research attention has been focused in the area of developmental control of gene expression and cell function related to the storage tissues and organs in plants, in particular mechanisms involved in the regulation of gene expression in response to the level of sugars and mechanisms involved in the targeting of storage proteins to the vacuole are studied.

I. Regulatory factors involved in the sugar-inducible expression of plant genes

Availability of sugars and inter-organ transport of sugars are essential in the growth and development of plants. Expression of a variety of genes is now known to be regulated, either positively or negatively depending on the gene, by the level of sugars at the sugar-importing sink sites as well as at the sugar-exporting source sites of the plant body. Thus sugars are not only important as sources for cellular energy and macromolecules but also as signalling molecules controlling the growth and development by changing the pattern of gene expression in plants.

Although genes coding for sporamin and β-amylase, two major proteins of the tuberous root, are expressed specifically in tuberous roots of sweet potato under the normal growth conditions, their expression is inducible by high levels of sugars in other vegetative tissues. Expression of GUS reporter genes under the control of promoters of the sporamin gene or the β-amylase gene is inducible by sugars in leaves of transgenic tobacco plants, and these fusion genes are expressed in tubers of transgenic potato plants. It is suggested that massive transport of sugars to developing tuberous roots plays an important role in maintaining the high-level expression of sporamin and β-amylase genes. The sugar-inducible expression of sporamin and β-amylase genes requires synergistic action of two separate *cis* regulatory elements in each case, and one of the element is shared between the two genes. The activity to bind this conserved sequence motif in the nuclear extract increased upon treatment of the tissue with sucrose. A bZIP-type DNA binding protein and a novel DNA binding protein, which contains unique Zn-finger motif conserved among several plant DNA binding proteins, bind to other regulatory elements.

Experiments with various inhibitors suggested that the sugar-inducible expression of sporamin and β-amylase genes requires continuous dephosphorylation of proteins, Ca^{2+}-signalling and the activity of protein kinase. It was also found that in leaves of tobacco, the level of 54 kDa-protein with autophosphorylation acitivity in the plasma membrane increases upon treatment of the tissue with sucrose. The 54 kDa autophosphorylation protein was solubilized from the plasma membrane by 1% sodium deoxycholate (DOC), and it was purified to about 1,000-fold compared to the crude extract. The 54 kDa-protein phosphorylated histone IIIS in a Ca^{2+}-dependent manner and it cross-reacted with antibody raised against calcium-dependent protein kinase (CDPK) of *Arabidopsis thaliana*. By RT-PCR reaction with primers designed from sequence motifs highly conserved among plant CDPKs, we identified 8 cDNA fragments from leaves of tobacco which codes for different isoforms of CDPKs. Antibodies raised against kinase domains of two of these isoforms did not cross-reacted with the 54 kDa-protein. These results suggest that the 54 kDa-protein is a novel isoform of tobacco CDPK which is tightly associated with the plasma membrane.

II. Mutants of *Arabidopsis thaliana* with altered patterns of the sugar-inducible gene expression

Similar to the β-amylase gene of sweet potato, expression of the β-amylase gene of *Arabidopsis thaliana (ATβ-Amy)* occurs in response to high levels of sugars. We identified several mutants of *Arabidopsis* which showed altered response of the expression of *ATβ-Amy* to sugars in leaves. A recessive mutation, *lba1*, significantly reduced the level of expression of *ATβ-Amy* under high levels of sugars. By contrast, a recessive mutation, *hba1*, caused increased levels of expression of *ATβ-Amy* in response to lower levels of sugars, the results suggesting that *HBA1* might function to maintain low level expression of *ATβ-Amy* until the level of sugars reaches some high levels. It is suggested that the expression of *ATβ-Amy* is regulated by a combination of both positive and negative regulation depending on the level of sugars.

III. Derepression from negative regulation in the sugar-inducible expression of β-amylase gene of *Arabidopsis thaliana*

In addition to the *ATβ-Amy:GUS* fusion gene, in which the sequence from –1,587 to +136 of *ATβ-Amy* was fused with *gus*, the *ATβ-Amy[Δ-172]:GUS* fusion gene where the 5'-upstream of the promoter was truncated to –172, showed sugar-inducible expression in leaves of transgenic *Arabidopsis* plants in a manner similar to the endogenous *ATβ-Amy*. A sequence between –172 and –61 of *ATβ-Amy* was essential for the sugar-inducible expression and it conferred sugar-inducibility on the –89 core promoter of CaMV 35S. Linker scanning of the sequence between –172 and –62 in the *ATβ-Amy[Δ-172]:GUS* fusion gene identified two *cis*-regulatory elements. When sequence substitutions were made in the negative regulatory region

A, levels of GUS activity in non-treated leaves increased to those in sugar-treated leaves. On the other hand, substitutions in the positive regulatory region B abolished the expression. The region A contained a sequence which is highly homologous to the sequence motif that is conserved between sporamin and β-amylase genes of sweet potato and required for their sugar-inducible expression.

In contrast to *ATβ-Amy:GUS*, *ATβ-Amy[Δ-172]:GUS* did not show increased sensitivity to low levels of sugars in *hba1* mutant plants. In the wild type plants, the 5'-truncation of the promoter to –172, but not to –310, caused increased sensitivity to low levels of sugars similar to the pattern of expression of *ATβ-Amy* in *hba1* plants. These results suggest that the negative regulation by *HBA1* might require *cis*-element(s) located between –310 and –173, and further support that the sugar-inducible expression of *ATβ-Amy* might involve derepression from negative regulations.

IV. Screening of mutants of *Arabidopsis thaliana* with defective growth and development

Neither *lba1* nor *hba1* mutation affected the sugar-regulated gene expression in general suggesting that sugar-regulated expression of a variety of plant genes is mediated by multiple mechanisms. Nevertheless, *lba1* and *hba1* mutant plants showed pleiotropic effects on the sugar-inducible accumulation of anthocyanin. Furthermore, these mutant plants showed several characteristic growth properties. In particular, several growth and developmental properties of *Arabidopsis* plants, which in wild type plants occurs in response to changing levels of sugars, are affected in these mutant plants. To obtain further insights into the role of the sugar-regulated gene expression in the growth and development of plants, research attention will be focused on screening of mutants of *Arabidopsis thaliana* with defects or anomalies in the growth and organ development that are affected by the availability of sugars in the wild-type plants. To aid this purpose, we are constructing increasing number of mutagenized plants after transformation with multiple copies of enhancer fragments in the T-DNA.

DEPARTMENT OF REGULATION BIOLOGY

Chairman: Norio Murata

The Department is composed of two regular divisions and two adjunct divisions. The study of this department is focused on the molecular mechanisms for the development of central nervous systems in mice, chickens and *Drosophila*, and also on the molecular mechanisms for the response of plants toward external environments, such as light, temperature and salinity.

DIVISION OF MOLECULAR NEUROBIOLOGY

Professor: Masaharu Noda
Research Associates: Nobuaki Maeda
Masahito Yamagata
Eiji Watanabe
NIBB Fellow: Masakazu Takahashi
Graduate Students: Hiroki Hamanaka (– Sept. 30, 1996)
Junichi Yuasa
Takafumi Shintani
Taeko Nishiwaki
Chika Saegusa (Oct. 1, 1996 –)
Visiting Scientists: Ikuko Watakabe (Oct. 1, 1996 –)
Tatsunori Yamamoto (– Aug. 31, 1996)*
Technical Staffs: Akiko Kawai
Shigemi Ohsugi

*(*from Nagoya University)*

We study the molecular and cellular mechanisms underlying the development of the vertebrate central nervous system. Our experiments are designed to analyze molecules and structures involved in regulation of various cellular events in brain morphogenesis such as generation of neuroblasts, their migration to form laminar structures and various nuclei, elongation and path-finding of neural processes, and the formation and refinement of specific connections between neurons. These studies utilize various techniques, including molecular biological (*e.g.* cDNA cloning, site-directed mutagenesis), biochemical (protein, carbohydrate) and immunological methods (monoclonal-antibody production), in addition to neuroanatomy, cell and organotypic culture (immortalized-cell production), and embryo manipulation (classical embryology, gene transfer with viral vectors, and gene targeting).

I. Retinotectal projection map and synapse formation

Topographic maps of neuronal connectivity have been reported for various parts of the nervous system. In the visual system of birds, retinal ganglion cell axons from the nasal (anterior) retina connect to a caudal (posterior) part of the midbrain visual center, the optic tectum, and temporal (posterior) retinal axons connect to the rostral (anterior) part, thereby establishing a point-to-point projection map. To understand the development of the retinotectal projection map, Sperry formulated the chemoaffinity theory in 1963, which is generally accepted today. His notion of chemoaffinity consists of five elements: (1) neurons are intrinsically different from each other, (2) these differences are position-dependent, (3) the differences are acquired very early, independently of their connection partners, (4) the differences are biochemical in nature, and (5) presynaptic and postsynaptic cells with matching biochemical labels connect with one another in a specific manner. Subscribing to this hypothesis, we employed a subtractive hybridization technique to identify molecules that display asymmetrical distributions between the nasal and the temporal retina. We discovered several distinct transcripts which are topographically expressed in the retina. Among these position-specific molecules, two winged-helix transcriptional regulators named CBF-1 and CBF-2 were expressed in the nasal and temporal retina, respectively. The winged-helix domain is a DNA-binding motif found in a family of transcription factors such as *Drosophila* forkhead and vertebrate HNF-3. By *in situ* hybridization, it was revealed that CBF-1 and CBF-2 transcripts began to be detected topographically in the primordial retina by embryonic day (E) 2, before birth of the retinal ganglion cells. Their topographic expression in the retina ceased by E10, prior to the onset of retinotectal connections. Misexpression of each factor in the retina using a replication-competent retroviral vector caused misprojection on the tectum along the rostro-caudal axis, suggesting that they determine the naso-temporal axis of the retina, and consequently specify the topographic projection of the retinal ganglion cell axons to the tectum by controlling expression of their target genes (Fig. 1). Our results provide evidence for the chemoaffinity theory, and this approach is promising for systematic discovery of a series of topographic molecules, determination of their functional hierarchy, and elucidation of the molecular and cellular mechanisms that generate the topographic map.

After reaching their appropriate sites along the rostro-caudal and dorso-ventral axes of the tectum, retinal axons begin to seek their appropriate laminar termination sites among 15 distinct laminae within the tectum. Our second aim is to identify molecular and cellular cues for ingrowing axons to arborize in appropriate laminae, to recognize particular postsynaptic partners, and finally to establish functional synaptic connections. Molecular and cellular studies for this purpose are currently underway.

II. Receptor-like protein tyrosine phosphatases and brain development

Protein tyrosine phosphorylation is implicated in the various aspects of brain development. The pattern and level of tyrosine phosphorylation of the cellular proteins are controlled by a series of protein tyrosine kinases and protein tyrosine phosphatases (PTPs). In recent years, many classes of receptor-like PTPs (RPTPs) have been cloned and characterized. In 1994, we identified a brain-specific extracellular chondroitin sulfate proteoglycan, 6B4 proteoglycan/phosphacan as a splice variant of a proteoglycan-type RPTP, PTPζ/RPTPβ. PTPζ and 6B4 proteoglycan have been suggested to play some roles in neurite outgrowth, neuronal cell migration and synapse formation. To understand the functions of these molecules, it is crucial to dissect the molecular bases of the signal transductions. Therefore, we attempted firstly to identify the ligands of PTPζ.

