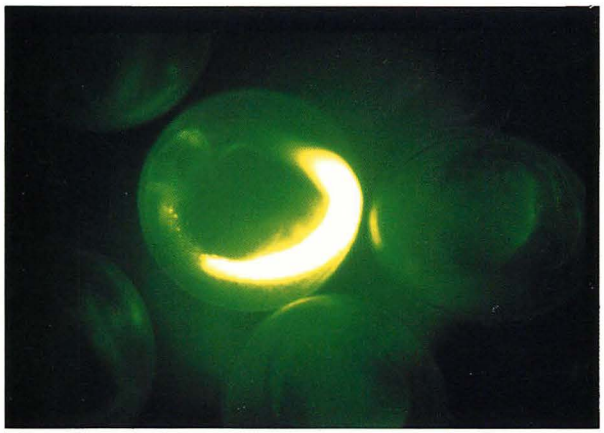
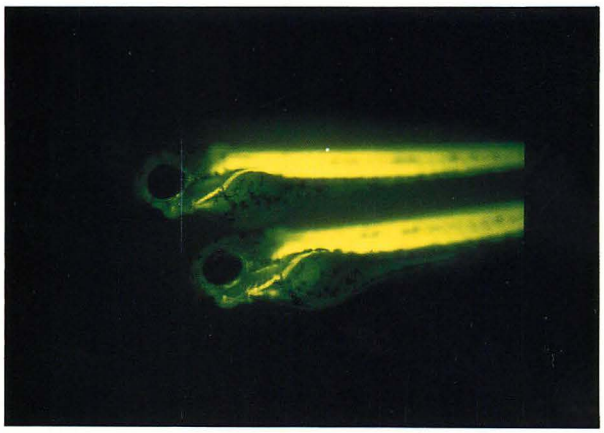
NATIONAL INSTITUTE FOR BASIC BIOLOGY

岡崎国立共同研究機構

基　礎　生　物　学　研　究　所





ANNUAL REPORT

1997

The cover photographs indicate GFP expression driven by α-actin-GFP promoter in live transgenic zebrafish embryos.

Post Doctral Fellow

1 NIBB Research Fellow
2 JSPS Postdoctral Fellow
3 JSPS Reserch Associate
4 JST Fellow

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INTRODUCTION

We present here the 1997 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.

In May of 1997, we celebrated the twentieth anniversary of the foundation of the NIBB together with the opening of the newly built Okazaki Conference Center on the campus of the Okazaki National Research Institutes. At the end of July, Professor Yoshiaki Suzuki of the Division of Cell Differentiation left the NIBB. Thus, the first professors of all Divisions, except those of the Laboratory of Gene Expression and Regulation which was established more recently have completely retired, and the NIBB has entered into a new era. We are now making up future plans for the NIBB including establishment of new divisions such as molecular environmental biology, neurobiology and mechanism of biodiversity. At the start of 1997, the activities and the future plan of the NIBB were subjected to a peer review and highly rated by authorities outside the institute.

The turnover of personnel was high during the past year. At the beginning of the year, Associate Professor M. Mimuro moved to Yamaguchi University as a professor. In May, Professor Naoto Uneno of Hokkaido University was appointed as Professor of the Division of Morphogenesis, the position formerly occupied by Professor Goro Eguchi. As Associate Professor of the same Division, Dr. H. Shibuya was appointed in July. At the end of March, Professor Hitoshi Sakano (University of Tokyo) of the Adjunct Division of Cell Fusion and Professor Kimiyuki Sato (Okayama University) of the Adjunct Division of Biological Regulation completed their terms of office. The former position was filled by Professor Issei Mabuchi (University of Tokyo) in April. Drs. I. Nishimura, N. Maeda, K. Mikami, K. Owaribe (Nagoya University), H. Abe (Chiba University) and Y. Ozeki (Tokyo University of Agriculture and Technology) were respectively appointed as Associate Professors in the Division of Cell Mechanism, the Division of Molecular Neurobiology, the Division of Cellular Regulation, the Adjunct Division of Cellular Communication, the Adjunct Division of Cell Fusion and the Adjunct Division of Behavior and Neurobiology. On the other hand, Adjunct Associate Professors T. Takai and H. Wada left the NIBB. In addition, we recently appointed 5 research associates and 2 institute research fellows, while 3 research associates and

2 institute research fellows moved to other institutions.

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 Cell." Based on these programs, the NIBB held the 38th Conference entitled "Plasticity in Differentiation and Morphogenesis" (organized by Professor G. Eguchi and Dr. R. Kodama) and the 39th Conference "Dynamic Aspects of Seed Maturation and Germination " (organized by Professor Mikio Nishimura). In September, the Director-General of the NIBB and the President of the Korea Basic Science Institute (KBSI) signed a "Memorandum of Understanding between the NIBB and the KBSI" in order to promote international cooperation between the two institutions.

In addition, the Institute sponsors interdisciplinary symposia and study meetings on current topics by inviting leading scientists national and international in various related fields. In 1997, an International Symposium "Dynamic Aspects of Lysosomal/Vacuolar System " (organized by Professor Yoshinori Ohsumi) was held at the Okazaki Conference Center, sponsored by the Ministry of Education, Science, Sports and Culture. The NIBB also provided a training course in biological sciences for young investigators.

Our efforts in the future will continue to be directed

at supporting leading research in various fields of biological sciences. We welcome suggestions and criticisms concerning the activities of the NIBB.

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 Professors: *Shigeru Itoh*
Ryuji Kodama
Research Associate: *Katsunori Aizawa (on leave)*

Evolution of photoysnthesis and the mechanism of electron transfer

Shigeru Itoh

We study the evolution of molecular mechanism of plant and bacterial photosynthesis. Anoxygenic photosynthesis of bacteria seems to have evolved in the Precambrian Earth just after the evolution of life. Oxygen-evolving photosynthesis was then established by cyanobacteria 3.5-2.7 billion years ago and increased atomospheric oxygen. Symbiosis of cyanobacteria inside the larger cells produced the first plant about 2 billion years ago.

What can we study to explore the past? We take three approaches (1) Survey of electron transfer mechanism in photosyntheic reaction center (RC) pigment-protein complexes. We replaced cofactors in the complex to see what happens. We studied light reactions by the ps-ns laser and the spin-echo ESR spectroscopy between 4 and 280 K in the cofactor-replaced RCs and found that the molecular architecture of plant and bacterial RCs are highly optimized in different directions. The result that was the first evidence of the energy-gap dependence of the ultra-fast electron tunnnelling in plant, also suggeted us the design of prototype RC. (2) Site-directed mutagenesis of electron transfer proteins in attempts to construct prototype electron transfer protein. (3) Survey of ever-unknown photosynthesis as well as the comparative studies of photosynthesis in purple, green sulfur, hellio-, cyanobacteria and plant. A recent topic was the discovery of a new bacterium, *Acidiphilium rubrum* that undergoes photosynthesis with Zn-bacteriochlorophyll, instead of Mg-chlorophylls used in all the ever-known plant and bacterial photosynthesis. This finding was a supprise by itself, and led to the study of another, completely new oxygen-evolving photosynthesis now.

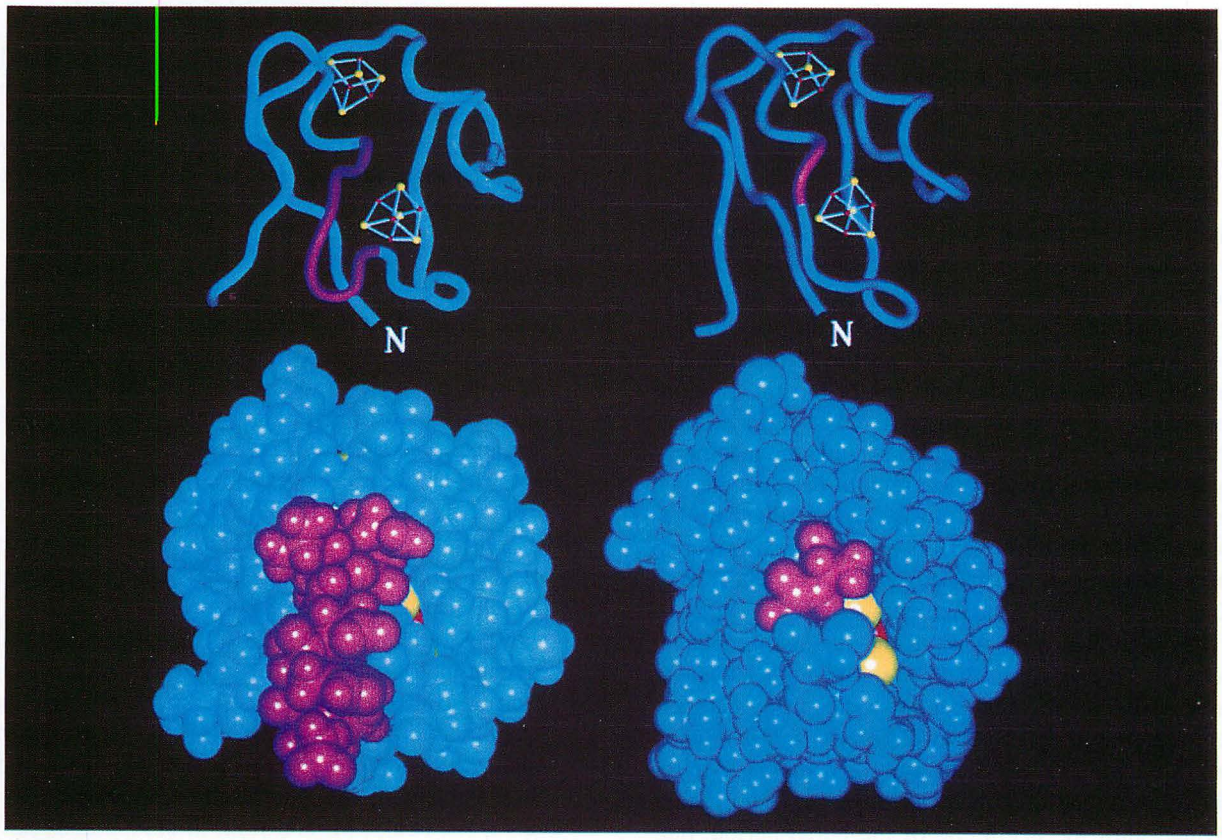


Fig. 1 Space-filling (lower) and wire-frame (upper) models of *Rhodopseudomonas capsulata* ferredoxin (FdxN). Pink-coloured moiety in native (left) protein was altered (right) by site-directed mutagenesis. The mutation exposed the Fe-S cluster (yellow and red) and decreased its reducing power detectable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

Mechanisms determining the outline shape of the adult lepidopteran wings

Ryuji Kodama

Wings of the lepidopteran insects (butterflies and moths) develop from the wing imaginal disc, which is a hollow sac made of simple epithelium. When the pupariation is completed, the wing, which was hidden inside the body wall of the larvae, is exposed on the surface of the pupa, which gradually turns into the adult wing. The outline shape of the adult wing is often different from that of the pupal wing. This difference is brought about by the programmed cell death of the marginal area of the pupal wing, while the internal area develops as adult wing blade. The marginal dying area is called the degeneration region and the internal area is called the differentiation region, hereafter.

The cell deaths in the degeneration region proceeds very rapidly and completes in a half to one day period in *Pieris rapae* or several other species examined. It was shown that the dying cells in the regeneration region have two characteristics common with the apoptotic cell death in mammalian cells. These are i) the presence of apoptotic bodies, which are heavily condensed cells or their fragments engulfed by other cells or macrophages, shown by transmission electron microscopy and ii) the presence of conspicuous accumulation of fragmented DNA evidenced by the TUNEL histological staining (Kodama, R. et al., Roux's Arch. Dev. Biol. 204, 418-426, 1995).

The cells in the degeneration region are actively engulfed by the macrophages in the cavity beneath the wing epithelium. Moreover, the macrophages seem to be concentrated beneath the degeneration region by the strong adhesion between basal surfaces of the dorsal and ventral epithelium in the differentiation region. By injecting the india ink or ferritin solution to the body cavity of the pupa, we have confirmed that this adhesion is tight enough to exclude the macrophages from the differentiation region, because the injected probes was found mostly concentrated in the degeneration region when observed several minutes later (Yoshida, A. (Biohistory Research Hall) and Kodama, R., unpublished).

These studies were done using the small cabbage butterfly, *Pieris rapae*, and the silk moth, *Bombyx mori*. We have recently begun studies using another lepidopteren species, *Orgyia recens approximans*, provided by Drs. Y. Arita and K. Yamada (Meijo University). In this species, the wing is normally formed until the beginning of the pupal period, but becomes conspicuously degenerated only in the female adult (Fig. 2). In our preliminary study, it was shown that the pupal wing is normally formed both in male and female pupa, but after about two days, female pupal wing starts degeneration on its margin, as if the degeneration region is continuously formed deep into the center of the wing (Kodama, R. et al., unpublished). It is thus suggested that the control mechanism which demarcates the region to be degenerated is defective in the female in this species. Further investigation using this species might give important insight into such mechanisms.

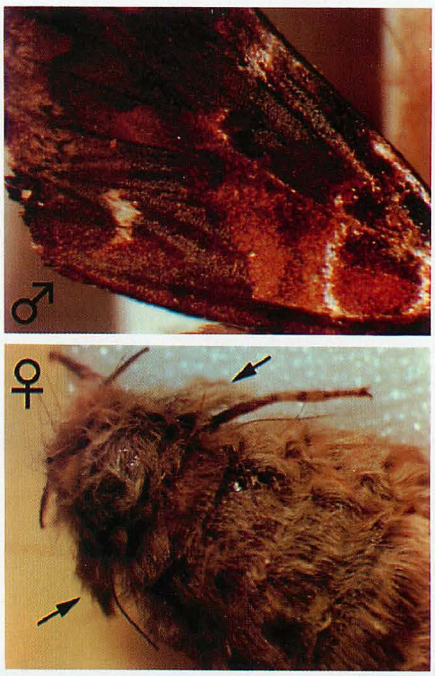


Fig. 2 The adult wing of the male (upper) and female (lower) moth, *Orgyia recens approximans*, are shown at the same magnification. Only a part of the left forewing is shown for the male wing. In the female, wings are degenerated to small protrusions (arrows) covered with scales similar to those covering the body.

Publication List:

Dzuba, S. A., Hara,H., Kawamori, A., Iwaki, M., Itoh, S. and Tsvetkov, Yu. D. (1997) Electron spin echo of spin-polarized radical pairs in the intact and quinone-reconstituted plant photosystem I reaction centers. *Chem. Phys. Lett.* **264**, 238-244.

Hara, H., Dzuba, S.A., Kawamori, A., Akabori, K., Tomo, T., Satoh, K., Iwaki, M. and Itoh, S. (1997) The distance between P680 and QA in Photosystem II determined by ESEEM spectrocscopy. *Biochim. Biophys. Acta* **1322**, 77-85.

Ogawa, K. and Mohri, H. (1997) Establishment of a dynein motor superfamily. In "Recent Advances in Marine Biotechnology. Vol. 1. Endocrinology and Reproduction" (M. Fingerman, R. Nagabhushanam and M.-F. Thompson, eds.), pp. 249-281. Oxford & IBH Publishing Co., New Delhi, Calcutta.

Ohoka, H., Iwaki, M. and Itoh, S. (1997) Viscosity dependence of the electron transfer rate from bound cytochrome c to P840 in the photosynthetic reaction center of a green sulfur bacterium *Chlorobium tepidum*. *Biochemistry* **36**, 9267-9272.