Since 6B4 proteoglycan was considered to be identical to the extracellular portion of PTPζ including

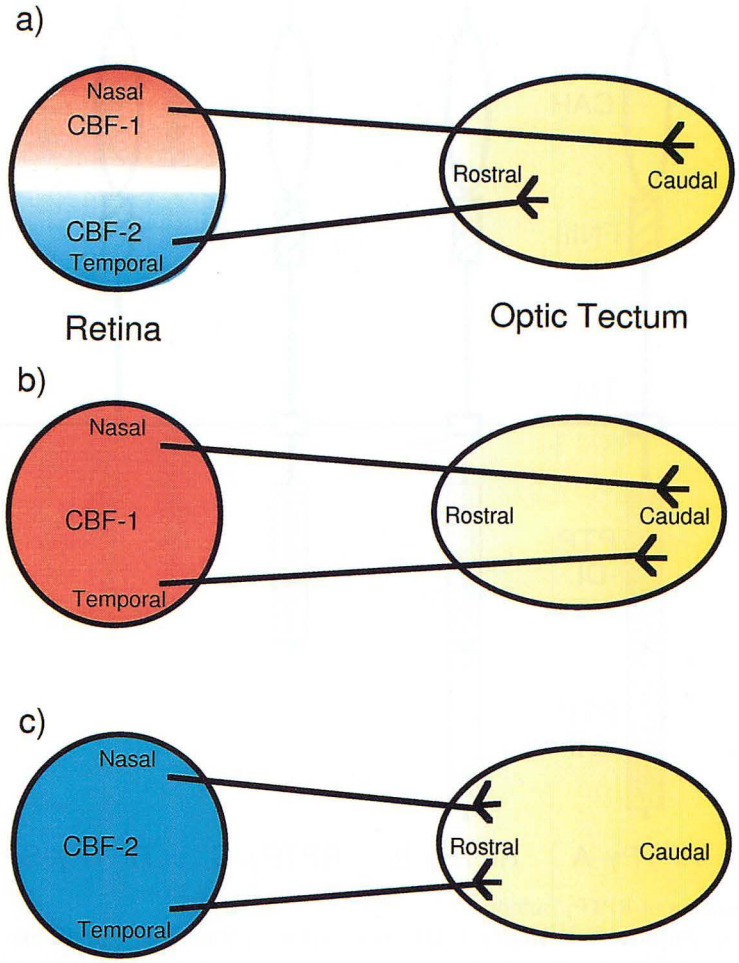

Fig. 1. CBF-1 and CBF-2 specify the retinotectal projection map
(a) CBF-1 and CBF-2 are expressed in the nasal and temporal retina, respectively. (b, c) Recombinant retrovirus-mediated misexpression of each factor changed the retinotectal projection map.

carbohydrate modifications, we used this molecule for affinity chromatography to purify PTPζ-binding proteins. From the CHAPS extract of rat brain microsomal fractions, 18-, 28-, and 40-kDa proteins were specifically isolated using 6B4 proteoglycan-Sepharose. N-terminal amino acid sequencing identified the 18-kDa protein as pleiotrophin/heparin-binding growth-associated molecule (HB-GAM), which was originally isolated from the brain as a mitogenic and neurite-promoting factor. Scatchard analysis of 6B4 proteoglycan-pleiotrophin binding revealed low (Kd = 3 nM) and high (Kd = 0.25 nM) affinity binding sites. Chondroitinase ABC digestion of the proteoglycan reduced the binding affinities to a single value (Kd = 13 nM) without affecting the number of binding sites. This suggested the presence of two subpopulations of the proteoglycan with different chondroitin sulfate structures. The binding of 6B4 proteoglycan to pleiotrophin was inhibited differently by various chondroitin sulfates. Interestingly, in contrast to chondroitin sulfate C which strongly inhibited binding of 6B4 proteoglycan to pleiotrophin (IC_{50} = 400 ng/ml), chondroitin sulfate A had almost no effect. These results suggested that chondroitin sulfate chains participate in the ligand-receptor interaction.

Immunofluorescence analysis indicated that both 6B4 proteoglycan and PTPζ are localized on cortical neurons especially at the growth cones of their extending neurites. Anti-6B4 proteoglycan antibody added to the culture medium strongly suppressed pleiotrophin-induced neurite outgrowth of cortical neurons. Chondroitin sulfate C, but not chondroitin sulfate A, potently inhibited pleiotrophin-induced neurite outgrowth, consistently with observations regarding inhibition of the ligand-receptor interaction. These results suggested that pleiotrophin is a functional ligand for PTPζ.

PTPζ has another family member, RPTPγ, which shows a high degree of structural similarity to PTPζ. Pleiotrophin also has another family member, midkine, which also has mitogenic and neurite-outgrowth promoting activities. Therefore, the ligand-receptor relationship between pleiotrophin, midkine and PTPζ, RPTPγ is an important question which should be addressed. As the first step, we cloned RPTPγ cDNAs to reveal its molecular diversity. cDNA clones

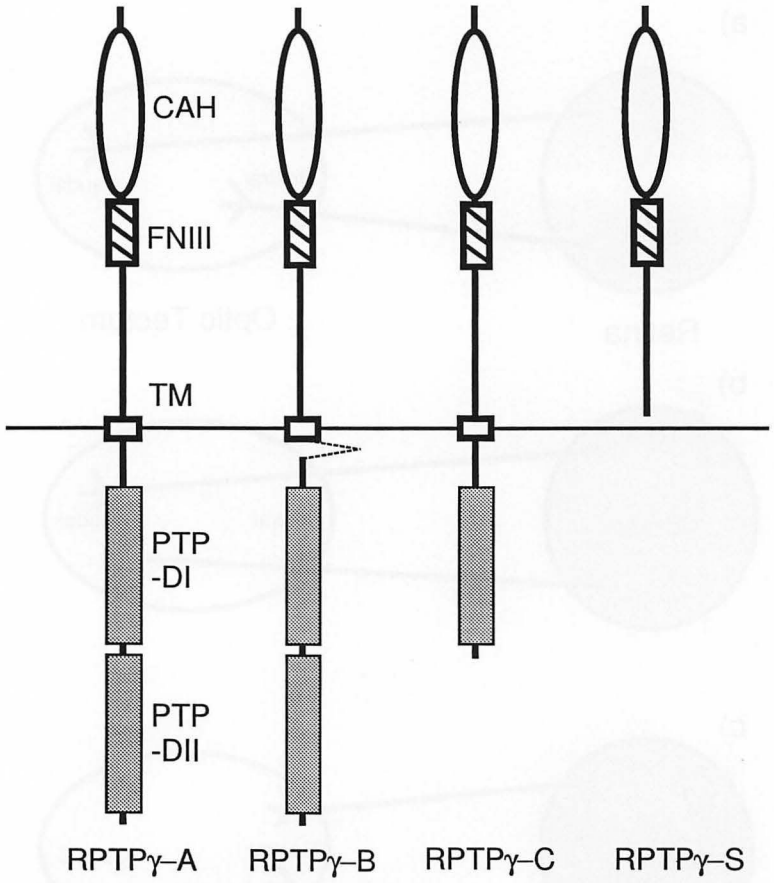

Fig. 2. Schematic representation of RPTPγ isoforms
RPTPγ consists of a carbonic anhydrase-like domain (CAH; open circles), a fibronectin type III-like domain (FN III; hatched boxes), a transmembrane segment (TM; open boxes) and two tyrosine phosphatase domains (PTP-DI and -DII; shaded boxes). The dashed line indicates the deleted sequence in RPTPγ-B. RPTPγ-C possesses only a single tyrosine phosphatase domain, and RPTPγ-S is an extracellular variant of RPTPγ.

for the four splicing variants of RPTPγ were isolated from rat brain cDNA libraries (Fig. 2). We designated these molecules as RPTPγ-A, -B, -C and -S. RPTPγ-A was the longest form and had a similar structure to human and mouse RPTPγ. RPTPγ-B was devoid of the intracellular juxtamembrane 29 amino acids of RPTPγ-A. Recently, this type was independently found in a human kidney-derived cell line, ACHN, by Sorio et al. The other two variants were novel: RPTPγ-C had a single phosphatase domain, and RPTPγ-S was a secretory type of RPTPγ. mRNAs of these four species were expressed in the brain, kidney, lung and heart. Transfection of RPTPγ-A and -S expression plasmids into COS7 cells resulted in the expression of membrane-bound 190- and secretory 120-kDa proteins, respectively. RPTPγ is thus comparable to PTPζ with regard not only to structure but also to the presence of both secretory and transmembrane forms. However, any RPTPγ variants ectopically expressed in these cells did not exist as proteoglycans contrasting to PTPζ variants.

III. Gene targeting of Na-G, a voltage-gated sodium channel expressed in glial cells

Glial cells have been considered to be inexcitable. Despite the lack of electrical excitability, they express voltage-gated sodium channels with properties similar to the sodium channels in excitable cells. The cellular function of these voltage-gated sodium channels is not clear. An *in vitro* study raised the possibility that glial sodium channels serve as a pathway for sodium-ion entry to fuel Na-K ATPase, which requires three sodium ions for transport of two potassium ions. A drop in the intracellular concentration of sodium ion concentration through these channels may hamper ATPase activity. To test this hypothesis and clarify the roles of glial sodium channels *in vivo*, we are currently attempting to generate knock-out mice deficient in the glial voltage-gated sodium channel, Na-G, the cDNA of which was recently cloned. We have successfully produced chimeric mice which contain the targeted embryonic stem-cells contribution.

By applying gene targeting technology to other novel genes expressed in the brain, we intend to investigate the molecular mechanisms underlying the development and function of the brain.

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DIVISION OF CELLULAR REGULATION

Professor: Norio Murata
Associate Professor: Hajime Wada [1] (adjunct)
Research Associates: Atsushi Sakamoto
Yoshitaka Nishiyama
Iwane Suzuki
Monbusho Foreign Scientists: Malay Kumar Ray [2]
Zoltan Gombos [3]
NIBB Fellow: Akira Katoh
JSPS Visiting Scientists: Ding-Ji Shi [4]
Éva Hideg [3]
P. Pardha Saradhi [5]
Diego de Mendoza [6]
Richard A. Dilley [7]
Peddisetty Sharmila [5]
JSPS Postdoctoral Fellows: Alia [5]
Michael P. Malakhov [8]
EC Fellow: Ana Maria Otero Casal [9]
NIBB Visiting Scientists: Yasushi Tasaka
Oxana Malakhov [10]
Monika Debreczeny [3]
Prasanna Mohanty [11]
Byong Yong Moon [12]
Leonid Kislov [8]
Kostas Stamatakis [13]
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Julian Eaton-Rye [17]
Suleyman I. Allakhverdiev [18]
Graduate Students: Sayamrat Panpoom
Masami Inaba
Hiroshi Yamamoto
Yuji Tanaka
Fumiyasu Yamaguchi
Technical Staffs: Sho-Ichi Higashi
Hideko Nonaka

[1] *from Kyushu University, Fukuoka, Japan*
[2] *from Centre for Cellular and Molecular Biology, Hyderabad, India*
[3] *from Biological Research Center, Szeged, Hungary*
[4] *from Institute of Botany, Academia Sinica, Beijing, China*
[5] *from Jamia Millia Islamia University, New Delhi, India*
[6] *from Universidad Nacional de Rosario, Rosario, Argentina*
[7] *from Purdue University, Lafayette, IN, USA*
[8] *from Plant Physiology Institute, Moscow, Russia*
[9] *from Universidad de Santiago, Santiago de Compostela, Spain*
[10] *from Institute of General Genetics, Moscow, Russia*
[11] *from Jawaharlal Nehru University, New Delhi, India*
[12] *from Inje University, Kimhae, Korea*
[13] *from National Centre for Scientific Research "Demokritos", Athens, Greece*
[14] *from International Institute for Genetics and Biophysics, Naples, Italy*
[15] *from Bulgarian Academy of Sciences, Sofia, Bulgaria*
[16] *from Oregon State University, Corvallis, OR, USA*
[17] *from Otago University, Dunedin, New Zealand*
[18] *from Institute of Soil Science and Photosynthesis, Moscow, Russia*

The research effort of this division is aimed at establishing a full understanding of the molecular mechanisms by which plants can acclimate to and tolerate stresses that arise from changes in environment conditions, with particular emphasis on temperature stress and salt stress. In 1996, significant progress was made in research on the following topics in studies with cyanobacteria and higher plants as experimental materials.

I. The temperature-regulated expression of genes for acyl-lipid desaturases in a cyanobacterium

Most living organisms are exposed to changes in the temperature of their environment. In response to a decrease in temperature, an increase occurs in the extent of unsaturation of membrane lipids, which enhances the fluidity of the membranes. This mechanism provides compensation for the decrease in the molecular motion of membrane lipids, which is caused by the decrease in ambient temperature. Individual acyl-lipid desaturases introduce a double bond into fatty acids that are esterified to the glycerol moiety of membrane lipids. The cyanobacterium *Synechocystis* sp. PCC 6803 has four genes, *desA*, *desB*, *desC* and *desD*, that encode the Δ12, ω3, Δ9 and Δ6 acyl-lipid desaturases, respectively. We investigated the regulation of the expression of the genes for these desaturases in response to changes in temperature. We found that low temperatures enhance the steady-state levels of mRNAs for all the desaturases with the exception of the Δ9 desaturase. Functional analysis of promoters, using a bacterial gene for luciferase as a reporter, revealed that, to some extent, it is the activation of the promoters of the genes for the Δ12 and ω3 desaturases that is responsible for the increases in the levels of their mRNAs after a downward shift in temperature. In addition, the lifetimes of the mRNAs for the Δ6, Δ12, and ω3 desaturases were extended at lower temperatures, indicating that the stability of mRNAs might also be involved in the control of the levels of the desaturases. Western blotting analysis demonstrated that the levels of the Δ6, Δ12 and ω3 desaturases increase at low temperatures, while the level of the Δ9 desaturase remains constant. These results indicate that the expression of the gene for the Δ9 desaturase is basically independent of temperature, while the expression of the genes for the Δ6, Δ12, and ω3 desaturases is regulated by temperature.

II. Localization of acyl-lipid desaturases in cyanobacterial cells

An immunocytochemical study was performed in an attempt to localize the four acyl-lipid desaturases in *Synechocystis* sp. PCC 6803 using antibodies raised against synthetic oligopeptides that corresponded to the carboxyl-termini of the individual desaturases. The individual preparations of antibodies were specific to the respective desaturases. Immunogold labeling and electron microscopy revealed the specific

distribution of the desaturases in regions that corresponded to both the cytoplasmic and the thylakoid membranes, suggesting that all four desaturases are located in both types of membrane. Localization of the desaturases in thylakoid membranes was further confirmed by Western blotting of proteins from isolated membranes. These findings indicate that the desaturation of the fatty acids of membrane lipids occurs within the thylakoid membranes as well as within the cytoplasmic membranes in the cyanobacterial cells.