Tomo, T., Mimuro, M., Iwaki, M., Kobayashi, M., Itoh, S. and Satoh, K. (1997) Topology of pigments in the isolated photosystem II reaction center studied by selective extraction. *Biochim. Biophys. Acta* **1321**, 21-30.

DEPARTMENT OF CELL BIOLOGY

Chairman: Mikio Nishimura

The department consists of two regular divisions and three adjunct divisions. An adjunct division was started in 1997. The department conducts studies on molecular dynamics of the cell in higher plants and animals such as organelle differentiation, autophagy, cell motility, cytokinesis and expression of cell cycle genes.

DIVISION OF CELL MECHANISMS

Professor: *Mikio Nishimura*
Associate Professor: *Ikuko Hara-Nishimura*
Associate Professor (adjunct): *Masayoshi Maeshima*
Research Associates: *Makoto Hayashi*
Tomoo Shimada
Post doctoral fellows: *Akira Kato* [1]
Tetsu Kinoshita [2]
Nagako Hiraiwa [3]
Yasuko Ishimaru [3]
Kanae Shirahama [3]
Graduate Students: *Shoji Mano*
Hiroshi Hayashi
Kenji Yamada
Daigo Takemoto (Nagoya University)
Yuki Tachibe (Hiroshima University)
Technical Staff: *Maki Kondo*
Katsushi Yamaguchi
Visiting Scientists: *Luigi De Bellis (Pisa Univ. Italy)*
Claus Schnarrenberger (from Free Univ. of Berlin, Germany)
Yasuko Koumoto
Miwa Kuroyanagi

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. The dynamic transformation of organellar function (differentiation of organelles) is responsible for flexibility of differentiation in higher plant cells. Elucidation of regulatory mechanisms underlying organelle transformation are currently 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 the greening that occurs during 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.

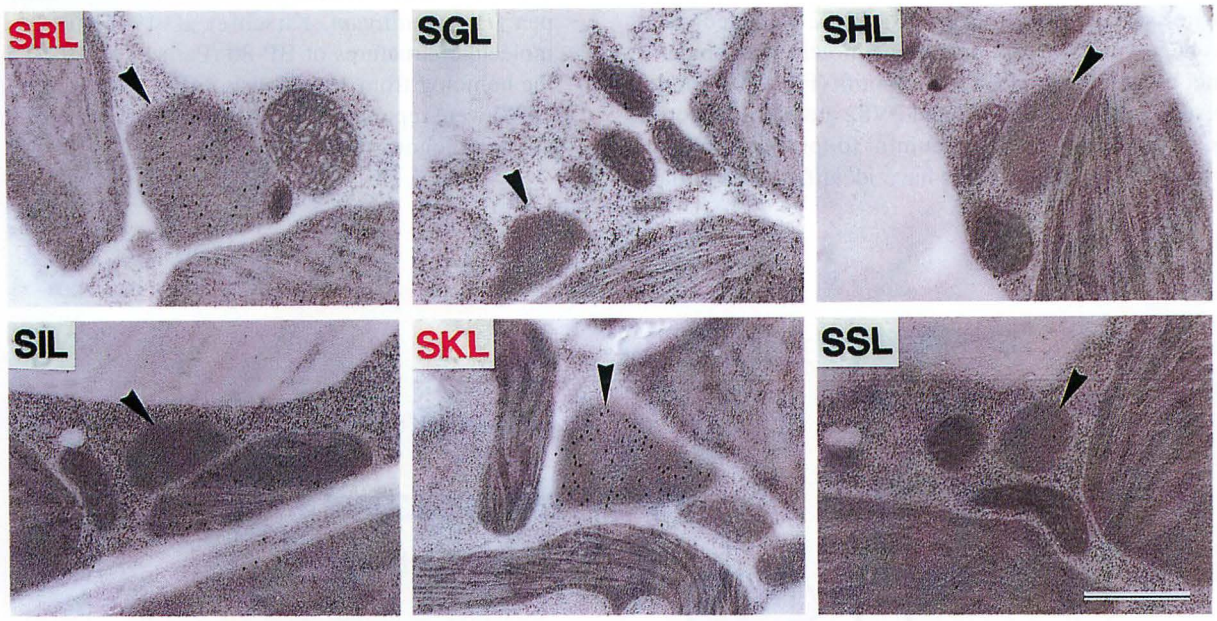
Microbody proteins are synthesized in the cytosol on free polysomes and are transported post-translationally into microbodies. Two types of targeting signals to microbodies have been reported. One type of targeting signal is part of the mature protein. One such signal, the tripeptide Ser-Lys-Leu, occurs 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, we have examined the ability of 24 carboxy-terminal amino acid sequences to facilitate the transport of a bacterial protein, β-glucuronidase (GUS) into microbodies in green cotyledonary cells of transgenic Arabidopsis. Immunocytochemical analysis of the transgenic plants revealed that carboxy-terminal tripeptide sequences of the form [C/A/S/P]-[K/R]-[I/L/M] function as a microbody-targeting signal, although tripeptides with proline at the first amino acid position and isoleucine at the carboxyl terminus show weak targeting efficiencies (Fig. 1). All known microbody enzymes that are synthesized in a form similar in size to the mature molecule, except catalase, contain one of these tripeptide sequences at their carboxyl terminus. These carboxyl tripeptides function as a targeting signal to the microbodies, not only to glyoxysomes but also to leaf peroxisomes. Therefore, it is unlikely that glyoxysomes and leaf peroxisomes possess different targeting machineries.

II. Light dependent alternative splicing in higher plants.

We previously showed that two different forms of hydroxypyruvate reductase which are localized in microbodies and in the cytosol, are produced by alternative splicing. In 1997, we found that stromal (sAPX) and thylakoid-bound ascorbate peroxidases (tAPX) are also produced by alternative splicing. cDNA for sAPX and tAPX were isolated and characterized. The cDNA for sAPX encodes a polypeptide with 372 amino acids and shares complete sequence identity with tAPX, except for the deletion of a putative membrane domain of tAPX. Southern blot hybridization and analysis of intron structure indicated that the mRNAs for sAPX and tAPX, whose suborganellar localizations in chloroplasts are different, are produced by alternative splicing. Immunoblot analysis showed that the accumulation of sAPX and tAPX was differently regulated and that the alternative splicing is regulated developmentally and by light.

A



B

Substitutions of first amino acid		Substitutions of second amino acid		Substitutions of C-terminal amino acid	
LRL	-	SIL	-	SRI	+
FRL	-	SGL	-	SRV	-
CRL	++	SSL	-	SRL	++
ARL	++	SHL	-	SRF	-
GRL	-	SKL	++	SRM	++
SRL	++	SRL	++	SRS	-
YRL	-			SRE	-
PRL	+			SRK	-
ERL	-				
KRL	-				

Fig. 1. Effects of Substitutions at the Carboxy-terminal Tripeptide on Subcellular Localization of Chimeric Proteins.

A) Subcellular localization of chimeric proteins in the cells of transgenic Arabidopsis was analyzed by immunoelectron-microscopy. Label on each photograph represents the carboxy-terminal tripeptide sequence of the chimeric protein. Arrowheads indicate microbodies. Bar = 1 μm.

B) Summary of the targeting efficiencies of carboxy-terminal tripeptides. Amino acid sequences of carboxy-terminal tripeptides are shown by single letter codes. Targeting efficiencies of proteins containing these tripeptides at the carboxy terminus were presented by ++ (efficient), + (detectable) and - (inefficient).

III. A pumpkin 72-kDa membrane protein of precursor-accumulating vesicles has characteristics of a vacuolar sorting receptor.

Seed proteins are synthesized on the rough endoplasmic reticulum and are then delivered to the protein-storage vacuoles. Sorting and targeting them to the vacuoles requires the presence of a specific signal and a receptor. Previously we isolated transport vesicles that mediate the transport of the precursor of major storage proteins to protein-storage vacuoles in developing pumpkin cotyledons. We designated them precursor-accumulating (PAC) vesicles, as shown in Fig. 2A. The vesicles might contain a receptor protein for the storage protein. We characterized two homologous proteins from PAC vesicles, a 72-kDa protein (PV72) and an 82-kDa protein (PV82). PV72 and PV82 showed an ability to bind to peptides derived

from both an internal propeptide and a C-terminal peptide of a proprotein precursor of 2S albumin, a major storage protein (Fig. 2B). PV72 was predicted to be a type I integral membrane protein with epidermal growth factor (EGF)-like motifs (Fig. 2A). These results suggest that PV72 and PV82 are potential sorting receptors for 2S albumin to protein-storage vacuoles. Previous studies have identified a potential receptor protein, BP-80, from the membranes of clathrin-coated vesicles of developing cotyledons of pea (*Pisum sativum*, Kirsch et al. 1994). Recently molecular structures of BP-80 (Paris et al. 1997) and the homolog from Arabidopsis (AtELP; Ahmed et al. 1997) were reported. They are membrane proteins that are homologous to PV72.

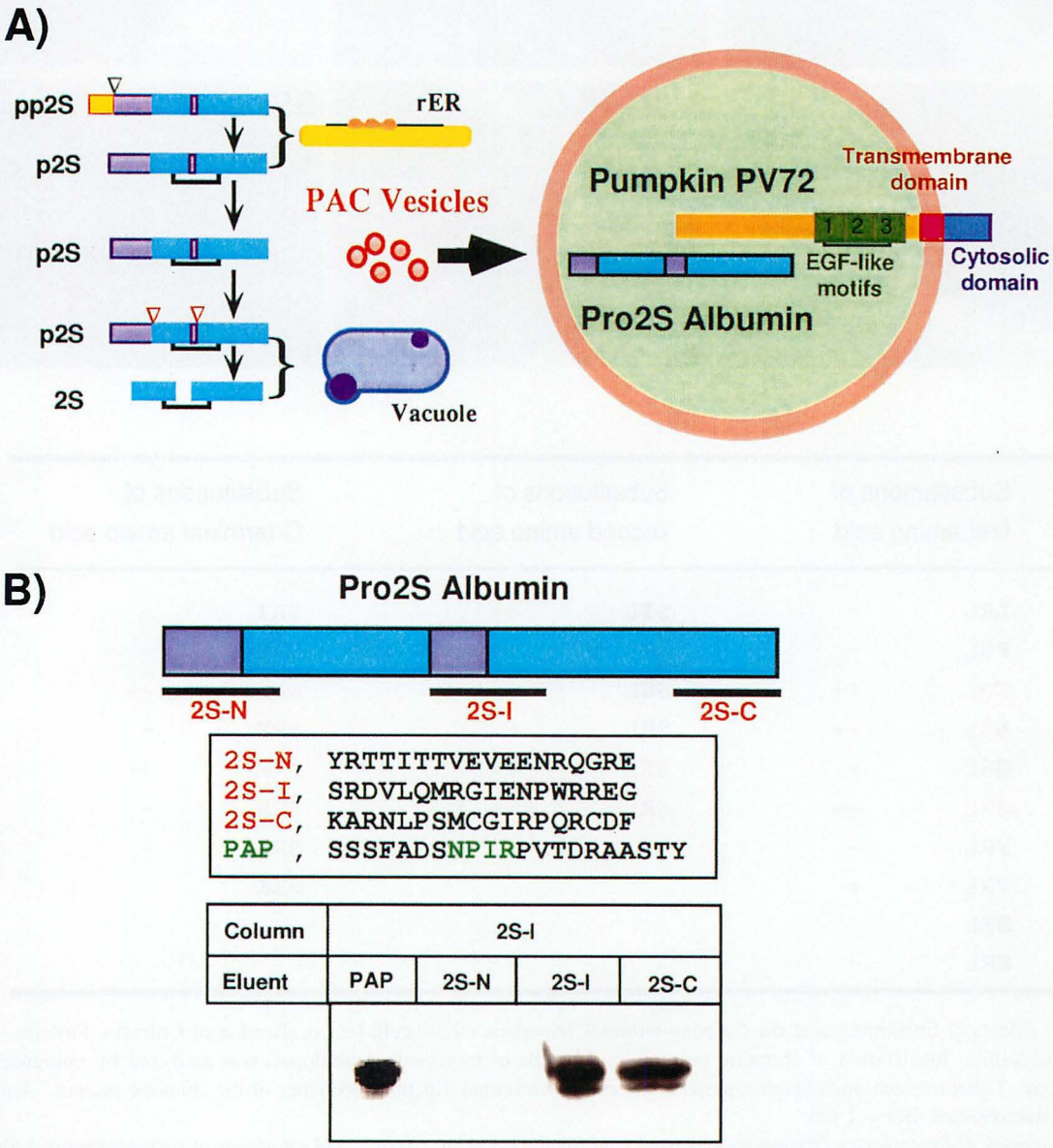


Fig. 2. PV72 functions as a sorting receptor for intracellular transport of pro2S albumin.

A) A hypothetical scheme for the transport and processing of pro2S albumin. Pro2S albumin is synthesized on rER and transported to protein storage vacuoles via precursor-accumulating (PAC) vesicles. PV72 and pro2S albumin co-exist in the PAC vesicles. PV72 is predicted to be a type I integral membrane protein with three repeats of epidermal growth factor (EGF)-like motifs.

B) PV72 binds the peptides derived from pro2S albumin. PV72 binds to an affinity column with 2S-I peptide as a ligand and is specifically eluted with an excess amount of each peptide of 2S-I, 2S-C or PAP, but not 2S-N peptide. PAP is a peptide derived from a propeptide of barley aleurain, a thiol proteinase, that contained a vacuolar targeting signal. An NPIR (Asn-Pro-Ile-Arg) has been shown to be a consensus signal sequence for vacuolar targeting that is found in both propeptides of barley aleurain and sweet potato sporamin.

IV. Expression and activation of the vacuolar processing enzyme in Saccharomyces cerevisiae.

Vacuolar processing enzymes (VPE) are cysteine proteinases responsible for maturation of various vacuolar proteins in plants. A larger precursor to VPE synthesized on the rough endoplasmic reticulum is converted to an active enzyme in the vacuoles. We expressed a precursor to castor bean VPE in a *pep4* strain of the yeast *Saccharomyces cerevisiae* to examine the mechanism of activation of VPE. Two VPE proteins with 59 kDa and 46 kDa were detected in the vacuoles of the transformant. They were glycosylated in the yeast cells, although VPE is not glycosylated in plant cells in spite of the presence of two *N*-linked glycosylation sites. During the growth of the transformant, the level of the 59-kDa VPE increased slightly until a rapid decrease occurred after 9 h. By contrast, the 46-kDa VPE appeared simultaneously with the disappearance of the 59-kDa VPE. Vacuolar processing activity increased with the accumulation of the 46-kDa VPE, but not an accumulation of the 59-kDa VPE. The specific activity of the 46-kDa VPE was similar to that of VPE in plant cells. The 46-kDa VPE mediated the conversion of procarboxypeptidase Y to the mature form instead of proteinase A. This indicated that proteinase A which is responsible for maturation of yeast vacuolar proteins, can be replaced functionally by plant VPE. These findings suggest that an inactive VPE precursor synthesized on the endoplasmic reticulum is transported to the vacuoles in the yeast cells and then processed to make an active VPE by self-catalytic proteolysis within the vacuoles.

V. An aspartic proteinase is involved in the maturation of storage proteins in concert with the vacuolar processing enzyme.

A 48-kDa aspartic proteinase was purified from protein bodies of dry seeds of castor bean. Immunocytochemical and cell fractionation analyses of the endosperm of maturing castor bean seed showed that the aspartic proteinase was selectively localized in the matrix of the protein storage vacuoles, where a variety of seed storage proteins were also present. To determine whether the aspartic proteinase is responsible for maturation of seed proteins, we prepared [^{35}S]proproteins that were localized in the endoplasmic reticulum in pulse-labeled endosperm cells and used the authentic proproteins as substrate for *in vitro* processing experiments. The purified aspartic proteinase was unable to convert any of three endosperm proproteins into their mature forms, while the purified vacuolar processing enzyme could convert all three proproteins. We further examined the activity of aspartic proteinase on the cleavage of an internal propeptide of Arabidopsis pro2S albumin, that was post-translationally removed. The aspartic proteinase cleaved the propeptide at three sites at pH 3.0, but not at pH 5.5. These results suggest that aspartic proteinase cannot directly convert pro2S albumin into the mature form, but it may play a role in trimming the C-terminal propeptides from the subunits that are produced by the action of the vacuolar processing enzyme. However, the activity of the aspartic proteinase in the protein storage vacuoles might be limited.

VI. Role of molecular chaperones in organelle differentiation

Molecular chaperones are cellular proteins that function in the folding and assembly into oligomeric structures of certain other polypeptides 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 translocation of proteins into chloroplasts. In addition to mitochondrial chaperonin10 homologues, we isolated a cDNA for chloroplastic chaperonin 10 homologues from *Arabidopsis thaliana*. The cDNA was 958 bp long and encoded a polypeptide of 253 amino acids. The deduced amino acid sequence showed that the protein contained an N-terminal chloroplast transit peptide and the mature region which was comprised of two distinct GroES-domains. The two halves of the Cpn20 show 42 % amino acid identity to each other. A Northern blot analysis revealed that the mRNA for the Cpn10 homologue was abundant in leaves and was increased by heat treatment. A histidine-tagged construct lacking the transit peptide was expressed in *E. coli* and the functional and structural characterization is in progress.

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DIVISION OF BIOENERGETICS

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This division aims to elucidate the mechanism and its regulation of intracellular protein degradation in a lytic compartment. Recently it was realized that degradation process plays essential roles for various physiological function. 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.

Little is known about the mechanism of protein degradation in contrast with that of 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 (Fig.1). Since cellular lytic compartment, lysosome, was identified, biogenesis of lysosomal enzymes and their enzymatic characterization have been studied thoroughly. However, the mechanisms of delivery of proteins to the lysosomes are not known at a molecular level. Biochemical analysis of lysosome/vacuole system is quite difficult because of its complexity and dynamic feature. Autophagic process has been studied mostly by electron microscopy, and molecular mech ıism of autophagy remains to be elucidated.

Yeast Induces Autophagy as Mammalian Cells

Recently we discovered yeast, *Saccharomyces cerevisiae*, induces bulk protein turnover in the vacuoles under starvation conditions. This whole process corresponds to that of macroautophagy in higher eukaryotic cells. By electron microscopic analyses we succeeded in detecting double membrane structures in the cytoplasm enclosing a portion of cytosol. These yeast autophagosomes immediately fuse with vacuoles, delivering single inner membrane structures, autophagic bodies in the vacuole. When vacuolar proteinase activities are blocked genetically or by specific inhibitor such as PMSF autophagic bodies are accumulated in the vacuoles. They moves around vigorously in the vacuoles by Brownian motion, and are easily detectable by light microscope. Thus we can follow the progression of autophagy as the accumula-

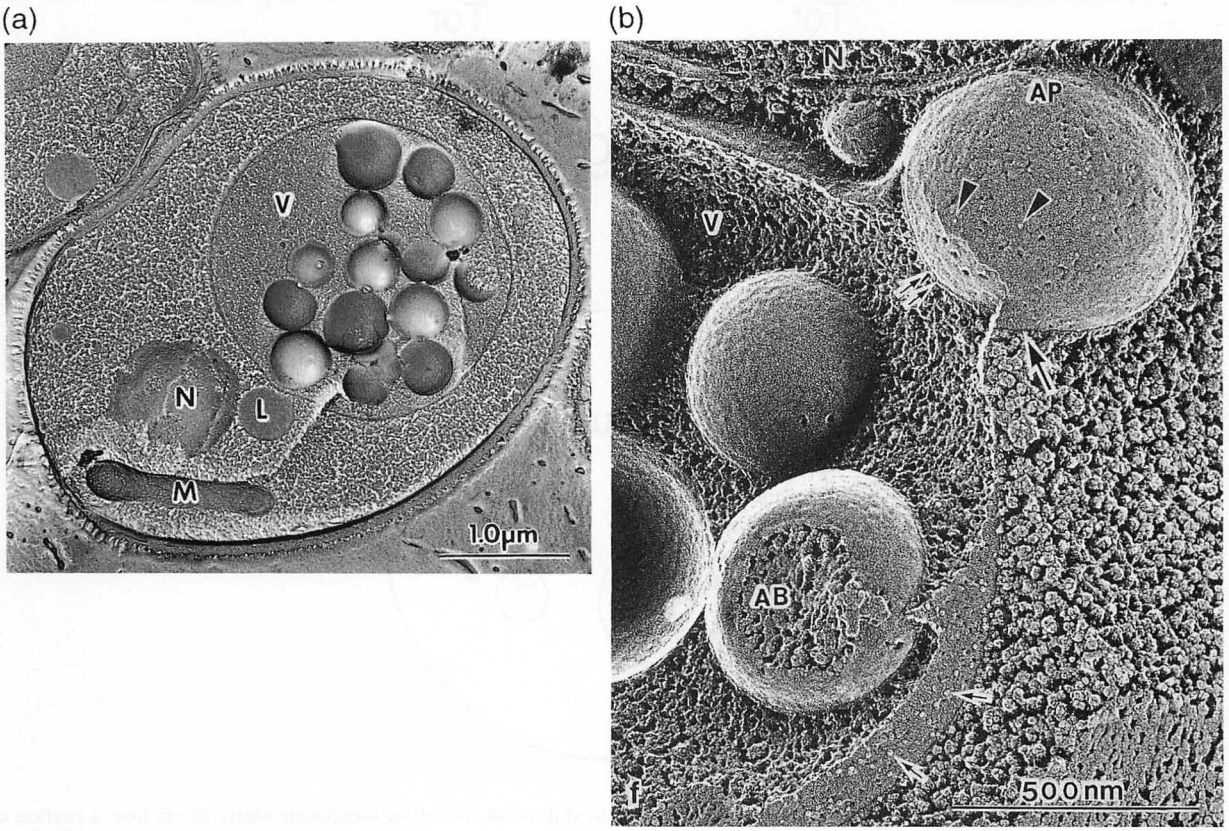

Fig. 1. (a) Freeze-Fracture Image of Yeast under Nitrogen Starvation.
When vacuolar proteinases are blocked, spherical membrane structures, autophagic bodies accumulate in the vacuole.
(b) Fusion of Autophagosome to the Vacuolar Membrane.
Double membrane structure, autophagosome (AP), encloses a portion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

tion 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 randomly to the vacuoles to be degraded.

Autophagy in yeast is a kind of general stress response to adverse environmental condition and is induced by various starvation conditions such as nitrogen, carbon, sulfate, and even single amino acid depletion. Signal transduction of starvation for induction of autophagy is a key question to be solved. Recently we found rapamycin induces autophagy in the cell growing in a rich medium and that Tor, phosphatidyl inositol kinase homologue plays essential role for the regulation of autophagy (Fig.2).

Another crucial and controversial question is the mechanism of formation of autophagosome. Still nothing is clear about the origin of the membrane. Freeze-fracture electron microscopy showed that autophagosomal membrane has quite characteristic feature and the density of intramembrane particles is extremely low as compared with other intracellular membrane.

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 autophagy defective mutants, *agp*, were isolated. They cannot induce protein degradation upon shift from growth medium to the starvation medium. 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* mutant is sporulation-defective as expected.

Analyses of *APG* genes

Now we are focusing on characterization of these *APG* genes, essential for autophagy. So far we have finished cloning and identification of twelve *APG* genes. *APG1* codes a novel Ser/Thr protein kinase essential for the induction of autophagy. This pro-

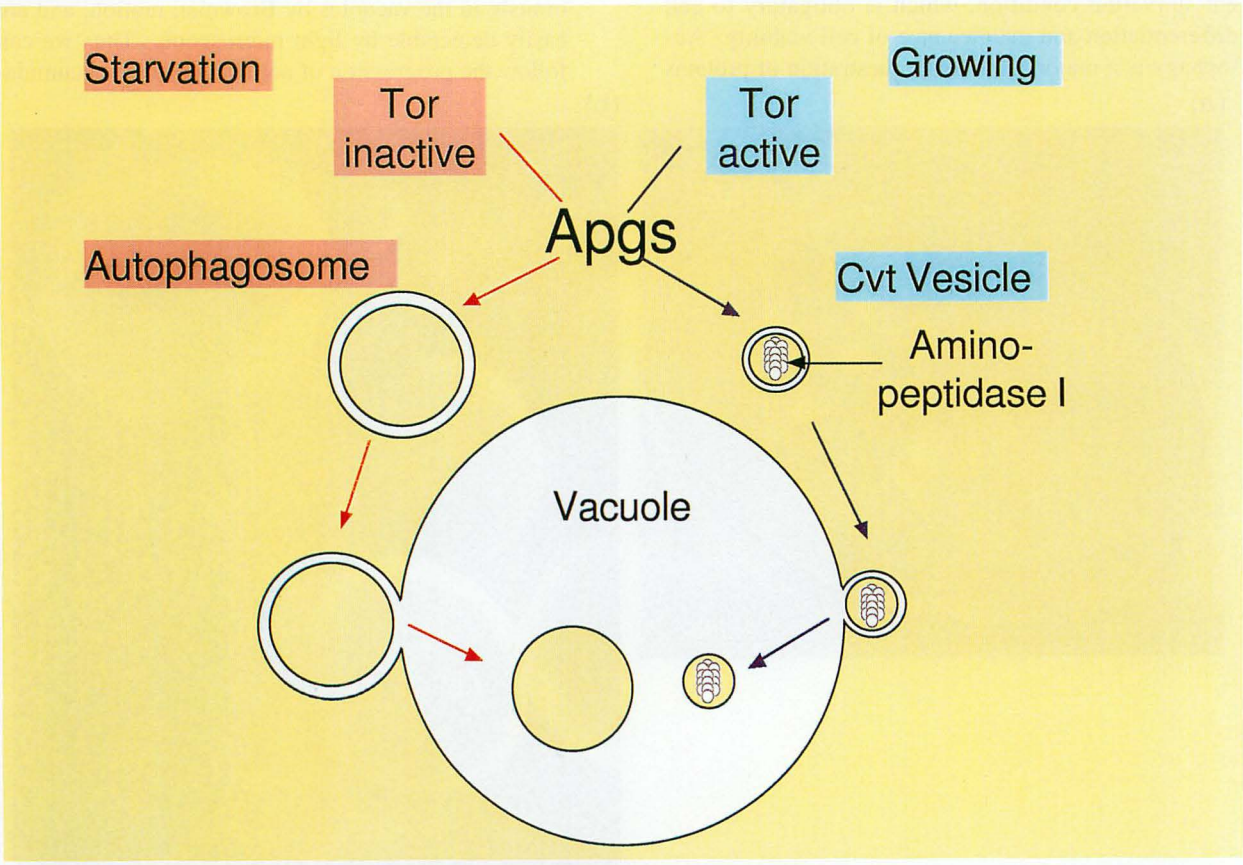

Fig. 2 Schema of autophagy in yeast. When yeast cells face to starvation, isolation membrane starts to enclose a portion of cytosol and form an autophagosome. Autophagosome fuses to the vacuolar membrane, delivering autophagic body in the vacuoles (Autophagic pathway). Under growing condition one of vacuolar enzymes, API is selectively sequestered to the vacuole (Cvt pathway). Two distinct pathways share most molecular machinery.

vides the first direct evidence for involvement of protein phosphorylation in the process of autophagy. Apg13p interacts with Apg1p and might function as a regulator of its kinase activity. So far most *APG* genes analyzed are novel and non-essential for vegetative growth. Using the assay system of autophagy we developed, we are studying genetic interaction among *APG* genes. Some *APG* gene products are inducible or change phosphorylation state by nutrient starvation. Analyses of intracellular localization of these Apgps may provide us specific marker to elucidate the membrane dynamics during autophagy.

Diverse pathways of autophagy

Autophagy is ubiquitous and fundamental physiological response of every eukaryotic cell. We found yeast cells induce macroautophagy under starvation, which is a main route for bulk and non-selective protein degradation. While it is known that excessive amount of organelles are also degraded by autophagic process. 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 lysosome/vacuoles.

Recently it was found that all *APG* genes are required for targeting of one of vacuolar enzyme, API, from cytosol to the vacuole. Electron microscopic analyses of API transport process revealed that API first forms a complex in the cytosol and delivered to the vacuole by the distinct but topologically similar mechanism to the macroautophagy (Fig.2). However, API transport is selective and constitutive process. It is interesting that biosynthetic pathway shares the machinery with degradative process. Some selective degradation may also be mediated by autophagy-related mechanism.

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. Molecules involved in the membrane dynamics are well conserved from yeast to higher eukaryotes. We realized some *APG* genes in yeast show homologues in mammals or higher plants. Knowledge in yeast must give us key to uncover the mechanism of autophagy in higher eukaryotic organisms. We are now developing systems for studying autophagy in mammalian cell culture.

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DIVISION OF CELL PROLIFERATION (ADJUNCT)

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	Satsuki Okamoto

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 relevant to the regulation of meiosis in animals and plants, by isolating genes that either complement *Schizosaccharomyces pombe* meiotic mutants or affect the meiotic behavior of this fission yeast. We have isolated a number of animal and plant genes in this manner, and analysis of the cloned genes is in progress. In addition, efforts have been also paid to elucidate the regulatory mechanisms of meiosis in fission yeast in more detail, so that the above strategy may be reinforced. Some examples of the analysis are presented below.

I. Microtubule-binding protein which functions specifically during meiosis.

In fission yeast, an RNA species named meiRNA is specifically required for the promotion of the first meiotic division. To dissect the function of this RNA and its partner RNA-binding protein Mei2p, we screened for high-copy-number suppressors of the arrest prior to the first meiotic division caused by loss of meiRNA. Analysis of one of the suppressors thus isolated, named *ssm4*, suggested that it encodes a coiled-coil protein carrying a microtubule-binding motif at its N-terminus. Expression of *ssm4* was restricted to cells undergoing meiosis. Disruption of *ssm4* affected neither vegetative growth nor conjugation, but resulted in frequent generation of asci carrying less than four spores. Tagged Ssm4p localized at spindles during both the first and the second meiotic division. The microtubule-binding motif was essential for the association of Ssm4p with microtubules and for its function during meiosis, but not for the suppression of loss of meiRNA. Ssm4p appeared to possess a potential to migrate to the nucleus. Thus, Ssm4p is a microtubule-colocalizing protein that plays a role specifically in meiosis. Ssm4p appears to modify the structure or the function of nuclear microtubules in order to promote the meiotic nuclear division.