III. The importance of polyunsaturated membrane lipids in photosynthesis

We demonstrated previously that polyunsaturated membrane lipids are important in the ability of the photosynthetic machinery to tolerate low temperatures by targeted disruption of genes for desaturases in *Synechocystis* sp. PCC 6803, in which all polyunsaturated fatty acids of membrane lipids were replaced, as a result, by monounsaturated fatty acids. To confirm this finding using an alternative approach, we introduced the *desA* gene for the Δ12 desaturase of *Synechocystis* sp. PCC 6803 into cells of *Synechococcus* sp. PCC 7942 that contained monounsaturated fatty acids but no polyunsaturated fatty acids. As the result of this transformation, half of the monounsaturated fatty acids in the membrane lipids were replaced by diunsaturated fatty acids. Comparison of the transformed cells with the wild-type cells revealed that the increase in the number of double bonds in the membrane lipids enhanced the ability of the cells to resist photoinhibition at low temperatures by accelerating the recovery of the photosystem II complex from photoinhibitory damage. These findings indicate that polyunsaturated fatty acids are important in the protection of the photosynthetic machinery from damage by strong light at low temperatures.

IV. Acclimation to heat of the photosynthetic machinery

The evolution of oxygen is one of the reactions of photosynthesis that is most susceptible to high temperature. The molecular mechanism underlying the stabilization of the photosynthetic machinery against heat-induced inactivation has been studied in the cyanobacterium *Synechococcus* sp. PCC 7002. We demonstrated previously that cytochrome c_{550}, located in the lumen of thylakoids, is involved in the stability of the oxygen-evolving machinery at high temperatures. We searched for another factor that might enhance the heat stability and recently identified a protein of 13 kDa as an important factor. The gene

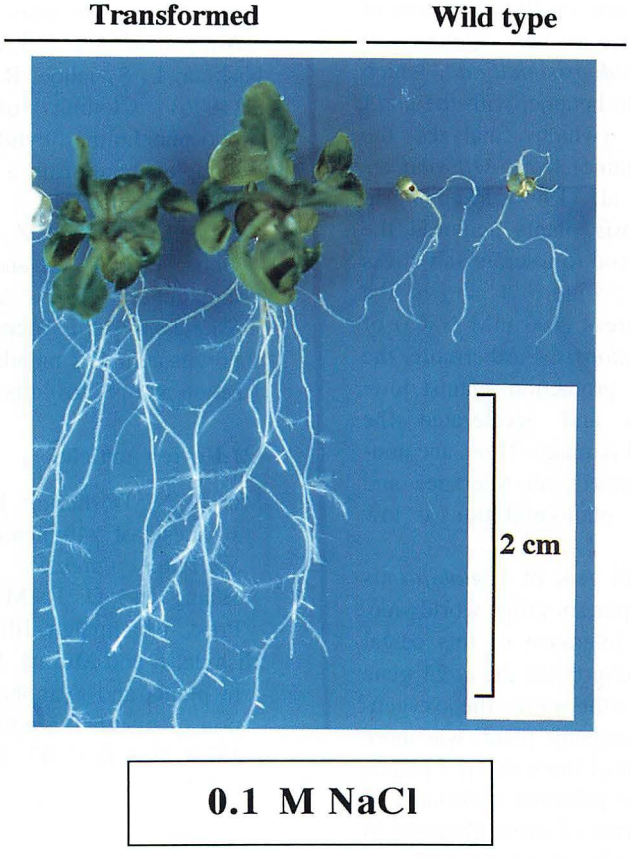

Fig. 1. Effects of salt stress on the growth of seedlings after germination of seeds of wild-type and transformed *Arabidopsis thaliana*. Seeds from wild-type and transformed plants were germinated on a plate of Murashige-Skoog medium supplemented with 0.1 M NaCl and solidified with Gellan gum, and incubated at 22°C for 20 days.

encoding the 13-kDa protein was cloned from *Synechococcus*, and the deduced amino-acid sequence revealed that this protein is homologous to the PsbU protein, an extrinsic protein of photosystem II, which has previously been found in thermophilic species of cyanobacteria. Inactivation of the gene in *Synechococcus* sp. PCC 7942, by insertion of an antibiotic-resistance gene cartridge, had no significant effect on the oxygen-evolving activity or on the photoautotrophic growth at temperatures within the physiological range. However, when mutant cells and wild-type cells that had been grown at a physiological temperature were exposed to a higher temperature, the oxygen-evolving activity was lost much more rapidly from the mutant cells than from the wild type. These results indicate that the PsbU protein plays an important role in stabilizing the oxygen-evolving machinery at high temperatures.

V. Genetic enhancement of stress tolerance in *Arabidopsis thaliana* and in rice plants by genetic engineering that allowed the biosynthesis of glycinebetaine

Glycinebetaine (hereafter abbreviated as betaine) is a compatible solute that is found in a number of halotolerant species of plants and bacteria. It has been implicated in the protection of cellular functions against salt and other types of environmental stress. To examine the effect of betaine on the protection of the photosynthetic machinery *in vivo* against salt stress, we transformed *Arabidopsis thaliana*, which does not normally accumulate betaine, with the *codA* gene for choline oxidase (which catalyzes the conversion of choline to betaine) from *Arthrobacter globiformis* (H. Hayashi et al. (1997) *Plant J.*, in press). Transgenic *Arabidopsis* plants acquired the ability to synthesize betaine and to tolerate salt stress throughout their life cycle. Figure 1 shows the enhanced tolerance to salt stress (100 mM NaCl) of seedlings of transgenic *Arabidopsis*. Furthermore, the transformation enhanced the protection against low-temperature photoinhibition and accelerated the recovery from photo-induced damage. Thus, accumulation of betaine both enhanced salt tolerance and contributed to resistance to photoinhibition at low temperature.

We also introduced the *codA* gene of *A. globiformis* into rice, one of the most important crops worldwide, aiming to enhance the salt tolerance of this cereal crop. Transgenic rice plants expressed the *codA* gene and accumulated betaine. Furthermore, the oxygen-evolving machinery of the transgenic plants was more tolerant to salt stress than that of the wild-type plants. These results demonstrate the potential usefulness of the *codA* gene in the engineering of stress tolerance in a wide variety of agronomically important crops.

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DIVISION OF BIOLOGICAL REGULATION AND PHOTOBIOLOGY (ADJUNCT)

Professor (adjunct): Kimiyuki Satoh
Associate Professor (adjunct): Hirokazu Kobayashi
Research Associate: Noritoshi Inagaki
NIBB Fellow: Hiroshi Kuroda
*Graduate Students: Yoshihiro Narusaka**
*Tatsuya Tomo**
*Akihiko Tohri**

*(*from Okayama University)*

From an energetic point of view, the conversion of radiant energy into chemical energy in photosynthesis is the most important biological process on our planet. The highly efficient energy conversion in this process is ensured by the highly ordered organization of molecules in the photochemical reaction center, in a physical, chemical and biological sense. The project in this division is aiming to elucidate the dynamic as well as static organization of molecules in the photosystem II (PSII) reaction center (RC) of oxygenic photosynthesis which has a unique property to generate a strong oxidant leading to the extraction of electrons from water for the synthesis of organic compounds.

In the first approach, molecular organization of the PSII RC, which has been identified in our study, will be analyzed by biochemical and biophysical techniques which include crystallographic analysis, chemical modification & cross-linking analysis and optical & EPR spectroscopies. Structure-functional analysis for the constituent subunits will also be conducted using random and site-directed mutants of transformable algae, *Synechocystis* PCC 6803 and *Chlamydomonas reinhardtii*. The principal target of these analyses is the structure and molecular environment of the RC chlorophylls, P-680, which determine the redox potential of this photosystem.

In the second approach, the effort will be focused on the elucidation of molecular mechanism of light-regulated metabolic turnover of a subunit of the PSII RC, D1 protein. Some of unique phenomena are involved in this metabolic process; *i.e.*, photo-damage of the function of photochemical RC, specific degradation by proteases of a photo-damaged protein subunit, light-regulated gene expression at the translational level, post-translational cleavage of the C-terminal extension and incorporation of cofactors and subunits into multi-component pigment-protein complexes.

I. Structural organization of photosystem II reaction center

(1) The purified PSII RC contains D1 and D2 proteins, α and β subunits of cytochrome *b*-559 (Cyt*b*-559) and the *psbI* gene product. It also contains a number of cofactors important in the primary processes of PSII, such as chlorophyll *a* (Chl *a*), pheophytin *a* (Pheo *a*) and β-carotene (β-Car), as well as cytochrome *b*-559 heme. Taking advantage of biased distributions of amino acids in the constituent protein subunits, we have conclusively demonstrated that they are present in an equimolar ratio in the isolated complex. We also have re-evaluated the pigment stoichiometry in the isolated PSII RC, by HPLC and ICP emission spectroscopy, to be 6/2/2/1 for Chl *a*/Pheo *a*/β-Car/Cyt*b*-559.

(2) Pheo *a* molecule on the inactive branch of photochemical electron transport system in the isolated PSII RC was identified by fluorescence excitation spectroscopy at 77K, and confirmed by absorption, linear dichroism (LD) and magnetic circular dichroism spectra (in collaboration with Dr. Mimuro). When the emission was monitored at 740 nm, photochemically active Pheo *a* was detected as peaks at 418, 513, 543, and 681 nm, as described in previous reports. However, when the emission was monitored at 665 nm, two bands were observed at 414 and 537 nm; these bands were insensitive to photochemical reduction of the primary acceptor and thus appear to be due to photochemically inactive Pheo *a*. Two β-Car molecules in the complex were also discriminated by fluorescence excitation and LD spectra. These observations were discussed in the light of current understanding of the molecular organization of pigments and the relationship of pigment arrangement to the optical properties in the PSII RC.

(3) Pigments in the purified PSII RC were extracted with diethyl ether containing varied amount of water. As mentioned above, the preparation originally contained approximately six Chl *a* two β-Car and two Pheo *a* per one photochemically active Pheo *a*. The treatment with 30 - 50% water-saturated ether solution extracted one β-Car, as well as one Chl a absorbing at 677 nm, remaining 60% of the photochemical activity to reduce Pheo *a*. With 60 - 80% water-saturated ether, almost all of the β-Car in the RC was extracted. However, nearly 50% of the photochemical activity was retained in the complex, after the ether treatment. The absorption, fluorescence excitation and LD spectra demonstrated the presence of two spectral forms of β-Car in the PSII RC. The short-wavelength form of β-Car, with absorption peaks at 429, 458 and 489 nm, was selectively extracted with ether at the low water content, whereas the long-wavelength form, with peaks at 443, 473 and 507 nm, was extracted only at the higher water content. The extraction of carotenoid enhanced the photobleaching of Chl. Based on the photobleaching experiments it was suggested that Chl *a* forms with peaks at 667 and 675 nm are located close to the short-wavelength form of β-Car that can transfer excitation energy to the photoactive Pheo *a* on the D1 protein. Based on these observations, the topology of pigments in the PSII RC was discussed.

(4) Isolated PSII RC contains no functional plastoquinone, Q_A and Q_B. However, externally added quinones, such as dibromomethylisopropyl benzoquinone (DBMIB), serve as the electron acceptor. The

present study provided evidence that DBMIB fully replaces the function of Q_A and rapidly oxidized $Pheo^-$ even at 77 K (in collaboration with Dr. Itoh). The use of DBMIB in the nanosecond spectroscopy at 77 K enabled us to inspect the spectrum of the $P680^+(DBMIB^-)$ state that is fully reversible and contains neither the absorption change of the excited states nor the irreversible bleaching of the other pigments. The feature of the spectrum was discussed in relation to the difference spectra of $^3P680/P680$ and $Pheo^-/Pheo$ couples measured at 77-280 K.

II. Dynamic aspects of molecular organization

(1) The light-regulated synthesis of the D1 protein in PSII RC was analyzed using isolated pea chloroplasts. In the presence of externally added ATP in darkness, isolated chloroplasts accumulated two proteins of about 22 and 24 kDa which precipitate with specific antibodies raised against the D1 protein. By chasing in the light, these proteins disappeared concomitant with the appearance of the precursor and the mature forms of D1 protein. The pulse-chase experiment in the light and the polysome analysis unequivocally concluded that these two components were the paused intermediates of D1 protein. An unexpected observation was that these translational intermediates accumulated in illuminated chloroplasts by the combined addition of ATP and inhibitors of photosynthetic electron transport, such as atrazine, 3-(3,4-dichlorophenyl)-1,1-dimethylurea and 3,5-dibrome-4-hydroxybenzonitrile. This indicates that the synthesis of the full-length D1 protein requires a factor(s) generated by the operation of photosynthetic electron transport. The accumulation of the full-length D1 protein, however, was induced even in the presence of inhibitory concentration of atrazine in the light when both 2,6-dichlorophenolindophenol and ascorbate, a system donating electrons to the photosystem I (PSI), were also present, and in darkness as well upon the addition of a reductant, dithiothreitol (DTT), suggesting that the translation of D1 protein is regulated via redox change of a component(s) around the PSI.