II. Arabidopsis 14-3-3 isoform.

14-3-3 proteins constitute a highly conserved family among eukaryotes, and diverse biochemical activities have been ascribed to them. We isolated a variety of *Arabidopsis* 14-3-3 genes in a screening for cDNAs that could block ectopic meiosis driven by the *pat1* mutation in fission yeast. In addition to suppressing the *pat1* mutation, these cDNAs could cure the deformed morphology and raised UV sensitivity of fission yeast cells defective in rad24, which encodes a 14-3-3 protein. This indicates that most *Arabidopsis*

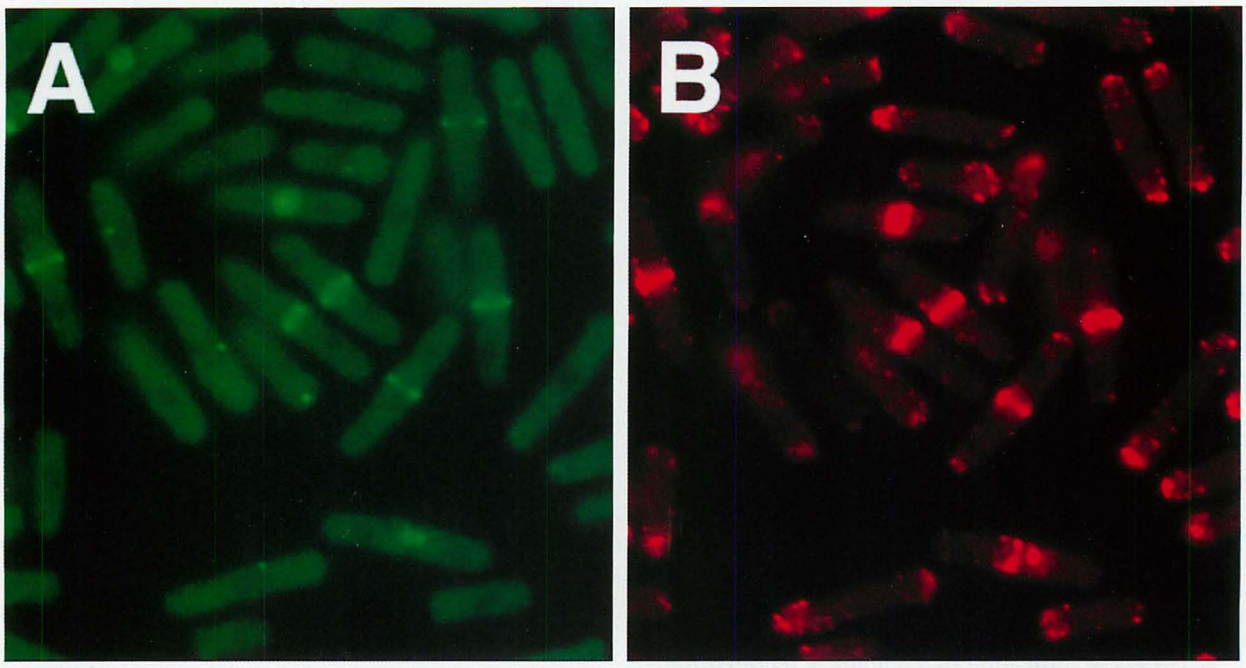

Fig. 1. Cellular localization of *S. pombe* type II myosin heavy chain Myo2p fused to the jellyfish green fluorescent protein (GFP) and that of *S. pombe* actin. Cells grown to the mid-log phase were fixed, immediately stained with Phalloidin-TRITC, and observed under the microscope as rapidly as possible. Micrographs show GFP fluorescence of Myo2p (A) and phalloidin-TRITC-stained actin (B).

14-3-3 proteins can execute the function of the fission yeast 14-3-3 protein encoded by *rad24*, at least partially. We analyzed one of the *Arabidopsis* 14-3-3 clones in more detail, as it was not fully characterized in literature. This 14-3-3 gene turned out to be expressed in all plant organs examined.

III. Identification of fission yeast type II myosin heavy chain.

We cloned the *myo2* gene of *Schizosaccharomyces pombe*, which encodes a type II myosin heavy chain, by virtue of its ability to promote meiosis in certain mutants. The *myo2* gene encoded 1526 amino acids, and deduced Myo2p showed homology to the head domains and the coiled-coil tail of the conventional type II myosin heavy chain, and carried putative binding sites for ATP and actin. Disruption of *myo2* inhibited cell proliferation. *myo2*-defective cells showed normal punctate distribution of interphase actin, but they produced irregular actin rings and septa, and were impaired in cell separation. Overproduction of Myo2p was also lethal, apparently blocking actin relocation. Nuclear division proceeded without actin ring formation and cytokinesis in cells overexpressing Myo2p, giving rise to multinucleated cells with dumbbell morphology. Analysis using tagged Myo2p revealed that Myo2p colocalizes with actin in the contractile ring, suggesting that Myo2p is a component of the ring and responsible for its contraction. Furthermore, genetic evidence suggested that the acto-myosin system may interact with the Ras pathway, which regulates mating and the maintenance of cell morphology in *S. pombe*.

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DIVISION OF CELLULAR COMMUNICATION (ADJUNCT)

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Graduate student: *Itsushi Minoura (University of Tokyo)*

The research in this laboratory, started in November, 1966, 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 actin and flagellar dynein. 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 significantly differ in function. For example, the outer arm heavy chains are important for flagellar beating at high frequency, whereas the inner-arm heavy chains are important for producing proper waveforms. Indirect evidence also suggests that the properties of force generation by different heavy chains differ greatly. We are currently carrying out micro-physiological experiments to assess the force production by each species of dynein.

The inner dynein arms are known to contain actin as a subunit. Hence the two independent motility systems of eukaryotes - the actin-based and microtubule-based systems - should somehow cooperate in the inner arm dynein, although the function of actin as a subunit of dynein is totally unknown at present. Recently we found that the mutant *ida5*, lacking four out of the seven subspecies of inner-arm dyneins, has a mutation in the actin-encoding gene. Intriguingly, *Chlamydomonas* has only a single gene of conventional actin, and the mutant *ida5* was found to express no conventional actin at all. On close inspection, however, the cytoplasm and axoneme of this mutant were found to contain a novel actin-like protein (NAP) which has only a very low (64%) homology to conventional actin. Despite the lack of the conventional actin, *ida5* displays normal cell division and grows as rapidly as wild type. However, the mating efficiency of this mutant is much lower than in wild type, because it is deficient in the growth of the fertilization tubule, an F-actin-containing structure that facilitates mating. Thus conventional actin and NAP appear to overlap in some, but not all, cellular functions.

The actin-null mutant *ida5* should be of great use in elucidating the functional domains of actin. We have recently succeeded in transforming this mutant with a cloned actin gene, and found that inner arm dynein, as well as the fertilization tubule in gametic cells, became restored in the transformants. We are currently trying to transforming the mutant with artificially modified actin gene, to see if polymerization or interaction with myosin is important for the actin function in the dynein arms. We are also examining expression and localization of actin and NAP in various stages of cell cycle. Studies on those lines will enable us to determine whether actin or NAP is essential for cytokinesis, assembly and function of inner dynein arms, and other fundamental cellular phenomena in *Chlamydomonas* and other cells.

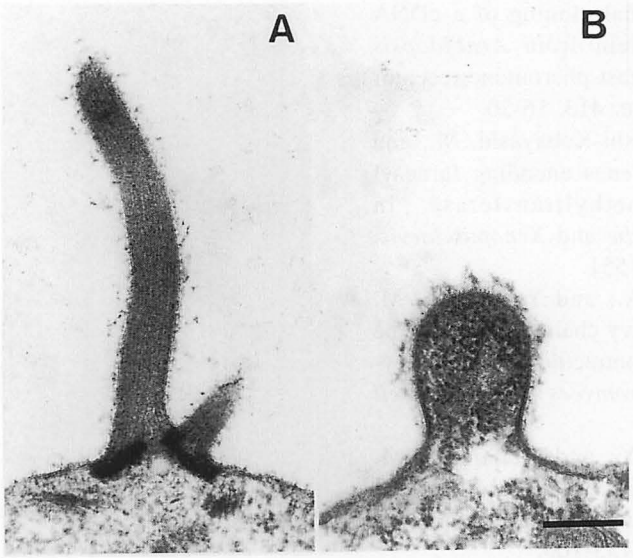

Fig. 1. Fertilization tubules in wild-type(A) and *ida5* (B) mt^+ gametes produced in response to a 1 hour exposure to 10 mM dibutyryl-cAMP and 1 mM IBMX. Bar, 0.3 μm. Wild-type fertilization tubules have been shown to contain F-actin bundles.

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DIVISION OF CELL FUSION (ADJUNCT)

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Research Associate: *Hirotaka Fujimoto*

Cytokinesis in animal and some primitive eukaryotic cells is achieved by the progressive contraction of the cleavage furrow. The cleavage furrow contains a contractile apparatus, called the contractile ring, which is composed of a bundle of actin filaments that lies in the furrow cortex beneath the plasma membrane. It has been established that the contractile ring contracts as the result of interaction between actin filaments and myosin. However, little is known about process of its formation, mechanism that controls its formation, protein constituents, and its ultrastructure. The goal of our research is to solve these problems and thereby clarify the molecular mechanism of cytokinesis. For this purpose, we use three kinds of cells, namely, sea urchin egg, *Xenopus* egg, and the fission yeast *Schizosaccharomyces pombe*.

The isolation of cleavage furrows from dividing cells is one of the most important methods for elucidating the molecular mechanism underlying cytokinesis. We hand-isolated furrows from newt eggs and found some unique proteins in the furrow preparation (Mabuchi, Tsukita, Tsukita, and Sawai, Proc. Natl. Acad. Sci. USA 85: 5966-5970, 1988). However, this method has the disadvantage that it is not easy to obtain a sufficient amount of the furrows for analysis of the protein constituents. We have also reported a mass isolation method for cleavage furrows from eggs of the sand dollar, *Clypeaster japonicus* (Yonemura, Mabuchi, and Tsukita, J. Cell Sci. 100: 73-84, 1991). However, this method has not been applicable to the regular sea urchins.

We have recently developed an isolation method for cleavage furrows by which we were able to isolate furrows from various sea urchin eggs. The contractile ring was included in the isolated cleavage furrows, as seen on rhodamine-phalloidin staining of actin filaments. When the furrows were isolated with the isolation medium containing both NaF and β-glycerophosphate, which are potent protein phosphatase inhibitors, the isolated furrows were found to be accompanied by the mitotic apparatus. When the isolation was carried out without both of them, cleavage furrows without the mitotic apparatus were obtained (Fig. 1).

The development of a method of isolation of cleavage furrows from regular sea urchin eggs enabled us to compare protein constituents among furrows from different sea urchin and sand dollar species. We found that 32, 36, 51 kDa proteins were concentrated in common in the cleavage furrows isolated from eggs of the sand dollars, *C. japonicus* and *Scaphechinus mirabilis*, and sea urchins, *Hemicentrotus pulcherrimus* and *Strongylocentrotus nudus*, on two-dimensional gel electrophoreses. We are currently investigating the nature of these proteins.

Next we investigated the role of myosin in cytokinesis of *S. pombe*. Recently, it has been shown by Kitayama et al. (J. Cell Biol. 137: 1309-1319, 1997) that Myo2, a type II myosin heavy chain plays a role in the F-actin ring formation in this organism. We found another myosin II called Myo3. Myo3 is the same protein as Myp2 reported independently by Bezanilla et al. (Mol. Biol. Cell, 8: 2693-2705, 1997).

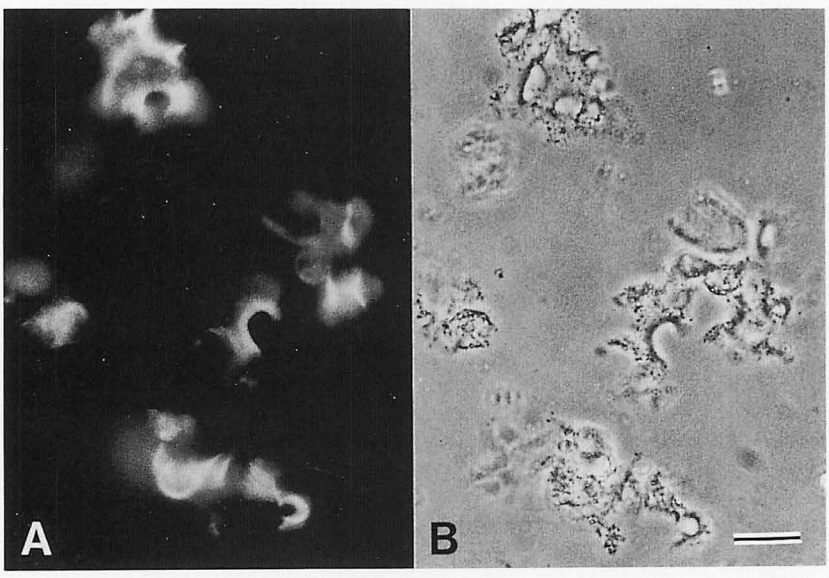

Fig. 1 Cleavage furrows of *Hemicentrotus pulcherrimus* eggs isolated with the isolation medium that does not contain protein phosphatase inhibitors. Fluorescence (A) and phase-contrast (B) micrographs of cleavage furrows stained with rhodamine-phalloidin are presented. Bar, 50 μm.

Overexpression of Myo3 in the cell leads to formation of aberrant F-actin ring, F-actin cable, and septum. We knocked out the myosin genes in *S. pombe*. Since Myo3 is not essential, but Myo2 is essential for its growth, we made a *myo2myo3* null strain containing pREP81-myo2 to control the expression of *myo2*. After shut off the myo2, no F-actin ring formation occurs during mitosis. Therefore, type-II myosin is necessary in the formation of the contractile ring in *S. pombe*.

Publication List:

Fujimoto, H. and Mabuchi, I. (1997) Isolation of cleavage furrows from eggs of regular sea urchins and identification of furrow-specific proteins. *J. Biochem.* **122**, 518-524.

DEPARTMENT OF DEVELOPMENTAL BIOLOGY

Chairman: Yoshitaka Nagahama

The Department is composed of three divisions and one adjunct division. Department members conduct molecular analysis on various aspects of developmental phenomena including: (1) differentiation and maturation of germ cells, (2) molecular basis of body plans, and (3) gene regulation in cell differentiation and growth.

DIVISION OF REPRODUCTIVE BIOLOGY

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Monbusho Foreign Scientist:	*Allen Schuetz (Johns Hopkins University)*
Visiting Scientist:	*Graham Young (University of Otago)*

The pituitary-gonadal axis plays an important role in regulating gametogenesis in vertebrates. In most cases, however, gonadotropins act through the biosynthesis of gonadal steroid hormones which in turn mediate various stages of gametogenesis. Studies using teleost fishes as experimental models provide new information about the endocrine regulation of gametogenesis including oocyte growth, oocyte final maturation, spermatogenesis, and sperm maturation. Our research focuses on (1) the identification of steroidal mediators involved in each process of gametogenesis, and (2) the mechanisms of synthesis and action of the mediators. These studies collectively demonstrate the appropriateness of using teleost fishes as valid models for examining hormonal influences on gametogenesis. Such models could also have applications and validity for vertebrates in general.

I. Endocrine regulation of oocyte growth and maturation

17α,20β-dihydroxy-4-pregnen-3-one (17α,20β-DP) was identified as the maturation-inducing hormone of several teleost fishes including salmonid fishes. 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 fishes. Two cell type models have been proposed for estradiol-17β and 17α,20β-DP production, with the thecal layer providing precursor steroids to the granulosa layer. 17α,20β-DP acts via a receptor on the plasma membrane of oocytes. A specific 17α,20β-DP receptor has been identified and characterized from defolliculated oocytes of several fish species. The concentrations of 17α,20β-DP membrane receptors increase immediately prior to oocyte maturation. The pertussis toxin-sensitive G-protein is involved in the signal transduction pathway of 17α,20β-DP. We have cloned two Giα cDNAs from a medaka oocyte cDNA library. 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.