The accumulation of these two intermediates was also observed by pulse-labeling in *in vitro* run-off translation system in darkness using thylakoid-bound polysomes and oxidized S100 fraction prepared from pea chloroplasts. However, the synthesis of full-length D1 protein was achieved in this system by the addition of DTT-reduced stroma fraction. The fractionation of DTT-reduced stroma by a gel-filtration column

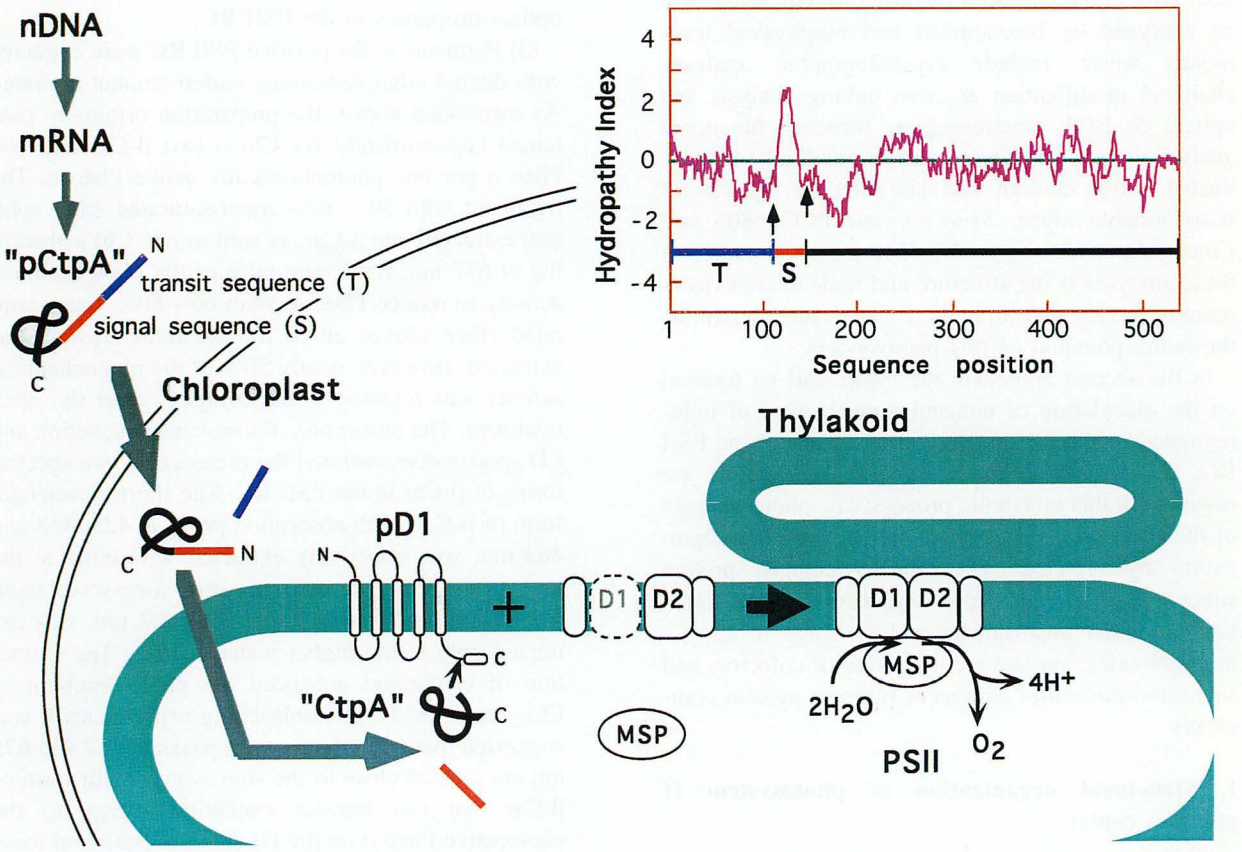

Fig. 1. A schematic drawing of maturation steps and function of C-terminal processing protease (CtpA) for the precursor D1 protein. The CtpA is synthesized in cytosol as a precursor with the N-terminal extension, consisting of a transit sequence (blue; T) and a signal sequence (red; S), for translocation into thylakoid lumen. The signals on the pCtpA are excised in two steps during the import, as shown in the figure. The mature CtpA cleaves off C-terminal extension of the pD1 protein as an integral event for the organization of oxygen evolving machinery in the PSII. Inset shows a hydropathy plot of the spinach pCtpA by a program of Kyte and Doolittle (1982). Two arrows indicate putative cleavage sites for the maturation of pCtpA.

demonstrated that the major activity corresponded to the molecular mass of about 40 kDa. The fractionation by DEAE anion-exchange chromatography and by ammonium sulfate precipitation of the active fraction indicated that the purification of this redox-responding regulatory factor is possible by the standard biochemical procedures, although further steps of purification is evidently required at present.

(2) The D1 protein of PSII RC is synthesized as a precursor that is processed by cleavage at the C-terminus. The C-terminal processing is necessary for the assembly of water-oxidizing machinery (Mn-cluster). The enzyme involved in this process was purified from sonicated extracts of thylakoids, by a method that includes chromatography on QAE-anion exchange, hydroxylapatite, Cu-chelating affinity and gel-filtration columns. Based on the amino acid sequence data of the purified protease, cDNA clones encoding the enzyme was identified and sequenced, from a spinach green leaf cDNA library. By these analysis, the full-length transcript was established to consist of 1906 nucleotides and a poly (A) tail, containing an open reading frame (ORF) corresponding to a protein with 539 amino acid residues. By comparing the amino acid sequence of the purified protease with that deduced from nucleotide sequence of the cDNA clones, the enzyme was shown to be furnished with an extra amino-terminal extension consisted of 150 amino acids which is characteristic of both a transit peptide and a signal sequence. This suggests that the protease is synthesized in the cytosol and translocated into the lumenal space of thylakoids (Fig. 1). The enzyme can be expressed in *E. coli* in its active form and purified in homogeneity.

Three sorts of compounds were used as substrate for the analysis of enzymatic activity, *i.e.*, (i) synthetic oligopeptides corresponding to the C-terminal sequence of the precursor D1 protein, (ii) *in vitro* transcribed/translated precursor D1 protein, and (iii) *in vivo* synthesized precursor D1 protein in thylakoid membranes. A series of substitutions at Ala-345, for example, were shown to have marked effects on the value of Vmax, in the previous analysis. Mixed culture experiments using genetically-engineered *Chlamydomonas* mutants substituted at this position (from Ser to Val or Gly) demonstrated that these substitutions seriously affect the viability of this organism. No specific inhibitor, except for substituted C-terminal oligopeptides of precursor D1 protein, could be found for the enzyme. The identification of the catalytic center of this unique enzyme is now in progress.

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DIVISION OF BEHAVIOR AND NEUROBIOLOGY (ADJUNCT)

Professor: Masatoshi Takeichi
Research Associates: Akinao Nose
Kazuaki Tatei
Institute Research Fellow: Emiko Shishido
JSPS Postdoctoral Fellow: Takako Isshiki
Graduate Students: Hiroki Taniguchi
Takeshi Umemiya (from Kyoto University)

How individual nerve cells find and recognize their targets during development is one of the central issues in modern biology. The aim of our division is to elucidate the molecular mechanism of axon guidance and target recognition by using the simple and highly accessible neuromuscular system of *Drosophila*.

The musculature of *Drosophila* embryos consists of 30 identifiable muscle fibers per hemisegment. Each muscle fiber is innervated by a few motoneurons in a highly stereotypic manner. The high degree of precision and previous cellular manipulations of neuromuscular connectivity suggest the presence of recognition molecules on the surface of specific muscle fibers which guide the growth cones of motoneurons. We have previously isolated several enhancer trap lines that express the reporter gene β-galactosidase (β-gal) in small subsets of muscle fibers prior to innervation. By molecularly characterizing these lines, we are trying to identify genes that play roles in the specification of the muscles and neuromuscular connectivity. Previous studies showed that two of the lines are insertions in the *connectin* and *Toll* genes, that encode cell recognition molecules which belong to the leucine-rich repeat (LRR) family. We have been studying the function of these genes, and also characterizing other lines by molecular and genetic analysis.

I. Connectin can function as an attractive target recognition molecule

Connectin is expressed on a subset of muscle fibers (primarily lateral muscles) and on the axons, growth cones of the motoneurons which innervate these muscles (primarily SNa motoneurons) and on several associated glial cells. When coupled with its ability to mediate homophilic cell adhesion *in vitro*, these results led to the suggestion that Connectin functions as an attractive signal for SNa pathfinding and targeting.

To study the role of Connectin *in vivo*, we ectopically expressed Connectin on all muscles by using MHC (myosin heavy chain) promoter (*MHC-connectin*) in the P-element mediated transformants. In *MHC-connectin*, SNa nerves were observed to send extra axon branches that form ectopic nerve endings on muscles 12, muscles they would never innervate in wild type. This phenoype was highly penetrant and was observed in over 60% of the segments examined. Furthermore, the ectopic innervation on muscle 12 was dependent on the Connectin expression on SNa. These results showed that Connectin functions as an attractive and homophilic guidance molecule for SNa *in vivo*.

II. P750, a novel LRR cell surface molecule expressed on subsets of neurons and muscles

We have been conducting molecular and genetic analysis of other muscle enhancer trap lines. One of them, P750 expresses β-gal in subsets of neurons and muscles, including the RP5 motoneuron and its target, muscle 12. The cDNA cloning and sequencing revealed that *P750* encodes a novel transmembrane protein that belongs to LRR family. It is interesting that three of the five muscle enhancer trap lines that we have thus far characterized contain LRRs. Within the LRR family, P750 was found to be most related to the *Drosophila tartan* gene that have been implicated in neural and muscular development. We have recently found that in the loss-of-function mutant of *P750*, the synaptic arborization pattern on muscle 12, a *P750*-expressing muscle, is abnormal, suggesting that this molecule play some roles in neuromuscular target recognition and/or stabilizaion of the synapses.

III. *msh*, a homeobox containing gene essential for neural and muscular development

Another line rH96 was found to be a P-element insertion in the *muscle segment homeobox (msh)* gene, that was previously cloned as a homeobox containing gene. By generating and analysing both loss-of-function and gain-of-function (ectopic expression) mutants, we showed that *msh* is essential for neural and muscular development. During CNS development, *msh* is specifically expressed in the dorsal neuroectoderm (Fig. 1A) and subsequently in many neuroblasts and their progeny derived from this region (Fig. 1B). We found that the loss of *msh* results in the failure of the proper differentiation of many neural and glial progeny derived from the dorsal neuroectoderm. Conversely, ectopic expression of *msh* in the entire neuroectoderm severely disrupts the formation of midline structure and differentiation of neuroblasts located in the ventral neuroectoderm. These results suggest that *msh* plays crucial roles in the dorso-ventral (DV) specification of the CNS. The vertebrate homologues of *msh*, *Msxs* are also know to be expressed in the dorsal portion of the spinal cord. Our work on *msh* raises a possiblity that this family of genes may plays a conserved role during DV patterning of the CNS.

IV. M-spondin and G-spondin: a novel gene family of secreted molecules

By molecularly characterizing another enhancer trap line, AN34 which is also expressed in a subset of muscles and neurons, we identified a novel secreted protein, termed M-spondin, that is highly homologous to rat F-spondin. F-spondin is a secreted molecule expressed at high levels in the floor plate and has been shown to promote neural cell adhesion and neurite

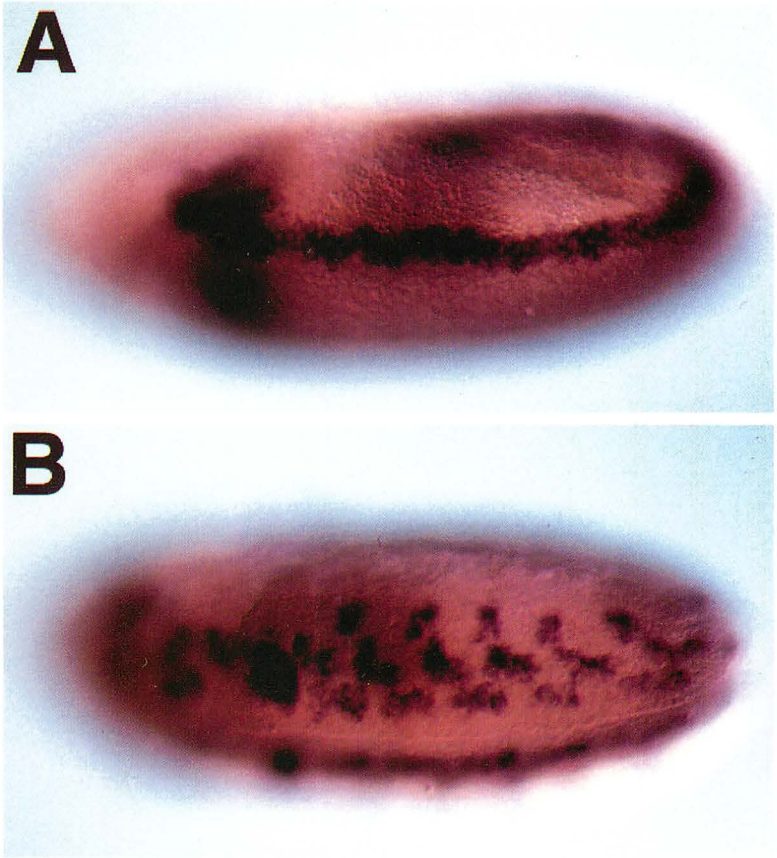

Fig. 1. The expression pattern of *msh* mRNA. Stage 8 (A) and stage 11 (B) whole mount *Drosophila* embryos hybridized in situ with *msh* riboprobe.

extension *in vitro*. We found three regions that are highly conserved between M-spondin and F-spondin. One of them is a known repeating motif called thrombospondin type I repeats (TSRs). The other two domains (termed FS1 and FS2) are novel conserved sequences that we identified. By using PCR, we cloned two more genes that share similar overall structure with M-spondin and F-spondin in that they possessed FS1, FS2 and one to six TSRs. The idintification of these genes thus defines a novel gene family of secreted protein with potential roles in cell adhesion. One of the newly cloned genes, termed G-spondin, is expressed in a subset of glia that sit along the longitudinal axon tracts in the CNS. The specific expression pattern of G-spondin suggests that it may play a role in the guidance of specific axons.