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), which consists of cdc2 kinase (34-kDa) and cyclin B (46- to 48-kDa). Goldfish immature oocytes contain only monomeric 35-kDa inactive cdc2 kinase and do not stockpile cyclin B, although immature oocytes contain mRNA for cyclin B. 17α,20β-DP induces oocytes to synthesize cyclin B, which in turn activates preexisting 35-kDa cdc2 kinase through its threonine 161 phosphorylation by a threonine kinase (MO15), producing the 34-kDa active cdc2. We examined goldfish oocyte proteins bound to cyclin B mRNA. Using oligo(dt)-cellulose affinity chromatograpy and Southwestern and Northwestern analyses, we found a 54-kDa cyclin B mRNA-binding protein (p54), which also bound to the Y box DNA element. We cloned cDNAs encoding Y box proteins, one of which is likely to encode p54 and is expressed specifically in germ cells. We suggest that the 54-kDa Y box protein may have a role in translational repression of cyclin B mRNA in immature oocytes, and 17α,20β-DP stimulation releases this repression, leading to the *de nove* synthesis of cyclin B.

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 the involvement of 26S proteasome in cyclin degradation through the first cleave on its N-terminus.

II. Endocrine regulation of spermatogenesis

Under cultivation conditions, male Japanese eels (*Anguilla japonica*) have immature testes containing only type A and early type B spermatogonia together with inactive Sertoli cells and Leydig cells. Using an organ culture system for eel testes, we have shown

that the hormonal regulation of spermatogenesis in eel testes involves the gonadotropin stimulation of Leydig cells to produce 11-ketotestosterone (11-KT), a potent androgen in fish. In turn, 11-KT activates Sertoli cells to stimulate premitotic spermatogonia to complete spermatogenesis. Gonadotropin (human chorionic gonadotropin, hCG)-induced spermatogenesis was accompanied by marked, rapid increase in 11-KT production. Northern blot analysis revealed that both 11β-hydroxylase and 11β-hydroxysteroid dehydrogenase mRNA transcripts were not present in testes of eels prior to gonadotropin treatment, but were abundant in those after gonadotropin treatment for 1 day. We used a subtractive hybridization method to clone genes that are expressed differentially in eel testes in the first 24 hr after gonadotropin treatment. One of the up-regulated cDNAs was identified as coding the activin βB subunit. Activn βB mRNA transcripts were absent in testes prior to gonadotropin treatment and were abundant in Sertoli cells in testes of eels treated with gonadotropin for 1-6 days. 11-KT induced a marked production of activin B in cultured testes, indicating that eel activn βB subunit production is largely for activin B formation. Addition of recombinant eel activin B to the culture medium induced proliferation of spermatogonia, producing late type B spermatogonia, within 15 days in the same manner as did 11-KT. Full-length cDNAs for eel activin type I and II receptors were isolated and sequenced. The type I receptor mRNA transcripts were present in testes prior to gonadotropin injection, with a marked increase in spermatogonia and spermatocytes after gonadotropin injection for 9 days. Taken together, these results indicate that activin B produced by Ser-

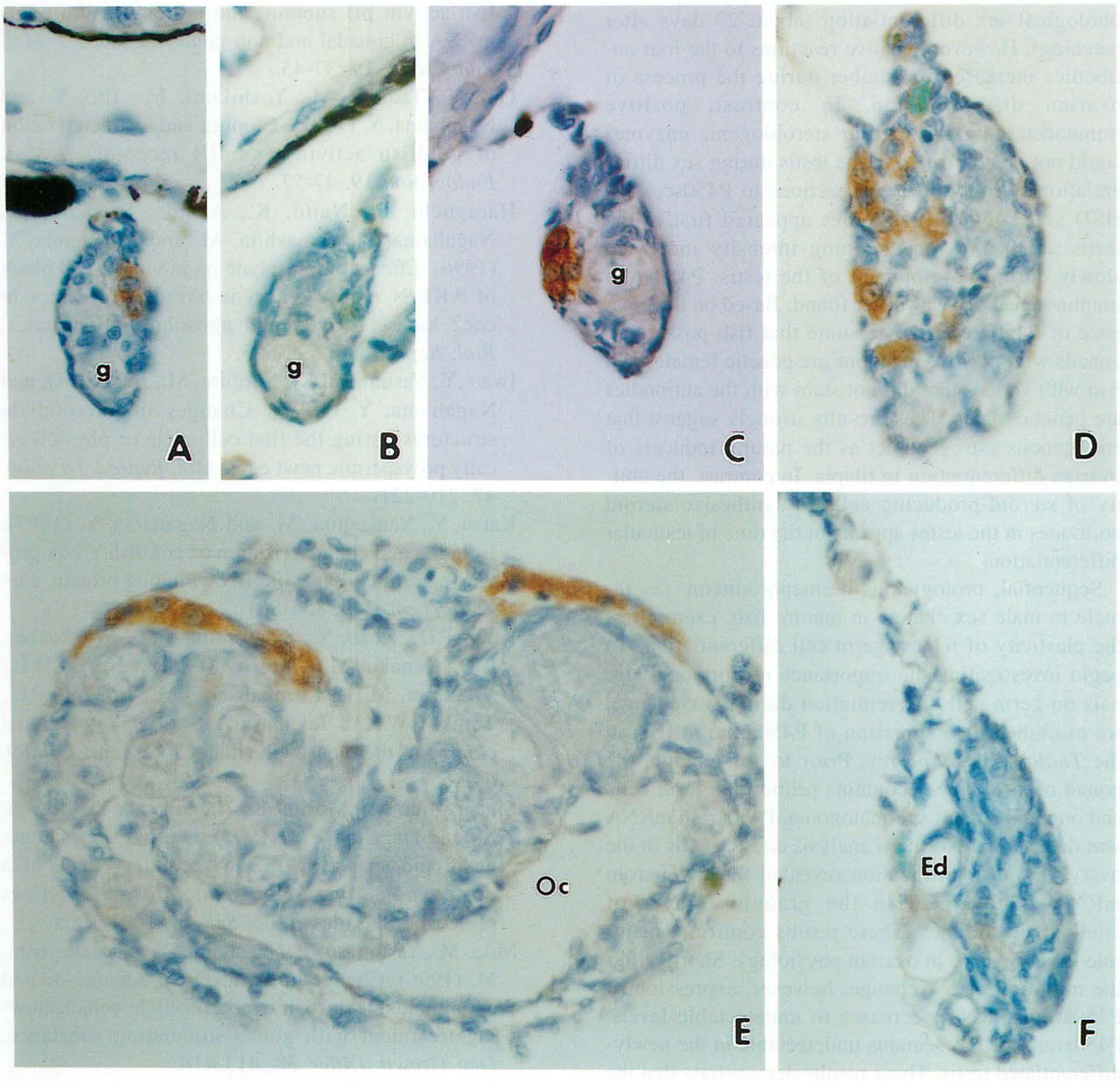

Fig. 1. Immunocytochemical localization of 3β-hydroxysteroid dehydrogenase and cytochrome P450 aromatase in tilapia gonads collected at 20 days (A, female; B, male), 23-25 days (C, female) , 30 days (D, female), 50 days (E, female), and 30 days (F, male) after hatching. A, B, D, E, and F, 3β-hydroxysteroid dehydrogenase. C, cytochrome P450 aromatase. g, gonium; Oc, ovarian cavity; Ed, efferent duct. X 800.

toli cells under the influence of gonadotropin and 11-KT plays an important role in the regulation of spermatogenesis in fish testes.

III. Endocrine regulation of gonadal sex differentiation

The initial differentiation and development of steroid-producing cells during gonadal sexual differentiation in tilapia, *Oreochromis niloticus*, were examined immunohistochemically using the antibodies of four enzymes essential for steroidogenesis (P450scc, 3β-HSD, P450c17, and P450arom)(Fig. 1). There are two patterns of immunoreactivity in gonads, with either positive reactions for all antibodies, or no reactions with any of the antibodies. Positive immunoreactions for all four antibodies became evident for the first time in the gonads of some fish in mixed sex group examined several days before morphological sex differentiation (about 23 days after hatching). However, positive reactions to the four antibodies increased in number during the process of ovarian differentiation. In contrast, positive immunostaining for all four steroidogenic enzymes could not be confirmed in the testis during sex differentiation. Weakly positive reactions to P450scc, 3β-HSD and P450c17 antibodies appeared first in the testis at 30 days and staining intensity increased slowly during development of the testis. P450arom immunoreactivity was never found. Based on the presence of P450arom, we presume that fish possessing gonads with positive reactions are genetic females and fish with gonads that did not stain with the antibodies are genetic males. These results strongly suggest that endogenous estrogens act as the natural inducers of ovarian differentiation in tilapia. In contrast, the ability of steroid-producing cells to synthesize steroid hormones in the testes appears at the time of testicular differentiation.

Sequential, protogynous hermaphroditism, i.e. female to male sex change in mature fish, exemplifies the plasticity of teleost germ cell differentiation. To begin investigating the importance of hormonal signals on germ cell differentiation during sex change, we examined the expression of P450arom mRNA in the *Thalassoma duperrey*. Prior to sex change, the gonad of *T. duperrey* contains primordial germ cells and oogonia but not spermatogonia. P450arom mRNA was detected by Northern analysis and RT-PCR in the ovary. *In situ* hybridization revealed that P450arom mRNA is localized in the granulosa layer of vitellogenic follicles. These results confirm a major role for aromatase in ovarian physiology. Shortly after the initiation of sex change, however, expression of P450arom mRNA decreases to undetectable levels. P450arom mRNA remains undetectable in the newly-differentiated testis. These results demonstrate that the loss of aromatase is a permanent and critical step in the sex change cascade. As such, they suggest that aromatase/estradiol is important for oogonial differentiation, detrimental to spermatogonial differentiation or a combination of both in *T. duperrey*.

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DIVISION OF CELL DIFFERENTIATION

Professor: *Yoshiaki Suzuki (until July)*
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Hiroki Kokubo (on leave)

The E complex is a homeotic gene complex which specifies the identities of abdominal segments in the silkworm, *Bombyx mori*. We have reported that the E complex is similar to the *Drosophila bithorax* complex. The E complex comprises three homeobox genes: *Bombyx Ultrabithorax* (*Bm Ubx*), *Bombyx abdominal-A* (*Bm abd-A*), and *Bombyx Abdominal-B* (*Bm Abd-B*). Since some types of mutations in the E complex cause abnormal development of abdominal legs, the E complex regulates development of abdominal legs. Analyses of two mutants, the E^{Ca}/E^{Ca} (additional crescent) and E^{N}/E^{N} (new additional crescent) mutants, which lack abdominal legs demonstrated that the *Bm abd-A* is deleted within the mutant chromosomes. Therefore, *Bm abd-A* may be an essential gene for development of abdominal legs.

The homeodomain proteins encoded by homeotic genes are proposed to act as transcriptional regulators on target genes. To determine the target genes of the *Bm abd-A* gene product, we biochemically analyzed the proteins that are expressed specifically in abdominal legs and found that a high molecular weight protein (p260/270) was expressed specifically in abdominal leg cells during early embryonic stages and disappeared by a late embryonic stage. p260/270 consists of two polypeptides with molecular weights of 260,000 and 270,000 daltons. We have established a purification procedure and have raised an antibody against p260/270. Immunoblot analysis of the E^{Ca}/E^{Ca} and E^{N}/E^{N} mutants demonstrated that the two mutants lacked p260/270.

cDNA cloning and sequencing demonstrated that p260 and p270 have structures similar to rat fatty acid synthase, which synthesizes palmitate. Most of enzymatic domains for palmitate synthesis were well conserved in the amino acid sequences of p260 and p270. Many viral, cytoskeletal and cell surface receptor proteins have been reported to be modified with palmitate at their cysteine residues. Some small GTP-binding, heterotrimeric G and G-protein-linked receptor proteins are known to be modified with palmitate via thioester linkages. Such modifications may be important in regulation of signal transduction. 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

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Technical Staff: *Chiyo Takagi*
Takamasa Yamamoto
Shunichiro Iemura
Yuko Takahashi

Our laboratory joined the NIBB in May of 1997 from Hokkaido University. We have been studying about the roles of growth factors in early embryogenesis.

Early developmental processes are regulated by a number of endocrine and paracrine factors that mediate cell-to-cell interactions. Particularly, polypeptide growth factors are believed to be essential for the regulation of cell proliferation and differentiation in morphogenesis during embryogenesis. One of the intriguing features of polypeptide growth factors is that their functions are well conserved in animal species. This allows us to study developmental mechanisms governed by growth factors using multiple animal models such as mouse, *Xenopus*, *Drosophila*, and *C. elegans*. We focus on a group of growth factors, the TGF-β superfamily and investigate molecular mechanisms for their actions in early embryogenesis. Our study involves biochemistry, embryology, molecular biology and genetics.

I. The role of follistatin in neural induction

It had been shown that bone morphogenetic protein BMP is a key regulator of mesoderm patterning by inducing ventral mesoderm in early *Xenopus* embryo. BMP also has an essential role in neural tissue formation. Presumptive ectoderm of *Xenopus* embryo gives rise to epidermis unless it is exposed to endogenous factors(s) from dorsal mesoderm or exogenous stimuli such as neural inducers. Therefore, it was believed that epidermis is a ground (default) state and neural tissue is induced by the neural inducers. However, it has been demonstrated by us and other groups that BMP specifies epidermis and inhibits neural fate. This anti-neural activity of BMP was later found to be conserved in animal species, from fly to mammals. More recently, it turned out that neural inducers chordin and noggin are BMP-binding proteins which inhibit BMP to interact with its receptor. These observations led to a model that not epidermal but neural fate is the default state. Follistatin, an activin-binding protein that inhibits activin activity had also been shown to act as a neural inducer. Because activin itself has no inhibitory action to neural induction, we questioned about the mechanism by which follistatin induces neural fate. Does follistatin mediate neural inducing signal through its own receptor yet unknown? Or, does it inhibit the neural inhibitor BMP as do chordin and noggin?

To address this problem, we hypothesized that binding specificity of follistain may be broader and bind BMP as well as activin. In fact, follistatin was found to inhibit ventralization caused by BMPs (Figure 1a, b). After a series of analyses employing an instrument called surface plasmon resonance (SPR) sensor, we have successfully demonstrated that follistatin interacts with three subtypes of BMP, namely homodimers of BMP-2, BMP-4, and BMP-7 and a heterodimeric BMP, BMP-4/7. Interestingly, we have also shown that follistatin bind to a complex of BMP and BMP receptor, thereby inhibiting receptor activation (Figure 1c). It was previously shown that there are two isoforms of follistatin generated by alternative splicing, one having an extended C-terminal. During the course of this study, we have found that the C-terminally extended form has much weaker inhibitory activity against BMP. We are currently undertaking a study to understand the structure-function relationship.

II. Intracellular signaling of BMP through a novel MAPKKK, TAK1

TGF-β superfamily members including BMP are known to elicit signals through simulation of a complex of serine/threonine kinase receptors consists of type I and type II receptors. Recent studies of this signaling pathway have identified two types of novel mediating molecules, the Smads and TGF-β activated kinase 1 (TAK1). Smads are cytoplasmic proteins that bind type I receptors but translocate to nucleus upon stimulation of ligands. They have been shown to mimic the effect of BMP, activin and TGF-β. In collaboration with groups of Dr. Matsumoto (Nagoya University) and Dr. Nishida (Kyoto University), we have identified TAK1 and TAB1 as a MAPKKK and its activator, respectively, which might be involved in the up-regulation of TGF-β family-induced gene expression.