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LABORATORY OF GENE EXPRESSION AND REGULATION

Head: Yoshitaka Nagahama

The laboratory consists of four regular divisions and conducts research into regulatory mechanisms of gene expression in higher plants and animals.

DIVISION OF GENE EXPRESSION AND REGULATION I

Professor: Shigeru Iida
Research Associates: Rie Terada
Yoshiki Habu
Graduate Students: Yasuyo Hisatomi
Atsushi Hoshino
Technical Staff: Sachiko Tanaka-Fukada

This group's move along the Tokaido line from Department of Biological Science and Technology, Science University of Tokyo, begun in April and its formation continued throughout the year. The main interest of the group is in understanding the biology of dynamic genome, namely, genome organization and reorganization and their impact on gene expression and regulation.

Although there are many elements affecting organization and reorganization of the genomes, we have currently focused on mobile genetic elements in general and plant transposable controlling elements in particular.

I. Identification and characterization of mutable alleles in the Japanese morning glory

The Japanese morning glory (*Pharbitis nil* or *Ipomoea nil*) is a traditional horticultural plant in Japan, and extensive physiological and genetical studies on the plant have been conducted. Among several mutable loci that condition variegated flower phenotypes, two mutable alleles, *flecked* and *speckled* have been chosen for molecular elucidation of the variegated phenotypes.

The *flecked* mutant bearing white flowers with colored flecks and sectors (**Fig. 1A**). The flecking was regarded as recurrent somatic mutation from the recessive white to the dominant pigmented allele, accompanied by changes in genotypes from the homozygous recessive to the heterozygous condition. We have molecularly characterized the *flecked* mutation in one of the *anthocyanin* genes, *A-3*, and found that the mutable *a-3*^{flecked} (*flecked*) allele carries a transposable element of 6412 bp, termed *Tpn1*, in the anthocyanin biosynthesis gene for dihydroflavonol 4-reductase (DFR). Among the three copies of the *DFR* gene in the genome of the Japanese morning glory, *Tpn1* resides within the second intron 9 bp upstream of the third exon of the *DFR-B* gene. The flower variegation is shown to be due to somatic reversion of the *DFR-B* gene by *Tpn1* excision (see Inagaki et al. (1996) Theor. Appl. Genet. **92**, 499-504). Structural characteristics revealed that *Tpn1* belongs to *En/Spm* family of elements and that it is likely to be a non-autonomous element deficient to produce active transposases.

It is known that the frequency and the timing of the flecking phenotypes in the mutable *a-3*^{flecked} line are generally heritable by their progeny but sometimes conversion of these phenotypes is also observed. Thus the flecking phenotypes caused by the excision of *Tpn1* from the *DFR-B* gene may be determined either by *trans*-acting activities of its related autonomous element or by heritable modifications of *Tpn1* itself. We are currently focusing on these lines of investigations.

Fig. 1B shows another flower variegation caused by the mutable *speckled* allele. Our genetic studies indicate that another element termed *speckled-activator* acting in *trans* on the *speckled* allele is necessary to confer the speckled variegation phenotype. Our current hypothesis is that the mutable *speckled* allele carries a non-autonomous transposable element and *speckled-activator* must be a related autonomous element. To test this hypothesis, we are trying to identify the mutable *speckled* allele.

II. Identification of the mutable alleles in the common morning glory

The mutable *a*^{flaked} line of the common morning glory (*Pharbitis purpurea* or *Ipomoea purpurea*) also bears white flowers with colored flakes and sectors (**Fig. 1C**). The mutable *a*^{flaked} allele is known to exhibit incomplete dominance. Interestingly, not only intensely colored flakes but also white spots and sectors were often observed in lightly colored flowers of morning glory with the heterozygous state *A/a*^{flaked}. To understand these allelic interactions in molecular terms, we are trying to identify the mutable *a*^{flaked} allele. Although several new mobile genetic elements have been found in the mutable line, we are still in process of identifying the mutable *a*^{flaked} allele.

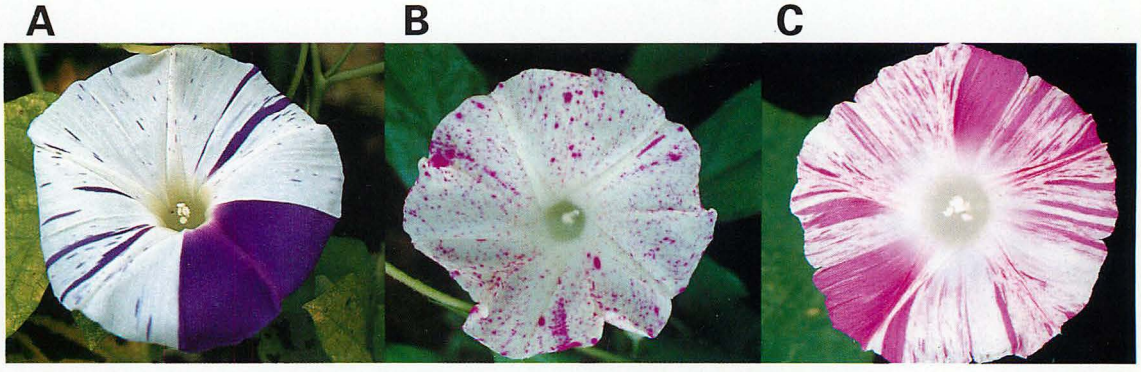

Fig.1 Flower variegation. (**A**) *a-3*^{flecked}; (**B**) *speckled*; (**C**) *a*^{flaked}.

DIVISION OF GENE EXPRESSION AND REGULATION II

Professor: Takashi Horiuchi
Research Associates: Masumi Hidaka
Takehiko Kobayashi
Ken-ichi Kodama
Katsuki Johzuka
Graduate Students: Katufumi Ohsumi
Keiko Taki
Hiroko Urawa
Technical Staffs: Kohji Hayashi
Yasushi Takeuchi

Homologous recombination which may occur in all organisms apparently involves exchange between two parent-derived chromatids plus the repair of DNA damage incurred by physical and chemical reagents. As deduced from our analyses of recombination hotspots *of E. coli* and *S. cerevisiae,* in particular the activity related to DNA replication fork blocking events, the physiological function of homologous recombination, especially in normally growing cells is better understood. In 1996, work on the three following interrelated subjects has advanced our knowledge of related factors.

I. Analysis of *E. coli* recombinational hot spots

In *E. coli* RNase H defective (*rnh*$^-$) mutants, we found specific DNA fragments, termed Hot DNA, when DNA in the ccc form is integrated into the *E. coli* genome by homologous recombination to form a directly repeated structure and a strikingly enhanced excisional recombination occurs between the repeats. We identified 8 groups (HotA-H) of such Hot DNA, 7 of which (HotA-G) were clustered in a narrow region, known as replication terminus region (about 280 kb) located on the circular *E. coli* genome. Analysis of HotA, B and C revealed that blocking of replication fork at the *Ter*, replication terminus, sites is responsible for these Hot activities. Further analysis led to design of a putative model, in which the ds (double stranded)-break occurs at the fork arrested at the DNA replication fork blocking (*Ter*) site. Through this site the RecBCD recombinational enzyme complex enters the ds-DNA molecule and enhances recombination between directly repeated Hot DNA when the enzyme complex meets an appropriately oriented recombinational hotspot sequence, Chi.

To determine how other termination event-independent Hots can be activated, we chose HotH, because it locates only outside of replication terminus region. HotH is a 11.2 kb *Eco*RI DNA fragment and locates at about 91 minutes on the *E. coli* genetic map. We found that it contains the 3' end region of an rRNA operon, *rrnD*, and also a Chi sequence at the downstream region of *rrnD*. HotH activity was abolished, when a mutation was introduced into the Chi sequence to destroy its activity, which means that Chi is probably required for HotH activity. We are now investigating events which occur near the HotH site and which probably provide an entrance site for RecBCD enzymes.

II. Atomic structure and functional model of *E. coli* replication terminator protein complexed with *Ter* DNA

Replication of prokaryotic chromosomes is terminated in a defined terminus region. In *E. coli*, this termination is mediated by a site-specific DNA-binding protein designated as Tus (or tau). The Tus protein (MW 36 kD) is a monomeric molecule that binds to six specific sequences (*Ter*) within the replication terminus region. The Tus-*Ter* complex halts passage of replication machinery in only one direction and allows passage in the other direction. This complex controls the passage of DnaB helicase, a constituent of the replication machinery. It is imperative to know the three-dimensional (3D-) structure to gain insight into the mechanism, by which the Tus protein recognizes *Ter* sites and blocks the replication fork, in a polar manner. Toward this end, we are doing crystallographic studies of the Tus protein in a complex with *Ter* DNA in collaboration with Drs. K. Kamada and K. Morikawa (Protein Engineering Research Institute, Osaka).

The most suitable crystals for X-ray analysis were grown from a mixture of the Tus protein and a 16 base long DNA duplex, by microdialysis against PEG solution. The crystal structure was determined at 2.7 A resolution by the multiple isomorphous replacement method and anomalous scattering (MIRAS) and has been refined to provide an R-factor of 17.0%.

Tus protein adopts an unique structure, as compared with other DNA binding proteins (Figure 1). The structure is divided in two domains, both of which are classified as $\alpha + \beta$ type. These domains form a positively charged central cleft which mainly accommodates the *Ter* DNA elements, as if two protruded α-helical regions on both sites and a central β-sheet region would embrace the DNA duplex. The most remarkable feature of the complex is an extensive protein-DNA interface which involves many direct and indirect polar interactions. The two β-sheets of both domains and the interdomain antiparallel β-strands are jointly responsible for recognition of the DNA in the major groove. This structure motif, while unique in detail, is similar to those of MetJ and Arc. The *Ter* DNA substantially deviates from the canonical B-DNA in the vicinity of central β-bulges of the interdomain β-strands inserted into the major groove. This local deformation of the *Ter* DNA makes for an overall bend of 20°.

Genetic approaches facilitate the isolation of mutant Tus proteins and most single mutations are mapped to the interdomain region. These mutants that affect efficiency of replication arrest partially or completely impair binding of the Tus protein with DNA. Therefore, the replication arrest and Tus-*Ter* DNA binding seem to be inseparable.

The overall structural features of the complex favor

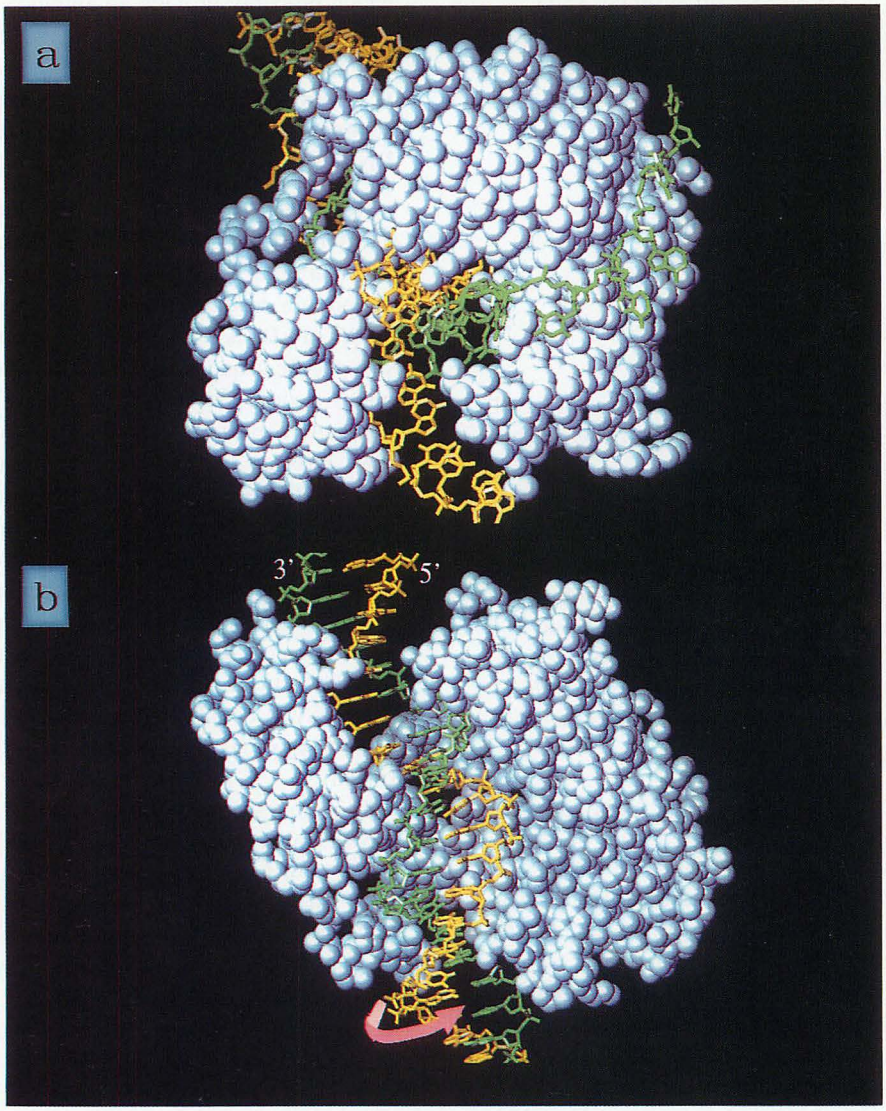

Figure 1. (a) Tus complexed with a putative Y-forked DNA, as viewed from the fork-block site. The single stranded DNAs near the terminus were hypothetically constructed to realize inhibition of fork progression. (b) A view from the free-passage side. Arrow (pink) denotes the putative unwinding motion of DNA. The unwinding of the helicase from the lower right would displace the yellow DNA strand.

the interpretation that the α-helical regions of both domains on the fork-blocking side act as a directional DNA clamp and prevent the passage of replication machinery along DNA (Figure 1). Presumably, the passing mechanism from the opposite side relies upon the structural disturbance of the β-sheet region, as induced by the unwinding motion of helicases. This interpretation fits the physical collision model that the intrinsic structure of the complex dominates the polar arrest of the replication fork.

III. The *E. coli* genome project

E. coli genome project in Japan was started in 1989, the objective being to analyze, in an independently living organism, nucleotide sequences from 0 min in a clockwise direction. In April 1995, the DNA sequences of the 0-12.7 min region in the genetic map were determined and they were published or registered. At that time I (T. H) was appointed leader of the Japan *E. coli* genome project and a new group of researchers was reorganized to analyze DNA sequences of the region from 12.7 min to 70 min, using Kohara lambda clones and a new protocol, including a shotgun sequence technique.

In January 1997, we determined about a 2.2 mega nucleotide sequence of a region corresponding to 12.7 -69.0 min (including DNA replication terminus region). Combining these data with those (69.0-100 min) provided by Fred Blattner in the USA, the entire *E. coli* genome was expressed in a single, continuous nucleotide sequence (4,636,552 bp), in which 3803 putative open reading frames were identified. Analysis of the genomic structure by computer is in progress. At this same time, Blattner's group determined independently the entire *E. coli* DNA sequence. Our sequence data, registered in DNA Data Bank of Japan (DDBJ) are also available through Web sites

(http://mol.genes.nig.ac.jp/ecoli/, http://www.ddbj.nig.ac.jp/, http://bsw3.aist-nara.ac.jp/).

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DIVISION OF SPECIATION MECHANISMS I

Professor: Tetsuo Yamamori
Research Associates: Satoshi Koike
Yuriko Komine
Akiya Watakabe
Institute Research Fellow: Takashi Kitsukawa
Visiting Scientist: Kimiko Yamamori
Graduate student: Kotarou Torii (Hokuriku Advanced Technology and Science University)
Technical Staffs: Hideko Utsumi
Miki Ida

Our research goal is to understand mechanisms underlying evolution of the nervous system. In order to approach this question, we are currently focusing on two systems.

I. Evolution of cytokine receptor families in the immune and nervous systems

It has been recognized that cytokines, defined as inter-cellular mediators in the immune system, have a variety of roles in the nervous system as well. One such a factor, LIF (leukemia inhibitory factor) known also as CDF (Cholinergic Differentiation Factor), is a pleiotropic factor which shows a remarkable repertoire of activities from embryonic stem cells to neurons (Yamamori, T., 1996). Recent study have revealed that CDF/LIF and its receptors belong to the IL-6 family and the receptor family.

Based on Bazan's model which predicted the cytokine receptor family as a member of immunoglobulin super gene family (1990) and the model of the interaction among the members of the IL-6 family (ligand) and the IL-6 receptor family (Taga and Kishimoto, 1992; Stahl and Yancopoulos, 1993), we proposed that the evolution of the IL-6/class IB receptor family may have occurred in at least two major steps (Yamamori and Sarai, 1994). Firstly, binding subunits of an IL-6 receptor and for a CDF/LIF receptor evolved and secondly, a third binding subunits of a CNTF receptor evolved. Our model predicts that the binding subunits generally determine the specificity of the receptors and it is possible that novel members of the cytokine family and their receptors exist in the nervous system.

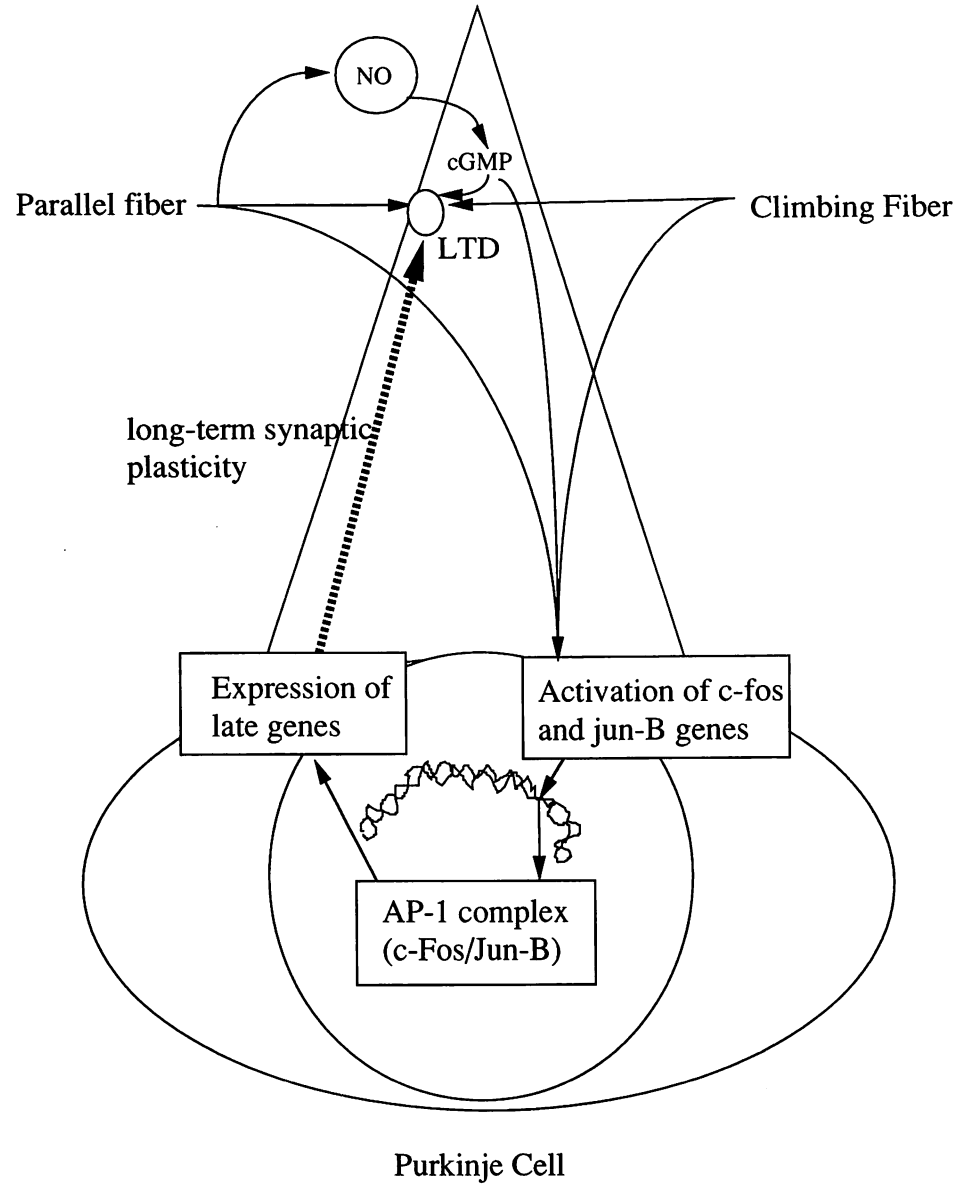
II. Gene expression and cerebellar long-term plasticity

In order to know roles of the genes involved in long-term memory, we choose the cerebellum as a model system. In the cerebellum the conjunctive stimuli of parallel fibers and a climbing fiber to a Purkinje cell induce prolonged reduction of a synaptic efficacy between the paralleled fiber to the Purkinje cell (LTD; long-term depression, Ito et al., 1982).

Previously, we examined the expression of 10 immediate early genes (IEGs) including all the known Fos and Jun family in cerebellar slices under the pharmacological condition that cause long-term desensitization of the Purkinje cell to AMPA (a glutamate analogue). Among the IEGs examined, Fos and Jun-B were predominantly induced under the conjunctive condition (Nakazawa et al., 1993).

Recently, we have examined Jun-B expression *in vivo* under a conjunctive protocol of AMPA, a pharmacological substitute for parallel fiber stimulation, and climbing fiber stimulation via electric Inferior Olive stimulation. June-B are predominantly induced around the local area where the AMPA and climbing fiber stimulation were conjunct (Yamamori et al., 1995). These results suggest that the coincidence mechanism may exist at gene expression level and lead to a cerebellar long-term plasticity (Fig. 1).

Toward further confirming this hypothesis, we are currently characterizing several molecules which are induced in Purkinje cells 3 hours after conjunction and following Jun-B induction in collaboration with Dr. Ryoji Yano's group (Frontier Research program, RIKEN). Most of these genes are unknown genes and we expect that the characterization will bring us new information on the late process of cerebellar long-term plasticity.



Publication List

Yamamori, T. (1996) Immediately early gene expression. In "Coincidence detection in the nervous system" Eds by A. Konnerth, R.Y. Tsien, K. Mikoshiba and J. Altman, Human Frontier Science Program, Strasbourg, 118-121.

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DIVISION OF SPECIATION MECHANISMS II

Professor: Ken-ichirou Morohashi
Associate Professor: Mitsuyasu Hasebe
Reserch Associate:
Graduate Students:
Technical Staff:

This division was newly established in 1996 with two research groups. These two groups managed by the professor, Ken-ichirou Morohashi (I), and the associate professor, Mitsuyasu Hasebe (II), have adopted animals and plants, respectively, as the materials for the research. Our attention has been and will be directed to understanding mechanisms underlying evolution of the reproductive system of animals and evolution of the morphology in plants. These efforts with a variety of the materials are expected to give us deeper insights into speciation mechanisms of organism.

I. Reproduction of animals

Because of the fundamental and pivotal function of the gonads, extensive efforts have been made to characterize the differentiation processes including sex-dependent differentiation. By focusing on the steroidogenesis as one of the gonad specific functions, we indentified a steroidogenic tissue specific transcription factor designated Ad4BP or SF-1. Recent studies with an antiserum to Ad4BP/SF-1 and a gene disrupted mice clearly demonstrated that the transcription factor is essential for differentiation of the steroidogenic tissues, the gonads and the adrenal cortex. Showing a good correlation with the observation above, a particular cell population identified as Ad4BP/SF-1 immunoreactive cells (AGP, adrenogenital primordium) was revealed to give rise to the gonads and adrenal cortex. These observations gave us many interesting issues as follows. What is the signal for the onset of the *Ad4BP/SF-1* gene transcription in the particular cells comprising AGP? What is the mechanism underlying the separation of the AGP into the adrenal and gonadal primordia? How is the mechanism employed for sex differentiation of the gonads? Why dose such sex dependent differentiation not occur in the adrenal cortex? To address the issues, the nuclear factors, WT1, SRY, SOX9, and DAX1 as well as Ad4BP/SF-1, critically implicated in gonadal and adrenocortical differentiation will be investigated from the molecular and morphological aspects.

II. Morphological Evolution in plants

Morphological diversity among taxa is the most prominent characteristics in the biodiversity. Our group aims to explore genetical and molecular changes which caused renovative morphological changes during the course of plant evolution. We selected *Arabidopsis* (angiosperm), *Gnetum* (gymnosperm), *Ginkgo* (gymnosperm), *Ceratopteris* (pteridophyte), *Physcomitrella* (bryophyte), and *Chlamydomonas* (green algae) as model plants to compare the genes related to morphogenesis. We are focusing on the following three topics; (1) Evolution of floral homeotic genes (MADS gene family and *LEAFY* gene homeologue, which is a regulator of the MADS genes), (2) Comparison of homeobox gene functions between sporophyte and gametophyte generations to reveal the evolution of the both generations, and (3) establishment of mutant library of *Physcomitrella patens* to get new morphological mutants which have not been obtained from the study of the higher plants having only reduced gametophytic generations. Some MADS, LEAFY and homeobox genes have been cloned from the gymnosperms, *Ceratopteris*, *Physcomitrella* and the analyses of the expression pattern are in progress. Some of those genes were observed to be expressed in both gametophyte and sporophyte generations and the regulation systems are studied.