To understand biological significance, we isolated *Xenopus* counterpart of TAK1 and TAB1 and examined their role in the dorsoventral patterning of early *Xenopus* embryo (Shibuya, H. et al. (1998) *EMBO J.*, 17, 1019-1028). Ectopic expression of TAK1 in early embryo induced cell death. Interestingly, however, concomitant overexpression of *bcl-2* with the acti-

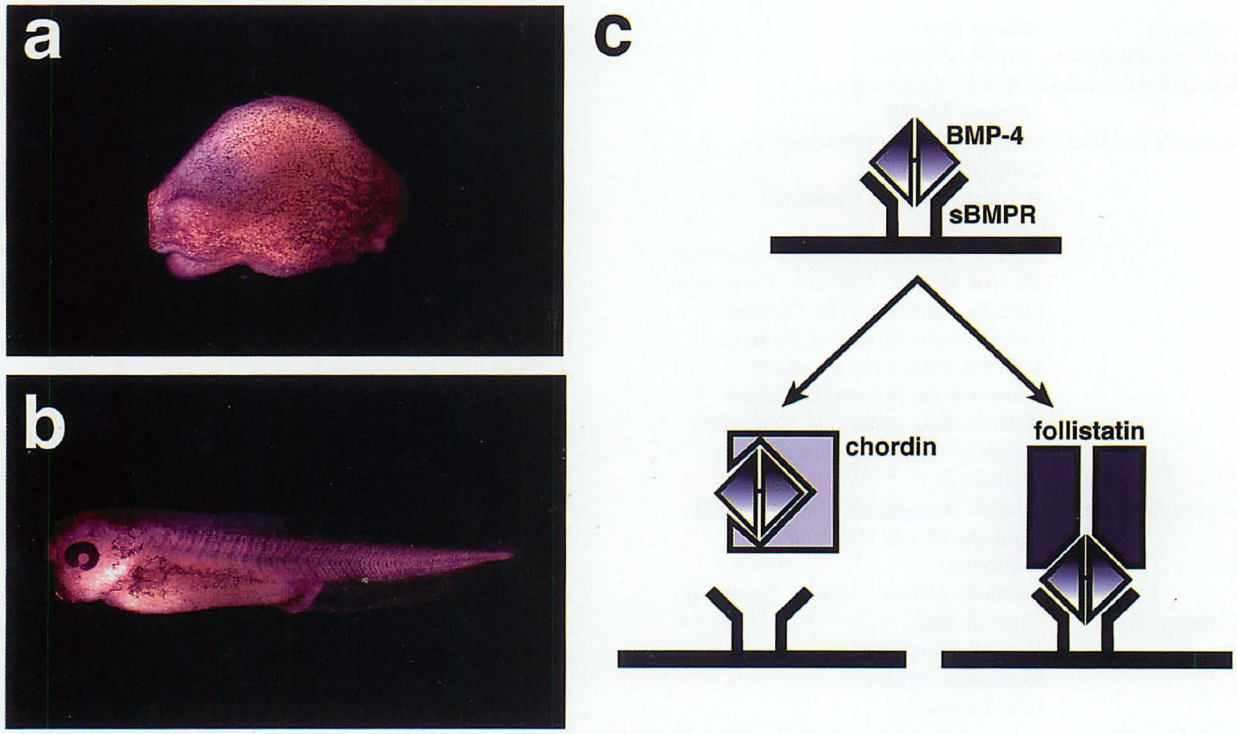

Figure 1. Inhibition of BMP-induced ventralization of *Xenopus* embryo by follistatin. (a), ventralized embryo caused by BMP-7 ectopic expression. (b), embryo rescued to normal by follistain coexpression with BMP-7. (c), proposed mechanism by which follistatin inhibits receptor activation by BMP. In contrast to chordin that competes with the BMP receptor for BMP-binding, follistatin forms a ternary complex with BMP and its receptor.

vated TAK1 or both TAK1 and TAB1 in dorsal blastomeres not only rescued the cells but also caused the ventralization of the embryo. In addition, a kinase-negative form of TAK1 (TAK1KN) which is known to inhibit endogenous signaling could partially rescued phenotypes generated by the expression of a constitutively active BMP-2/4 type I receptor. Moreover, TAK1KN could block the expression of ventral markers normally induced by Smad 1 or 5 that mediates BMP signal. Taken together, we proposed that TAK1 and TAB1 function in the BMP signal transduction pathway in *Xenopus* embryo in a cooperative manner. Further study is currently undertaken to identify downstream targets of BMP signals.

III. Use of zebrafish as a model animal to study gene function

In addition to *Xenopus*, zebrafish is a useful model animal because of several experimental advantages over other vertebrates. Function of BMP in early embryogenesis is also being studied using zebrafish. Taking advantage of transparency of zebrafish embryos, we have been able to show that not relay but direct diffusion is the mechanism by which BMP influences surrounding cells. Moreover, a visiting scientist Dr. Higashijima (PRESTO, STA) has succeeded in establishing transgenic technology for zebrafish (Higashijima, S. et al. (1997) *Dev. Biol.* **192**, 289-299). He used muscle actin promoter fused to green fluorescent protein (GFP) gene to show that the gene is transmitted to germ line and maintained in offspring of at least five generations. This technology will allow us to study how organs and tissues are formed, enabling us to observe promotor specific expression in live embryos and adult animals.

IV. TGF-β family in invertebrates

Nematode *C. elegans* and *Drosophila* provide powerful genetical approaches to understand the role of TGF-β family ligands and their signaling mechanism. We identified a BMP/nodal-like ligand in *C. elegans* and designated as *cet-1* (*C. elegans* TGF-β). To understand the function of *cet-1*, we isolated a null mutant for *cet-1* gene and found that the loss-of-function mutant worms have shortened body length, suggesting that *cet-1* encodes a ligand that regulates body length of *C. elegans*. The molecular basis of the regulation of body length is our current interest. We plan to identify genes that are up- or down-regulated in the mutant by a newly developed method Differential Panel Display (DPD), using arrayed cDNA of *C. elegans*.

In *Drosophila*, *decapentapledgic* (*dpp*) encodes a homologue of BMP. We have been studying the involvement of *dpp* in the formation of mechanosensory sensory organ. Ectopic activation of Dpp signal by a constitutively activated form of a dpp receptor tkv Dpp receptor tkv increased the number of machanosensory organ. It was found that Dpp signal

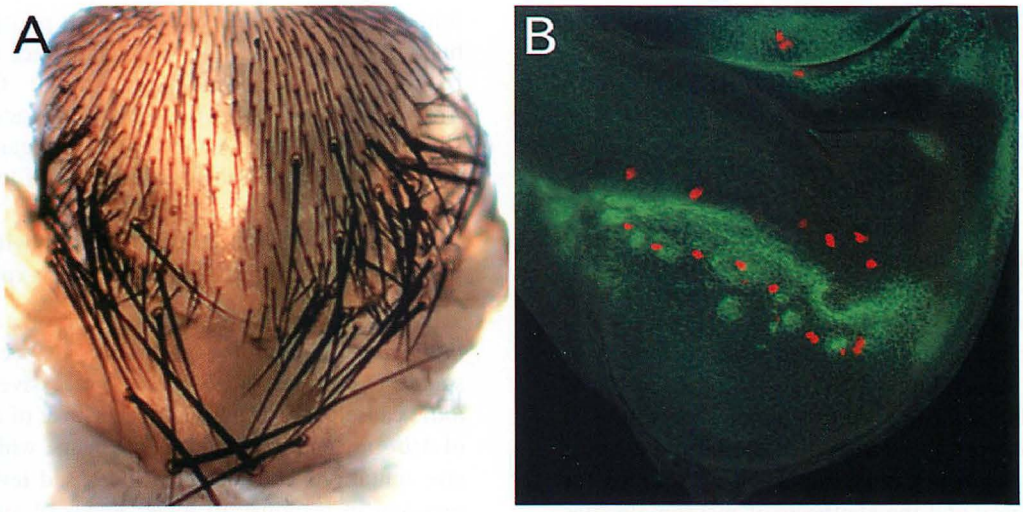

Figure 2. Interaction of *dpp* and *wingless* in the formation of the mechnosensory organ of *Drosophila* (a) increased mechanosensory bristles in the nortal region by ubiquitous expression of a constitutively active Dpp receptor Tkv. (b), induction of SOP cells (shown in red) nearby *wingless* expression domain (shown in green).

suppresses expression of another growth factor wingless. As a result, *dpp* induces sensory organ precursor (SOP) cells near by *wingless* expressing region. Position of SOP cells seems to be determined by the cooperation of at least two secreted growth factors, *dpp* and *wingless*.

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DIVISION OF DEVELOPMENTAL BIOLOGY

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Availability of sugars and the inter-organ transport and distribution of sugars are essential in the growth and development of the plant body. Expression of a variety of plant genes is 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 the synthesis of macromolecules but also as a signal controlling the growth and development of plants by changing the pattern of gene expression. Our research attention is focused to eludicate the mechanisms involved in the regulation of gene expression in response to sugars, especially the activation of gene expression by increased-levels of sugars, and the role of such regulation in the organ development in plants. In addition, mechanisms involved in the accumulation of storage proteins in plant vacuoles are also studied.

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

Expression of genes coding for sporamin and β-amylase, two major proteins of the storage roots of sweet potato, is inducible by high levels of sugars in various vegetative tissues. The GUS reporter genes under the control of the promoters of these genes are also inducible by sugars in leaves of transgenic tobacco plants, and these fusion genes are expressed in tubers of transgenic potato plants. Although the induction of expression of these fusion genes requires the activity of hexokinase, phosphorylation of hexose by hexokinase is not sufficient to cause the induction. The induction requires Ca^{2+}-signalling and the activity of protein kinase. Eight different cDNAs for the isoforms of calcium-dependent protein kinase (CDPK) were isolated from leaves of tobacco, and transcripts of two of them were found to be increased upon treatment of leaves with various metabolizable sugars. Antibodies against a fragment of one of these isoforms cross-reacted strongly with the 57 kDa-protein in the soluble fraction from the young leaves. The level of this 57 kDa-protein decreased significantly as leaf matures, while the level of this 57-kDa protein in mature leaves increased significantly after the treatment of leaves with sugars. The sugar-induction of the 57-kDa protein occured preceding the induction of expression of the β-amylase:GUS reporter gene. In addition, a 54 kDa-protein with autophosphorylation acitivity in the plasma membrane of mature leaves also increased significantly upon treatment of leaves with sugars. This protein was purified to about 1,000-fold compared to the crude extract. It phosphorylated histone IIIS in a Ca^{2+}-dependent manner and cross-reacted with an antibody against CDPK of *Arabidopsis thaliana*. These results suggest the possible involvement of CDPKs in the sugar-inducible gene expression and the development of leaves.

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

Expression of the β-amylase gene of *Arabidopsis thaliana* (*Atβ-Amy*) in vegetative tissues occurs in response to high levels of sugars. A recessive mutation, *lba1*, caused significantly reduced-level of expression of *Atβ-Amy* under high levels of sugars, while a recessive mutation, *hba1*, caused increased levels of expression in response to lower levels of sugars. It is suggested that *HBA1* might function to maintain low-level expression of *Atβ-Amy* until the level of sugars reaches some high levels and that the expression of *Atβ-Amy* is regulated by a combination of both positive and negative regulation depending on the level of sugars.

A sequence between -172 and -61 of *Atβ-Amy* was not only essential to drive the sugar-inducible expression of the GUS reporter gene but also able to confer sugar-inducibility to the core promoter of the constitutive gene. Linker scanning analysis identified two *cis*-regulatory elements between -172 and -62 of *Atβ-Amy*, namely the negative element A and the positive element B. The element 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. The gel-mobility shift assay of the nuclear extracts identified activities binding to these regions.

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. For example, *lba1* mutant plants showed defective leaf development under the carbohydrate-limiting growth conditions and altered patterns of root development. The flowering time of the Arabidopsis plants under different carbohydrate availability was altered in the *hba1* mutant plants. These results suggest that products of *LBA1* and *HBA1* genes might affect the organ development either directly or indirectly.

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

During the growth of plants, new organs develop as carbohydrate sink, and many vegetative organs shows sink to source transition after their maturation. Many aspects of the organ development in higher plants are

thought to be affected, to some degree, by the levels of sugars. Sugars seem to have influence on the meristematic transition in long day plants from vegetative to reproductive growth. To obtain insights into the role of sugar-regulated gene expression in the growth and organ development in plants, we are screening for mutants of *Arabidopsis thaliana* with defects or anomalies both in the sugar-regulated gene expression and in the developmental processes such as leaf development and the determination of the flowering time. To aid this purpose, we have established more than 4,000 independent lines of *Arabidopsis* plants transformed with T-DNA containing multiple copies of the enhancer sequence. We have identified several plant lines with defects in the development of leaves or anormalies in the flowering time which also show the altered patterns of the sugar-regulated gene expression (Fig. 1).

Selected Publications:

Mita, S., Murano, N.,Akaike, M. and Nakamura, K. (1997) Mutants of *Arabidopsis thaliana* with pleiotropic effects on the expression of the gene for β-amylase and of the accumulation of anthocyanin that are inducible by sugars. *Plant J.* **11**: 841-851.

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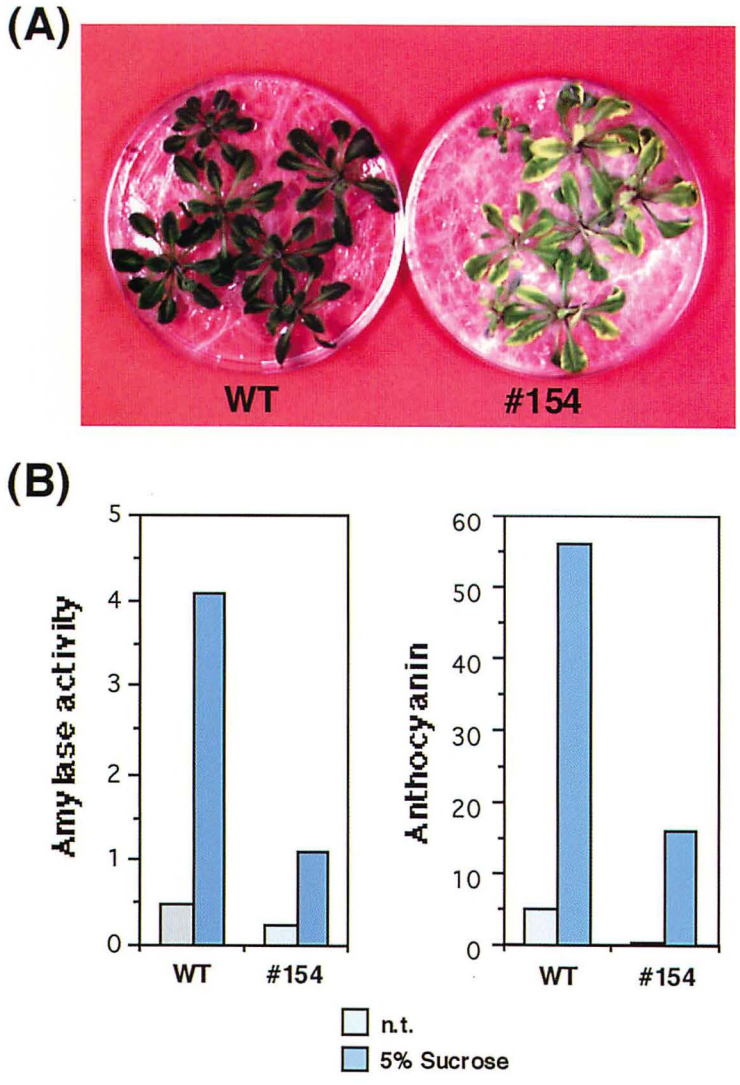

Fig.1 Phenotypes of the wild type plants (WT) and homozygous mutant line (#154) that were grown for 3 weeks. The chlorophyll content of the mutant plants is much lower than that of the wild type plants (Fig.1A). Mutant plants also showed the reduced levels of the sugar-inducible increase of β-amylase (left) and of anthocyanin (right) when leaf explants were treated with high levels (5%) of sucrose (Fig.1B).

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

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	Masakazu Takahashi [4]
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	Ryoko Suzuki (Oct. 1, 1997 ~)
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	Ikuko Watakabe
Technical Staff:	*Akiko Kawai*
	Shigemi Ohsugi
JST Technical Staff:	*Masae Mizoguchi (May 1, 1997 ~)*
	Megumi Goto (Jun. 11, 1997 ~)

Our efforts have been devoted to studying molecular and cellular mechanisms underlying the development of the vertebrate central nervous system. We are screening for molecules and structures that regulate various cellular events in brain morphogenesis such as generation of neuroblasts, their migration to form the laminar structure and various nuclei, elongation and path-finding of neural processes, and the formation and refinement of specific connections between neurons. Our research has been conducted using various techniques including molecular biology (*e.g.* cDNA cloning, site-directed mutagenesis), biochemistry (protein, carbohydrate), immunological methods (monoclonal-antibody production), neuroanatomy, cell and organotypic culture (immortalized cell production), and embryo manipulation (classical embryology, gene transfer with viral vectors, and gene targeting).

I. Topographic map and synapse formation in the retinotectal system

Topographic maps are a fundamental feature of brain organization. In the avian visual system, the temporal (posterior) retina is connected to the rostral (anterior) optic tectum, nasal (anterior) retina to the caudal (posterior) tectum, and likewise dorsal and ventral retina are connected to the ventral and dorsal tectum, respectively. About half a century ago, Sperry proposed that topographic mapping could be guided by complementary positional labels in gradients across pre- and postsynaptic fields. Although this concept is widely accepted today, the developmental mechanism has not been fully characterized at the molecular level. In 1996, using a subtractive hybridization technique, we discovered several distinct transcripts which are topographically expressed in the developing chick retina. Among these molecules, two winged-helix transcriptional regulators termed CBF-1 and CBF-2 were expressed in the nasal and temporal retina, respectively, and our misexpression experiments using a retrovirus vector suggested that these transcription factors direct the retinal ganglion cell axons to choose the appropriate tectal targets along the antero-posterior axis.

To examine topographic molecules which show asymmetrical distributions in the embryonic retina, we have undertaken a large-scale screen using a new cDNA display system called Restriction Landmark cDNA Scanning (RLCS) (Fig. 1). A number of molecules displaying asymmetrical expression along the naso-temporal axis or dorso-ventral axis in the retina have been identified. This approach seems to be promising because topographic molecules which are already known such as CBF-1, CBF-2, EphA3, aldehyde dehydrogenase etc. were included among those identified. Sequence analyses of the cDNA clones and examination of their expression patterns during development are currently underway. We expect that our studies will lead to systematic identification of a series of topographic molecules in the retina, and to elucidation of the molecular mechanisms underlying formation of the topographic retinotectal projection.

After reaching their appropriate sites along the rostro-caudal and dorso-ventral axes of the tectum, retinal axons begin to seek their appropriate termination sites among 15 distinct laminae within the tectum. The molecular and cellular bases of such discrete target choice are poorly understood, although our previous experiments suggested that molecular complementarity between retinal axons and tectal targets underlies lamina-specific synapse formation. We have generated a series of monoclonal antibodies that recognize one of these retinal termination laminae or a subset of retinal ganglion cells. We hope that characterization of these antigens and their cellular localizations in this relatively accessible system will provide insight into the complicated and still unresolved aspects of neural specificity.

II. Receptor-like protein tyrosine phosphatases and brain development

Protein tyrosine phosphorylation plays crucial roles in various aspects of brain development. The level of tyrosine phosphorylation is determined by the balance between the activities of protein tyrosine kinases and protein tyrosine phosphatases. Recently, many types of receptor-like protein tyrosine phosphatases (RPTPs) have been cloned and characterized. In 1994, we found that PTPζ/RPTPβ, a nervous system-specific RPTP, is expressed as a chondroitin sulfate proteoglycan in the brain. An RNA splice variant corresponding to the extracellular region of PTPζ is secreted as a major proteoglycan in the brain known as 6B4 proteoglycan/phosphacan. The extracellular region of PTPζ consists of a carbonic anhydrase-like domain, a fibronectin type III-like domain and a serine-glycine-rich region, which is considered to be the chondroitin sulfate attachment region. This unique structure of PTPζ has attracted a great deal of

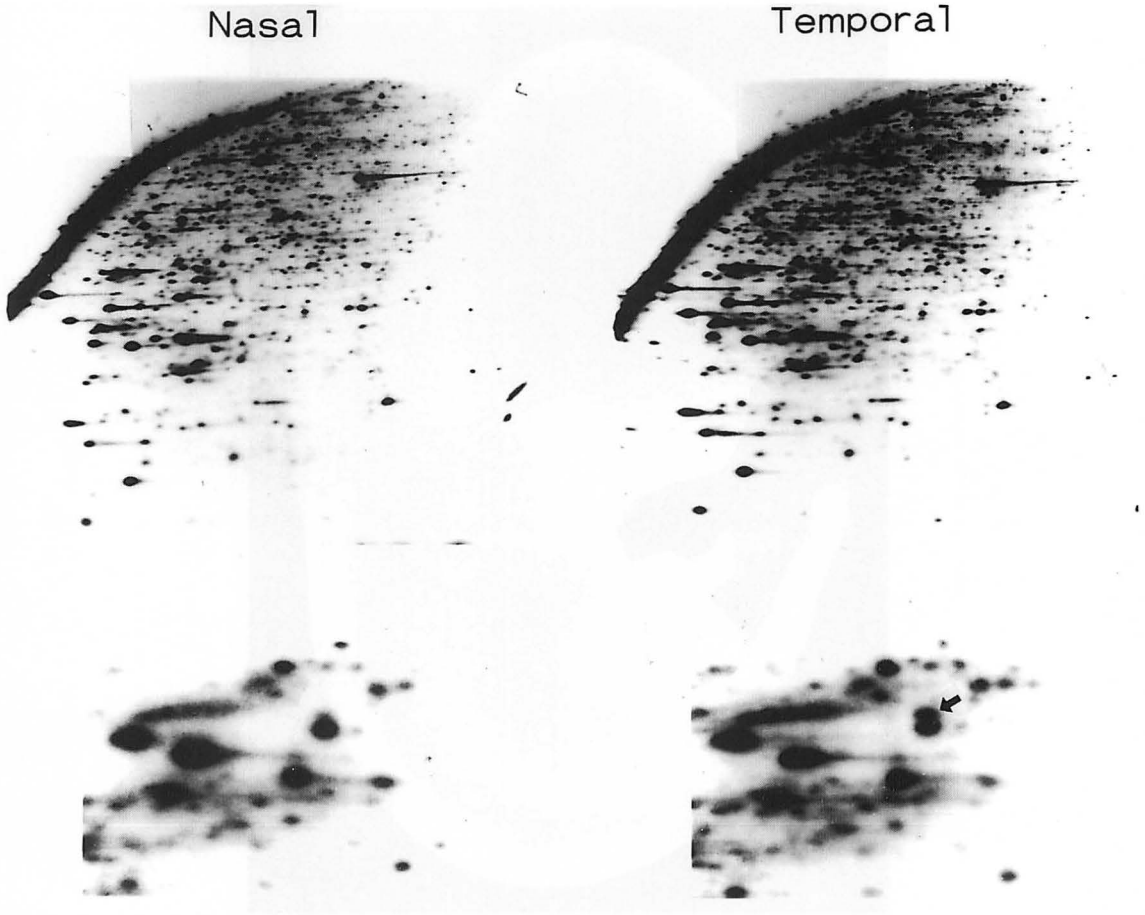

Fig.1 RLCS profiles for the chick nasal and temporal retina
Topographic molecules were detected as spots specifically found on one side. As an example, see the spot arrowed in the right below panel, the enlargement of a part of the above picture.

attention but little is known about the functional roles of this molecule.

In 1996, we found that PTPζ binds pleiotrophin/heparin-binding growth-associated molecule (HB-GAM). In the embryonic rat brain, pleiotrophin is localized along the radial glial fibers, a scaffold for neuronal migration. On the other hand, immunohistochemical studies of normal rat brain indicated that PTPζ is expressed in the migrating neurons, suggesting that the ligand-receptor relationship between PTPζ and pleiotrophin plays a role in migration of neurons during brain development. Thus, we examined the roles of pleiotrophin-PTPζ interaction in neuronal migration using the Boyden chamber cell migration assay. Pleiotrophin coated on the membranes stimulated migration of cortical neurons. Polyclonal antibodies against the extracellular domain of PTPζ, 6B4 proteoglycan, a secreted extracellular form of PTPζ, and sodium vanadate, a protein tyrosine phosphatase inhibitor, added into the culture medium strongly suppressed the pleiotrophin-induced neuronal migration. Furthermore, chondroitin sulfate C but not chondroitin sulfate A inhibited pleiotrophin-induced neuronal migration, in accordance with our previous findings that chondroitin sulfate constitutes part of the pleiotrophin-binding site of PTPζ, and PTPζ-pleiotrophin binding is inhibited by chondroitin sulfate C but not by chondroitin sulfate A. These results suggest that PTPζ is involved in neuronal migration as a neuronal receptor of pleiotrophin distributed along radial glial fibers.

Furthermore, to examine the functions of PTPζ in vivo, we generated PTPζ-deficient mice in which the PTPζ gene was replaced by the LacZ gene by gene targeting. Firstly, we examined the cell types expressing PTPζ by investigating the expression of LacZ in heterozygous PTPζ-deficient mice. Throughout development from the early stage of embryogenesis, LacZ staining was restricted to the nervous system. At embryonic day 12.5 (E12.5), LacZ staining was observed in the forebrain, midbrain, hindbrain and spinal cord (Fig. 2). In the adult heterozygous mice, LacZ expression was abundant in the olfactory bulb, cerebral cortex, hippocampus, thalamus and cerebellum. In the hippocampus, strong reporter gene expression was observed in the pyramidal neurons and in the granule cell layer of the dentate gyrus. We are currently examining the phenotype of homozygous PTPζ-deficient mice.

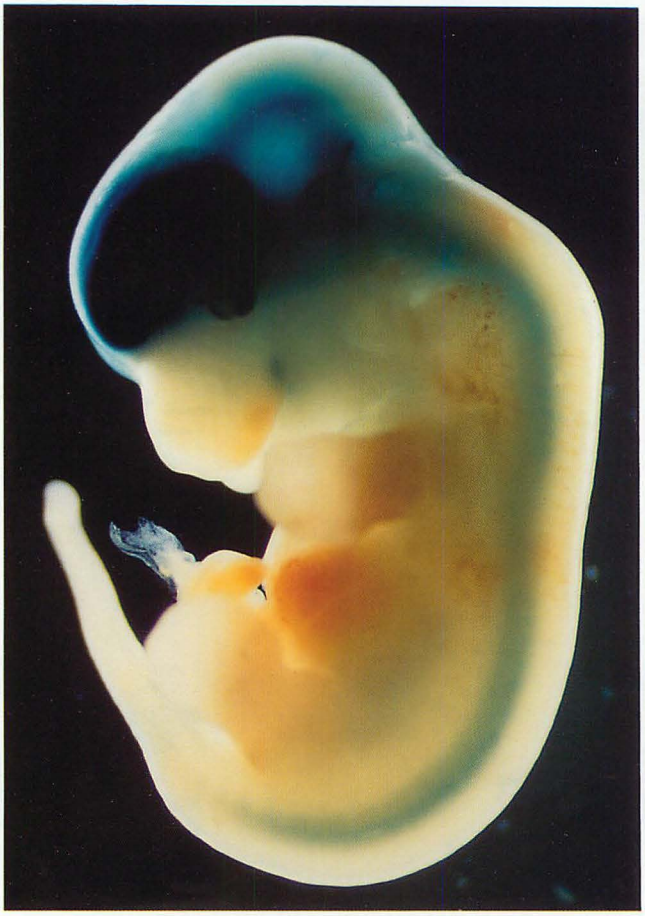

Fig.2 LacZ expression in E12.5 heterozygous PTPζ-deficient mice.

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 each pair of potassium ions transported: a drop in the intracellular concentration of sodium ions through this channel may hamper ATPase activity. To test this possibility and clarify the roles of the glial sodium channel *in vivo*, we are currently attempting to generate a knock-out mouse deficient in the glial sodium channel, Na-G. We have successfully produced chimeric mice which contain the targeted embryonic stem-cells.

By applying gene targeting technology to other novel genes expressed in the brain, we hope to shed light on the molecular mechanisms underlying the development and function of the brain.

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	P. Pardha Saradhi[5]
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The research effort of this division is aimed at developing a full understanding of the molecular mechanisms by which plants are able to acclimate to and tolerate stresses that arise from changes in environmental conditions, with particular emphasis on temperature and salt stresses. In 1997, significant progress was made in research on the following topics in studies with cyanobacteria and higher plants as experimental materials.

I. Improved performance of photosynthesis with polyunsaturated membrane lipids

We previously inactivated genes for fatty-acid desaturases by targeted mutagenesis in the cyanobacterium *Synechocystis* sp. PCC 6803 and we produced several mutant strains that contained abnormal numbers of unsaturated bonds in the fatty acids of their membrane lipids. By comparing these strains, we demonstrated that polyunsaturated membrane lipids are important in the ability of the photosynthetic machinery to tolerate low temperatures. In order to extend this finding by an alternative approach, we introduced the *desA* gene for the Δ12 fatty-acid desaturase of *Synechocystis* sp. PCC 6803 into cells of *Synechococcus* sp. PCC 7942 that normally contain monounsaturated fatty acids but no polyunsaturated fatty acids. This transformation resulted in replacement of half of the monounsaturated fatty acids in the membrane lipids 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 the photoinhibitory damage. These findings indicate that polyunsaturated fatty acids are important in protecting the photosynthetic machinery from damage caused by strong light at low temperatures.

To characterize biochemically the Δ12 desaturase of *Synechocystis* sp. PCC 6803, we overexpressed the enzyme in *Escherichia coli*. The overexpressed enzyme was active; it was associated with cell membranes and represented about 10% of the total cellular protein. The activity of desaturase was very stable in the presence of 2 M sorbitol between pH 7 and pH 8. Purification of the desaturase is in progress.

II. Enhancement of stress tolerance in plants by genetic engineering: transformation with a gene that confers 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 *in vivo* on the protection of the photosynthetic machinery against salt stress, we transformed *Arabidopsis thaliana*, which does not normally accumulate betaine, with the *codA* gene for choline oxidase (which catalyzes the conver-

sion of choline to betaine) from *Arthrobacter globiformis*. Transgenic *Arabidopsis* plants had the ability to synthesize betaine and to tolerate salt stress. Furthermore, transformation with the *codA* gene 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. Betaine was also accumulated in the seeds of transformed plants and, as a result, the seeds were more tolerant than seeds of wild-type plants to low temperatures during imbibition and germination. Furthermore, transformation with the *codA* gene accelerated the growth of young seedlings at low temperatures.