RESEARCH SUPPORT

TECHNOLOGY DEPARTMENT

Head: Hiroyuki Hattori

Common Facility Group
Chief: Kazuhiko Furukawa

Research Support Facilities
Mamoru Kubota (Subunit Chief)
Chieko Nanba
Toshiki Ohkawa
Masayo Iwaki
Kaoru Sawada
Tomoki Miwa

Radioisotope Facility
Yousuke Kato (Subunit Chief)
Yoshimi Matsuda

Center for Analytical Instruments
Akio Murakami (Unit Chief)
Sonoko Ohsawa
Yumiko Makino
Takeshi Mizutani
Yuki Nakashima (Technical Assistant)

Glassware Washing Facility
(Kazuhiko Furukawa)
(Toshiki Ohkawa)

Research Support Group

Cell Biology Group
Maki Kondo (Subunit Chief)
Yukiko Kabeya
Katsushi Yamaguchi

Developmental Biology Group
Hiroko Kobayashi (Unit Chief)
Chikako Inoue
Chiyo Takagi
Sanae Oka
Naomi Sumikawa

Regulation Biology Group
Shoichi Higashi (Subunit Chief)
Hedeko Nonaka
Akiko Kawai
Shigemi Ohsugi

Gene Expression and Regulation Group
Sachiko Tanaka
Tomoko Mori
Koji Hayashi
Miki Ida
Yasushi Takeuchi
Hideko Utsumi

The Technology Department is a supporting organization for researchers and research organization within the NIBB. The Department develops and promotes the institute's research activities and at the same time, maintains the research functions of the institute.

The department is organized into two groups: one, the Common Facility Group, which supports and maintains the institute's common research facilities and the other, the Research Support Group, which assists the research activities as described in individual reports.

Technical staffs participate, through the department, in mutual enlightenment and education increase their capability in technical area. Each technical staff is proceeded to the fixed division usually and they support the various research with their special biological and biophysical techniques.

The department hosts an annual meeting for technical engineers who work in various fields of biology at universities and research institutes throughout Japan. At this meeting, the participants present their own activities and discuss technical problems. The proceedings are published soon after the meeting.

RESEARCH SUPPORT FACILITY

Head of Facility: Takashi Horiuchi (April 1, 1996 –)
Associate Professor: Masakatsu Watanabe
Research Associates: Yoshio Hamada, (Tissue and Cell Culture)
Atsushi Ogiwara (Computer)
NIBB Fellow: Mineo Iseki (Large Spectrograph)
Technical Staffs: Masayo Iwaki
Mamoru Kubota
Chieko Nanba
Toshiki Ohkawa
Kaoru Sawada
Tomoki Miwa
Hideko Nonaka (– April 30, 1996)

The Facility provides large-and medium-scale instruments and facilities for biophysical, molecular biological, and computational analyses as well as for growing and maintaining biological specimens. The facility is shared among the research members, and has seven laboratories, among which the Large Spectrograph Laboratory and the Laboratory of Stress-Resistant Plants are dedicated to cooperative use under the NIBB Cooperative Research Programs.

On Nov. 12-14, 1996, the Research Support Facility organized an international symposium at NIBB on "New Prospects of Photobiology and the Future Plan of the Okazaki Large Spectrograph" participated by about 40 foreign and about 120 Japanese active scientists, and then got an overwhelming support for the idea of constructing a new generation spectrograph (Okazaki Super Sectrograph) providing about 2 orders of magnitude higher fluence rates (intensities) and versatile combinations of monochromatic irradiations by the use of over 50 tunable laser systems as the major light sources.

I. Facilities

1. *The Large Spectrograph Laboratory*

This laboratory provides, for cooperative use, the Okazaki Large Spectrograph (OLS), which is the largest spectrograph in the world, dedicated to action spectroscopical studies of various light-controlled biological processes. The spectrograph runs on a 30 kW-Xenon arc lamp and has a compound grating composed of 36 smaller individual gratings. It projects a spectrum of a wavelength range from 250 nm (ultraviolet) to 1,000 nm (infrared) onto its focal curve of 10 m in length. The fluence rate (intensity) of the monochromatic light at each wavelength is more than twice as much as that of the corresponding monochromatic component of tropial sunlight at noon (Watanabe et al., 1982, Photochem. Photobiol., 36, 491-498).

A tunable two-wavelength CW laser irradiation system is also available as a complementary light source to OLS to be used in irradiation experiments which specifically require ultra-high fluence rates as well as ultra-high spectral-, time-and spatial resolutions. It is composed of a high-power Ar-ion laser (Coherent, Innova 20) (336.6-528.7 nm, 20 W output), two CW dye lasers (Coherent, CR599-01) (420-930 nm, 250-1000 mW output), A/O modulators (up to 40 MHz) to chop the laser beam, a beam expander, and a tracking microbeam irradiator (up to 200 μm s^{-1} in tracking speed, down to 2 μm in beam diameter) with an infrared phase-contrast observation system.

2. *Tissue and Cell Culture Laboratory*

Various equipments for tissue and cell culture are provided. This laboratory is equipped with safely rooms which satisfy the P2/P3 physical containment level. This facility is routinely used for DNA recombination experiments.

3. *Computer Laboratory*

To meet various computational needs and to provide means of electronic communication in this institute, many kind of computers are equipped: UNIX servers and engineering workstations (SPARCstations, IRIS machines, NEWS machines, etc.), VAX mini computer system, and some personal computers (Macintosh's and Windows machines). All of these machines are connected each other with local area networks, which are also linked to the high performance multimedia backbone network of Okazaki National Research Institutes. Since this backbone network, called ORION, is joined to the Internet, the users can access various services and databases on the Internet.

The Computer Laboratory provides various computational services to the institute members: file servers for Macintosh and NetWare users, print servers for PC and UNIX users, computational servers that provides sequence analyses and database retrievals, communication servers to the Internet, and so on.

A new computational server with large scale disk system was introduced at the end of this year and started to provide database search service. The laboratory also provides an information dispatching service to the Internet using the World Wide Web (URL is http://www.nibb.ac.jp).

4. *Plant Culture Laboratory*

There are a large number of culture boxes, and a limited number of rooms with environmental control for plant culture. In some of these facilities and rooms, experiments can be carried out at the P1 physical containment level.

5. *Experimental Farm*

This laboratory consists of two 20 m^2 glass-houses with precise temperature and humidity control, a small farm, two greenhouses (45 and 88 m^2) with automatic sprinklers, two open aquariums (30 and 50 t) and several smaller tanks. The laboratory also includes a building with office, storage and work-space.

6. *Plant Cell Laboratory*

Autotrophic and heterotrophic culture devices and are equipped for experimental cultures of plant and microbial cells. A facility for preparation of plant cell cultures including an aseptic room with clean-

benches, is also provided.

7. *Laboratory of Stress-Resistant Plants*

This laboratory was founded to study transgenic plants with respect to tolerance toward various environmental stresses. It is located in the Agricultural Experimental Station of Nagoya University (30 km from the National Institute for Basic Biology). The laboratory provides a variety of growth chambers that precisely control the conditions of plant growth and facilities for molecular biological, and physiological evaluations of transgenic plants.

The laboratory is also a base of domestic and international collaborations devoted to the topic of stress-resistant transgenic plants.

II. Research activities

1. *Faculty*

The faculty of the Research Support Facility conducts its own research as well as scientific and administrative public services.

(1) Photobiology: Photoreceptive and signal transduction mechanisms of phototaxis of single-celled, flagellate algae are studied action spectroscopically (Watanabe 1995, In CRC Handbook of Organic Photochemistry and Photobiology) by measuring computerized-videomiceographs of the motile behavior of the cells at the cellular and subcellular levels (Erata et al. 1995, Protoplasma). Photo-receptive and signal tranduction mechanisms of algal gametogenesis are also studied by action spectroscopy.

(2) Developmental Biology: Notch is an integral cell surface membrane protein that is known to play a key role in developmental cell-cell interactions in Drosophila, particularly in lateral specification of neural versus epidermal cell fates, a process described thus far only in invertebrates. It is thought to act by a direct signaling pathway rather than through one of the classical signal transduction cascades. The mammalian genome is known to contain three Notch homologues but their developmental significance is not clear. To investigate their role in mammalian development, we have sequenced the murine Notch 2 cDNA, determined the primary sequence of its protein, and have investigated its genomic organization. We are now attempting to produce a mutant in which the ankyrin repeat region of Notch 2 is replaced by lacZ. Analysis of the mutant phenotype will provide us with insights about the significance of the repeat in Notch 2 signal transduction in relation to its developmental importance.

(3) Computational Biology: One of the main theme of computational biology is to predict biological functions from amino acids or nucleic acids sequences. Homology search is a popular method that assists to predict biological roles by inference. However, it is very time consuming process as the rapid growing of sequence libraries. Use of sequence motifs and signature patterns is a convenient and speedy method to predict directly the biological functions. A new method to extract sequence motifs from groups of related proteins has been studied and the methodology was applied to analyze unknown protein sequences. The method was implemented as a computer tool on a UNIX workstation.

Another important theme of bioinformatics is the problem of databases. Recently, many bacteria genomes have been completely sequenced. To store and to publish the results of sequencing projects, database systems are indispensable. Since the purpose of genome sequencing is to understand biological functions from genetic information, the genome sequence database must also oriented to the biological function. As a model case of such database, Bacillus subtilis open reading frames database is constructed in collaboration with Prof. N. Ogasawara (NAIST) and Human Genome Center of the University of Tokyo. As the completion of sequencing of 1.3 Mbase regions by Japanese researchers groups, the first step of database construction has been finished and is released using WWW.

2. *Cooperative Research Program for the Okazaki Large Spectrograph*

The NIBB Cooperative Research Program for the Use of the OLS supports about 30 projects every year conducted by visiting scientists including foreign scientists as well as those in the Institute.

Action spectroscopical studies for various regulatory and damaging actions of light on living organisms, biological molecules, and organic molecules have been conducted (Watanabe, 1995, In CRC Handbook of Organic Photochemistry and Photobiology).

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RADIOISOTOPE FACILITY

(Managed by NIBB)

Head: Takashi Horiuchi
Associate Professor: Kazuo Ogawa
NIBB Postdoctoral Fellow: Hiroyuki Takai
Technical Staffs: Kazuhiko Furukawa (Radiation Protection Supervisor)
Yosuke Kato (Radiation Protection Supervisor)
Yoshimi Matsuda (Radiation Protection Supervisor)

This Facility consists of a main center and two branch offices. The third branch office will open in the laboratory of Gene Expression and Regulation in May 1, 1977. The members of the Radioisotope Facility are engaged in maintaining and controlling both the center and two branch offices, and providing users an appropriate guidance for radioisotope handling.

The teaching staffs are also engaged in their own research. They are interested in clarifying the structure and function of dynein motor protein. Dyneins are a group of microtubule-activated ATPases that serve to convert chemical energy into mechanical energy. They have been divided into two large subgroups, namely, the axonemal and cytoplasmic dyneins. Figure 1 shows the localization of two dyneins in the outer arms (Ogawa et al., 1977) and the mitotic apparatus (Mohri et al., 1976) that have been visualized by the same antibodies directed against the motor domain of axonemal dynein (fragment A).

The native dyneins are very large. They range in molecular mass up to 1 to 2 mega daltons and they are complex proteins as shown in Figure 2. Each dynein contains two or three heavy chains (HCs) with ATPase activity, which range in molecular mass up to 500 kDa. The motor activity of dynein is associated with these chains. Some functional differences have been reported between HCs of outer arm dynein. Sea urchin outer arm dynein is a heterodimer of HCs (alpha and beta) and at least the beta-HC is able to induce gliding of microtubules *in vitro*. The Alpha-HC might amplify the function of beta-HC and it has been reported to have no motile activity. After the first cloning of beta-HC from sea urchin ciliary axonemes (Gibbons et al., 1991; Ogawa, 1991), the sequences of HCs of axonemal and cytoplasmic dyneins from a variety of organisms were determined in their entirety. Without exception, all the HCs cloned to date contain four P-loop (ATP-binding) sequences in the midregion of the molecule. Thus, they can be classified as a four P-loop family.

The outer arm dyneins contain two or three proteins that range in molecular mass from 70 to 120 kDa and copurify with HCs. ICs of sea urchin outer-arm dynein are abbreviated as IC1, IC2, and IC3. Those of *Chlamydomonas* are called IC78 and IC69, and ICs of cytoplasmic dynein are called IC74. *Chlamydomonas* IC78 and IC69 were cloned by Wilkerson et al. (1995) and Mitchell and Kang (1991), respectively. The sequences of sea urchin IC2 and IC3 were determined by Ogawa et al. (1995). Finally, the sequence of IC1 was determined this year by Ogawa et al. (1996). Thus, all the ICs found in the axonemal and cytoplasmic dyneins of the model organisms used for studies of dynein function have been completely sequenced. Comparison of amino acid sequences of IC2 and IC3 with those of IC78 and IC69 and with that of IC74 showed that, although all five ICs are homologous, IC2 is much more closely related to IC78, and IC3 is much more closely related to IC69, than either sea urchin chain or either *Chlamydomonas* chain is related to each other. Regions of similarity between all five ICs are limited to the carboxy-terminal halves of the molecules. Similarity are due primarily to conservation of the WD repeats in all of these chains. The WD repeats are involved in protein-protein interactions in a large family of regulatory molecules (Neer et al.,1994). A parsimony tree for these chains (Ogawa et al., 1995) shows that, although the carboxy-terminal halves of all of these chains contain WD repeats, the chains can be divided into three distinct subclasses (IC3 plus IC69, IC2 plus IC78, and IC78).

By contrast, sea urchin IC1 is not a member of the WD family. IC1 has a unique primary structure, the N-terminal part is homologous to the sequence of thioredoxin, the middle part consists of three repetitive

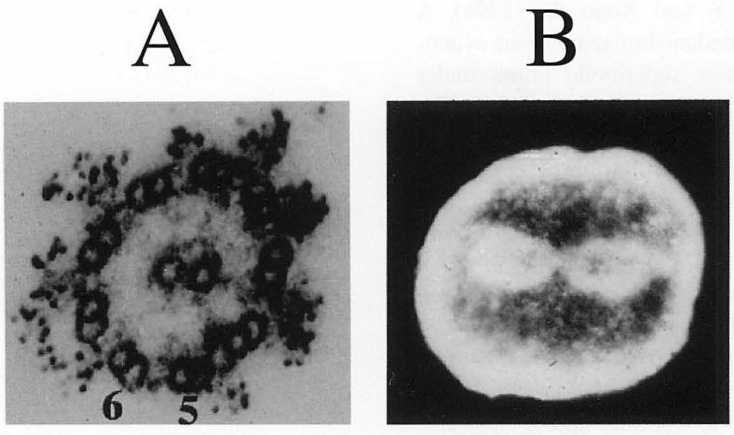

Figure 1. Localization of dynein in sea urchin sperm axoneme (A) and a cleaving egg (B).

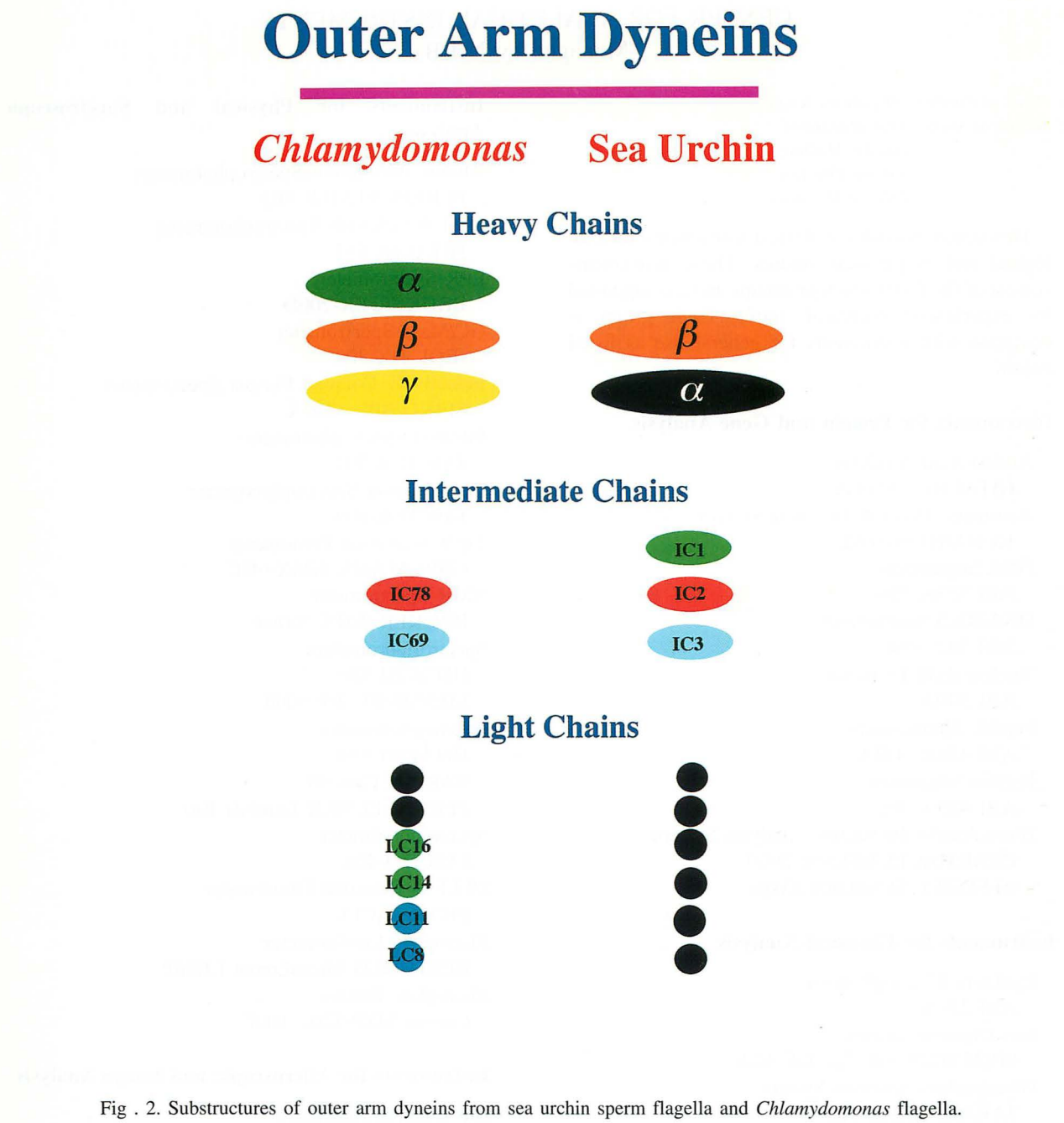

Fig . 2. Substructures of outer arm dyneins from sea urchin sperm flagella and *Chlamydomonas* flagella.

sequences homologous to the sequence of nucleoside diphosphate kinase, and the C-terminal part contains a high proportion of negatively charged glutamic acid residues. Thus, IC1 is a novel dynein intermediate chain distinct from IC2 and IC3 and may be a multifunctional protein. Then, a question arises as whether *Chlamydomonas* outer arm dynein contains IC1. The answer is "no". Because it consists of just two intermediate chains. Alternatively, the answer is "yes". Because the sequences of two light chains (LC16 and LC14) from *Chlamydomonas* outer arm dynein shows that they are members of novel family of thioredoxin (Patel-King et al. 1996). The thioredoxin-related part of IC1 is more closely related to those of two redox-active *Chlamydomonas* light chains than thioredoxin. Antibodies were prepared against the N-terminal and middle domains of IC1 expressed as His-tagged proteins in bacteria. These antibodies cross-reacted with some dynein polypeptides (potential homologues of IC1) from distantly related species. We propose here that the three intermediate chains are the basic core units of sperm outer arm dynein because of their ubiquitous existence.

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CENTER FOR ANALYTICAL INSTRUMENTS
(Managed by NIBB)

Head of Facility: Masaharu Noda
Technical Staffs: Akio Murakami
Yumiko Makino
Sonoko Ohsawa
Takeshi Mizutani

This center provides analytical instruments for biological and biophysical studies. These instruments consist of the following four groups and are supported by experienced technical staffs. Each group is equipped with instruments for general use as listed below.

Instruments for Protein and Gene Analysis

Amino Acid Analyzer
HITACHI L 8500A
Automatic Plasmid Isolation System
KURABO PI-100Σ
DNA Sequencers
ABI 373S, 310
DNA/RNA Synthesizers
ABI 392, 394
Nucleic Acid Extractor
ABI 340A
Peptide Synthesizers
ABI 431A, 432A
Portein Sequencer
ABI 473A, 492
Biomolecular Interaction Analysis System
PHARMACIA BIAcore 2000
AFFINITY SENSORS IAsys

Instruments for Chemical Analysis

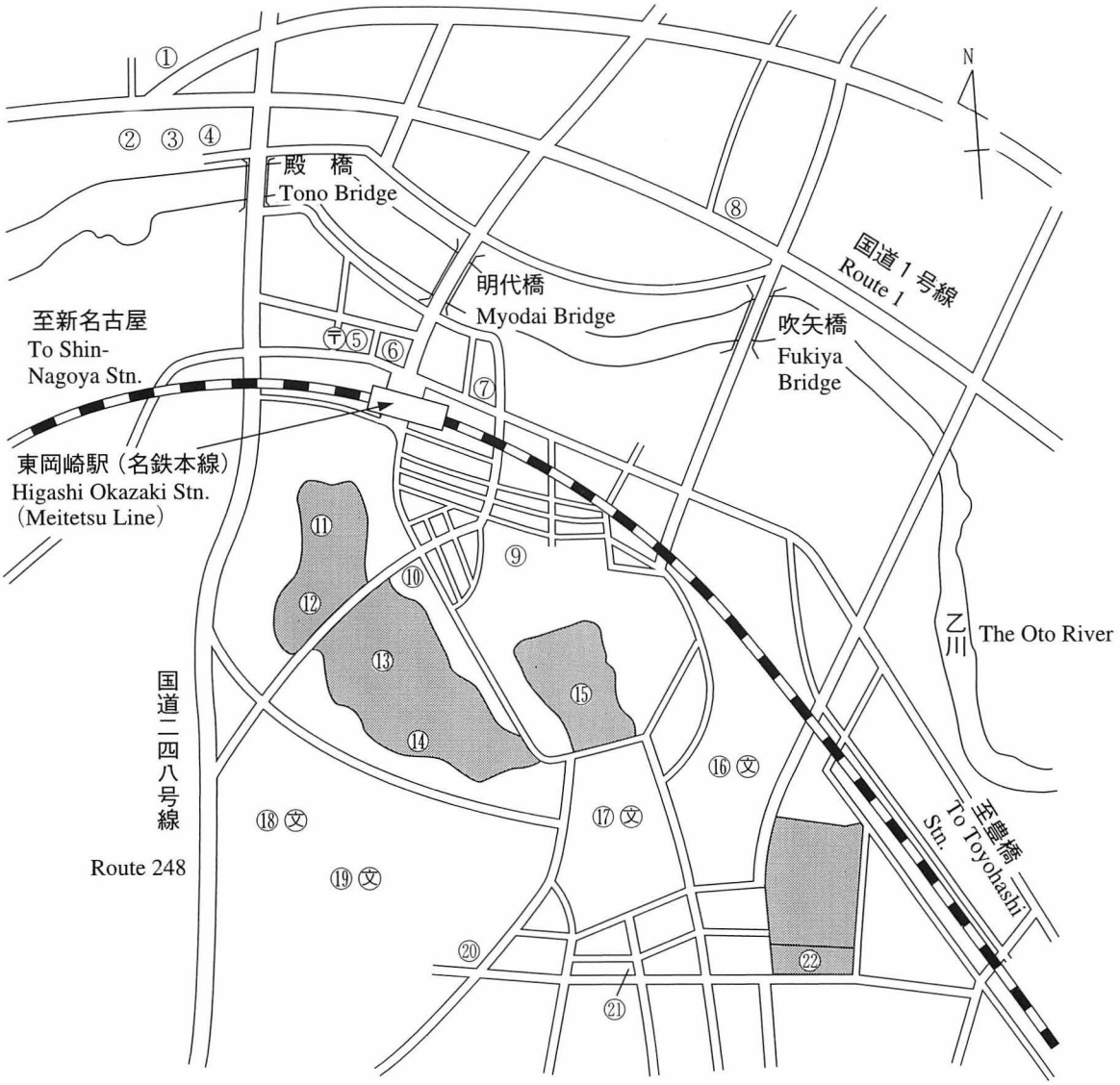
Capillary Electrophoresis
ABI 270A
Gas Chromatographs
SHIMADZU GC-7A, GC-14A
Glycoprotein Analysis System
TAKARA Glyco-Tag
High Performance Liquid Chromatographs
SHIMADZU LC-10AD, 6AD
Preparative Ultracentrifuges
BECKMAN L8-80, L5-75
Table-top Ultracentrifuges
BECKMAN TL-100
Micro Preparative System
PHARMACIA SMART System
Flow Cytometer
COULTER EPICS XL

Instruments for Physical and Spectroscopic Analysis

Atomic Absorption Spectrophotometer
PERKIN-ELMER 603
Dual Wavelength Spectrophotometer
HITACHI 557
EPR Spectrometer
BRUKER ER-200D
GC/Mass Spectrometer
JEOL DX-300
Inductively Coupled Plasma Spectrometer
SEIKO SPS 1200A
Infrared Spectrophotometer
JASCO A-302
Laser Raman Spectrophotometer
JASCO R-800
Light Scattering Photometer
CHROMATIX KMX-6DC
NMR Spectrometer
BRUKER AMX-360wb
Spectrofluorometers
HITACHI 850
SHIMADZU RF-5000
Spectrophotometers
HITACHI 330
VARIAN Cary5G
PERKIN ELMER Lambda Bio
Spectropolarimeter
JASCO J-40S
DELFIA Research Fluorometer
PHARMACIA
Microplate Luminometer
BERTHOLD MicroLumat LB96P
Microplate Reader
Corona MTP-120, -100F

Instruments for Microscopic and Image Analysis

Bio Imaging Analyzers
FUJIFILM BAS 2000
Imaging Analysing Systems
KONTRON IBAS-I & II
Electrophoresis Imaging System
PDI The Discovery Series
BIOIMAGE
Microscope
CARL ZEISS Axiophot, Axiovert
Microscope Photometer
CARL ZEISS MPM 03-FL
Microdensitometer
JOYCE LOEBL 3CS



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Meitetsu Okazaki Hotel

② 岡崎城
Okazaki Castle

③ 岡崎ニューグランドホテル
Okazaki New Grand Hotel

④ 岡崎グランドホテル
Okazaki Grand Hotel

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Post Office

⑥ 銀行
Bank

⑦ スーパーマーケット“ユニー”
Super Market "Uny"

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National Institute for Basic Biology
Okazaki 444, Japan