We also introduced the *codA* gene into rice plants by *Agrobacterium*-mediated transformation, aiming to enhance the tolerance of this important crop to salt stress. Transformed rice plants accumulated betaine and, consequently, exhibited enhanced ability to tolerate salt stress. Figure 1 shows wild-type rice plants and transformed plants after growth under salt-stress conditions. During the stress treatment with 0.15 M NaCl, growth of both wild-type and transformed plants was inhibited and obvious damage, such as wilting, bleaching of chlorophyll and necrosis, was visible. After removal of the salt stress, the growth rate of transformed plants returned to normal but that of wild-type plants did not recover. The oxygen-evolving machinery of the transformed plants was more tolerant to salt stress than that of the wild-type plants. Our results in *Arabidopsis* and rice demonstrate the potential usefulness of the *codA* gene in the engineering of stress tolerance in a wide variety of agronomically important crops.

III. A new factor involved in the heat stability of the photosynthetic machinery

The evolution of oxygen is one of the reactions of photosynthesis that is extremely 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

Fig. 1. Wild-type rice plants (WT) and rice plants (ChlCOD and CytCOD) transformed with the *codA* gene from *Arthrobacter globiformis* after growth under salt stress. Three-week-old plants that had been grown under normal conditions were subjected to salt stress by exposure to 0.15 M NaCl for one week. They were then allowed to grow under normal conditions for three weeks.

in the lumenal side of thylakoid membranes, is involved in the stability of the oxygen-evolving machinery at high temperatures. We then initiated a search for other factors that might enhance heat stability and we identified a protein of 13 kDa as an important factor. The gene for the 13-kDa protein was cloned from *Synechococcus*, and the deduced amino-acid sequence revealed that this protein was homologous to PsbU, an extrinsic protein of the photosystem II complex. Targeted mutagenesis of the *psbU* gene in *Synechococcus* sp. PCC 7002 resulted in cells in which oxygen-evolving activity was particularly susceptible to high temperature. These results indicate that PsbU plays an important role in stabilizing the oxygen-evolving machinery at high temperatures.

IV. Stress-dependent enhanced expression of cytochrome c_M and suppression of expression of cytochrome c_6 and plastocyanin

Cytochrome c_M is a *c*-type cytochrome with a molecular mass of 8 kDa. We identified and cloned the *cytM* gene for cytochrome c_M from *Synechocystis* sp. PCC 6803. In 1997, we cloned homologs of the *cytM* gene from other cyanophytes and a prochlorophyte, providing evidence that the *cytM* gene is probably distributed universally in oxygenic photosynthetic prokaryotes. Northern-blotting analysis revealed that the *cytM* gene of *Synechocystis* sp. PCC 6803 was barely expressed under normal growth conditions but that expression was induced when cells were exposed to low temperature and/or high-intensity-light. By contrast, expression of the *petJ* gene for cytochrome c_6 and the *petE* gene for plastocyanin, which are electron carriers that transport electrons from the cytochrome b_6/f complex to the photosystem I complex, was suppressed at low temperatures or under high-intensity-light. These observations suggest that regulation of the expression of the *cytM* gene was the mirror image of regulation of the *petJ* and *petE* genes under the stress conditions examined.

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(1) Original articles

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DIVISION OF BIOLOGICAL REGULATION AND PHOTOBIOLOGY (ADJUNCT)

Associate Professor (adjunct): *Hirokazu Kobayashi*
Research Associate: *Noritoshi Inagaki*

The conversion of light energy into chemical energy used for CO_2 assimilation is the process of photosynthesis, one of the most important biological events. The photosynthesis utilizing water as an electron donor occurs in cyanobacteria and in chloroplasts in all eukaryotic algae and plants. The architecture of machinery for photosynthesis is directed by both nuclear and plastid genes in plants. The extremely efficient energy conversion is ensured by the highly ordered organization of molecules in photochemical reaction centers. The projects in this division are aiming to elucidate the mechanisms underlying the biogenesis of the photosynthetic machinery and the dynamic organization of molecules in the photosystem II (PS II) reaction center.

I. Tissue-specific expression of plastid genes

(1) Mechanisms of suppressed levels of transcripts for plastid photosynthesis genes in nongreen tissues such as roots and calli were analyzed employing the higher plant *Arabidopsis thaliana* which is suited for further genetical dissection. A region encoding promoters of *rbcL* for the large subunit of ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) and *atpB/E* operon for β and ε subunits of coupling factor one was cloned and sequenced. Transcripts both for *rbcL* and *psbA* (gene for D1 protein in PS II reaction center) were undetectable and 16S rRNA was detected at a low level in roots of *A. thaliana*. The run-on transcription experiment revealed that expression of *rbcL* and *psbA* was regulated at transcription or upper steps in gene expression. The copy number of plastid DNA in roots was one-fifth of that in green leaves on the basis of total cellular DNA. Digestion of DNA with methyl-sensitive and -insensitive isoschizomeric endonucleases and subsequent polymerase chain reaction (PCR), as well as *in vitro* run-off transcription, resulted in no evidence of the regulation by DNA modification. In spite of predominant suppression of expression of *rbcL* and *psbA* at transcription in roots and calli, 16S rRNA levels there decreased in the result of low RNA stability.

(2) Genes for s-like factors of bacterial-type RNA polymerase have not been characterized from any multicellular eukaryotes, although they likely play a crucial role in the expression of plastid photosynthesis genes. We have cloned three distinct cDNAs designated *SIG1*, *SIG2*, and *SIG3* for polypeptides possessing amino acid sequences for domains conserved in σ^{70} factors of bacterial RNA polymerases from *A. thaliana*. Each gene is present as one copy per haploid genome without any additional sequences hybridized in the genome. Transient expression assays using green fluorescent protein (GFP) demonstrated that N-terminal regions of the *SIG2* and *SIG3* open reading frames could function as transit peptides for import into chloroplasts. Transcripts for all three *SIG* genes were detected in leaves but not in roots, and induced in leaves of dark-adapted plants in rapid response to light illumination. Together with results of the analysis of tissue-specific regulation of transcription of plastid photosynthesis genes, expressed levels of the genes may influence transcription by regulating RNA polymerase activity in a green tissue-specific manner.

II. Dynamic aspects of molecular organization of PS II

We have focused on elucidation of molecular mechanism of the C-terminal processing of the D1 protein known to be an integral subunit of the PS II reaction center. D1 protein is synthesized as a precursor form furnished with a C-terminal extension. The specific excision of the C-terminus is well characterized to be an essential event for construction of the photosynthetic oxygen-evolving machinery. Therefore, the protease, CtpA, involved in this process was characterized in biochemical and molecular biological techniques. However, the catalytic mechanism of the protease has not yet been demonstrated. We have constructed a series of systematic mutants of the cyanobacterium *Synechocystis* sp. PCC 6803, in which one of the putative catalytic residues of the protease was substituted to alanine by conventional site-directed mutagenesis. Biological and biochemical analyses of these mutants suggest that essential residues for the protease reaction are Ser313 and Lys338 in the *Synechocystis* CtpA sequence. It is speculated that the residues constitute Ser-Lys dyad in the tertiary structure and the Ser residue of the dyad hydrolyze a peptide bond with a similar manner of β-lactamase, a Ser-Lys dyad type hydrolase for cleavage of the β-lactam ring of antibiotics.

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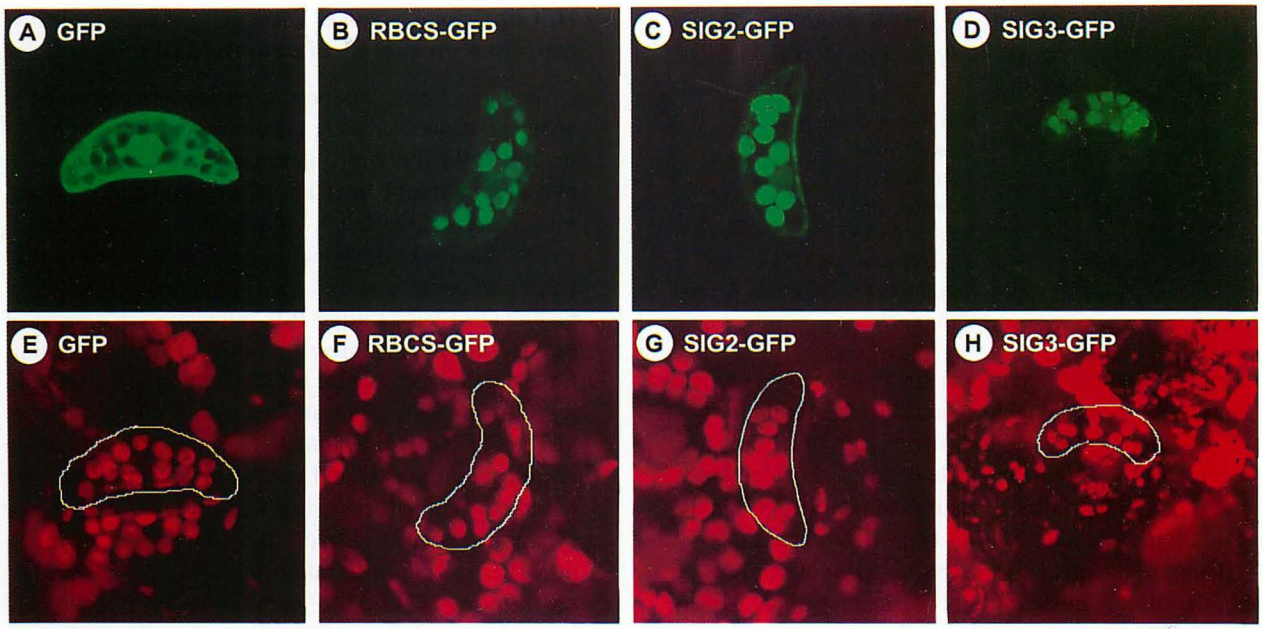

Fig. 1. Localization of GFPs fused to N-terminal regions of *SIG2* and *SIG3*. GFP fusion constructs with the N-terminal regions of *SIG2* ORF (SIG2-GFP, panels C and G) and *SIG3* ORF (SIG3-GFP, panels D and H), and the transit peptide of small subunit of Rubisco (RBCS-GFP, panels B and F), as well as GFP alone (GFP, panels A and E), were introduced into tobacco leaves by particle bombardment. Guard cells were observed using MRC-1024 Confocal Imaging System (X 480) with excitation at 488 nm and emission at 520 nm (panels A-D), as well as excitation at 647 nm and emission at 666 nm (panels E-H). The same objects are shown in each pair of upper and lower panels.

DIVISION OF BEHAVIOR AND NEUROBIOLOGY (ADJUNCT)

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Research Associate:	*Akinao Nose*
	Kazuaki Tatei
Postdoctoral Fellow:	*Emiko Shishido* [1]
	Takako Isshiki [2]
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. 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. Capricious, 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 the corresponding gene, termed *capricious* (*caps*), 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, Caps protein was found to be most related to the product of the *Drosophila tartan* gene that have been implicated in neural and muscular development. We found that in the loss-of-function mutants of *caps*, the synaptic arborization pattern on muscle 12, a *caps*-positive muscle, was abnormal (Fig.1A, B). The nerve terminal failed to stabilize on muscle 12, and instead extended to and arborized on the adjacent muscle, muscle 13. Ectopic expression of *caps* in all muscles by GAL4-UAS system also resulted in aberrant synapse formation on muscle 12 (Fig. 1C). Like in the loss-of-function alleles, the muscle 12 terminal axon branch often formed collaterals that turned back and innervated muscle 13. These results suggest that Caps is involved in neuromuscular target recognition and/or stabilizaion of the synapses.

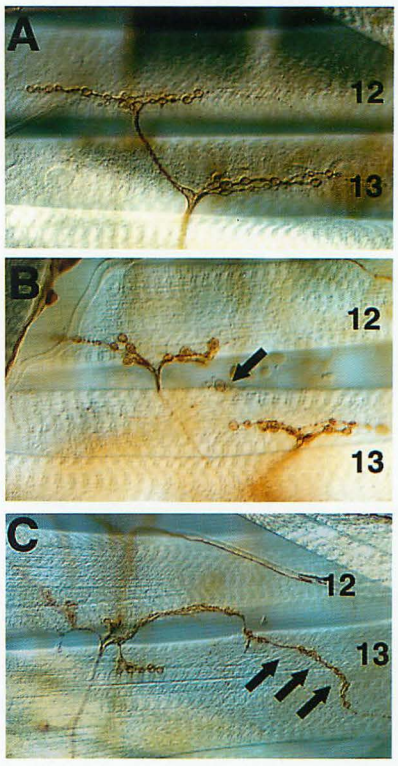

Fig. 1 Neuromuscular defects associated with loss-of-function and ectopic expression of *caps*. Body wall fillet of wild-type (A), *caps* loss-of-function (B) and *caps* misexpressing (C) 3rd instar larvae, stained with MAb 22C10 to visualize motor nerve projection and synaptic endings. Ectopic synapses formed by muscle 12 motoneurons are indicated by arrows.

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 analyzing 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 and subsequently in many neuroblasts and their progenies derived from this region. We found that the loss of *msh* results in the failure of the proper differentiation of many neural and glial progenies 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 possibility that this family of genes may play 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 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 identification 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: Takashi Horiuchi

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*
Associate Professor (adjunct):	*Yoshihiro Ozeki*
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	Yoshiki Habu
	Yoshishige Inagaki
Postdoctoral Fellow:	*Yasuyo Hisatomi* [2]
Graduate Student:	*Atsushi Hoshino*
Technical Staff:	*Sachiko Tanaka-Fukada*
	Tomoko Mori
	Yoshiko Inoue
Visiting Fellows:	*Ken-ichi Shiokawa*
	Yasumasa Morita
	Fumiyoshi Myoga
	Makiyo Yamagishi

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 bears 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 the transposable element, *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 of the *DFR-B* gene. The flower variegation is due to somatic reversion of the *DFR-B* gene by *Tpn1* excision. The 6.4 kb *Tpn1* element belongs to the *En/Spm* family and 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 are generally heritable by their progeny but sometimes conversion of these phenotypes is also observed. Thus the flecking 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 recessive *speckled* allele carries a non-autonomous transposable element and the dominant *speckled-activator* must be a related autonomous element. The plants carrying the *speckled* allele without active *speckled-activator* bear pale yellow flowers. Based on the chemical analysis of the pale yellow flowers, it was proposed that the mutation in the gene for flavanone 3-hydroxylase (F3H) is a likely candidate for the *speckled* allele. We have tested this hypothesis and shown that the *speckled* allele is neither *F3H* gene nor a regulatory gene acting on the *F3H* gene expression. Currently, we are characterizing the *CHI* gene which contains an insertion in the mutable *speckled* lines.

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 the morning glory with the heterozygous state *A/a*^{flaked} (Fig. 1D). To identify the mutable *a*^{flaked} allele, we have characterized DNA rearrangements found at the *DFR* and *ANS* (*Anthocyanidin synthase*) gene regions in the anthocyanin biosynthesis and identified several new mobile genetic elements. However, subsequent genetic studies revealed that these elements have not associated with the mutable *a*^{flaked} allele.

Based on amplified restriction fragment length polymorphism (AFLP) technology, we have also developed a new protocol termed AMF (AFLP-based mRNA fingerprinting) for the fingerprinting of mRNA to detect differentially expressed genes. Using both AMF and the differential display (DD) method, we were able to identified new *CHS* (*chalcone synthase*) genes, *CHS-D* and *CHS-E*, in the anthocyanin biosynthesis. Both *CHS-D* and *CHS-E* are expressed in the flower buds of both the common and the Japanese morning glory, and *CHS-D* is more abundantly expressed than *CHS-E*. In the mutable *a*^{flaked} line of the common morning glory, the *CHS-D* gene carries an about 4 kb insertion and its mRNA accumulation is drastically reduced. Further characterization of these newly identified *CHS* genes is in progress.

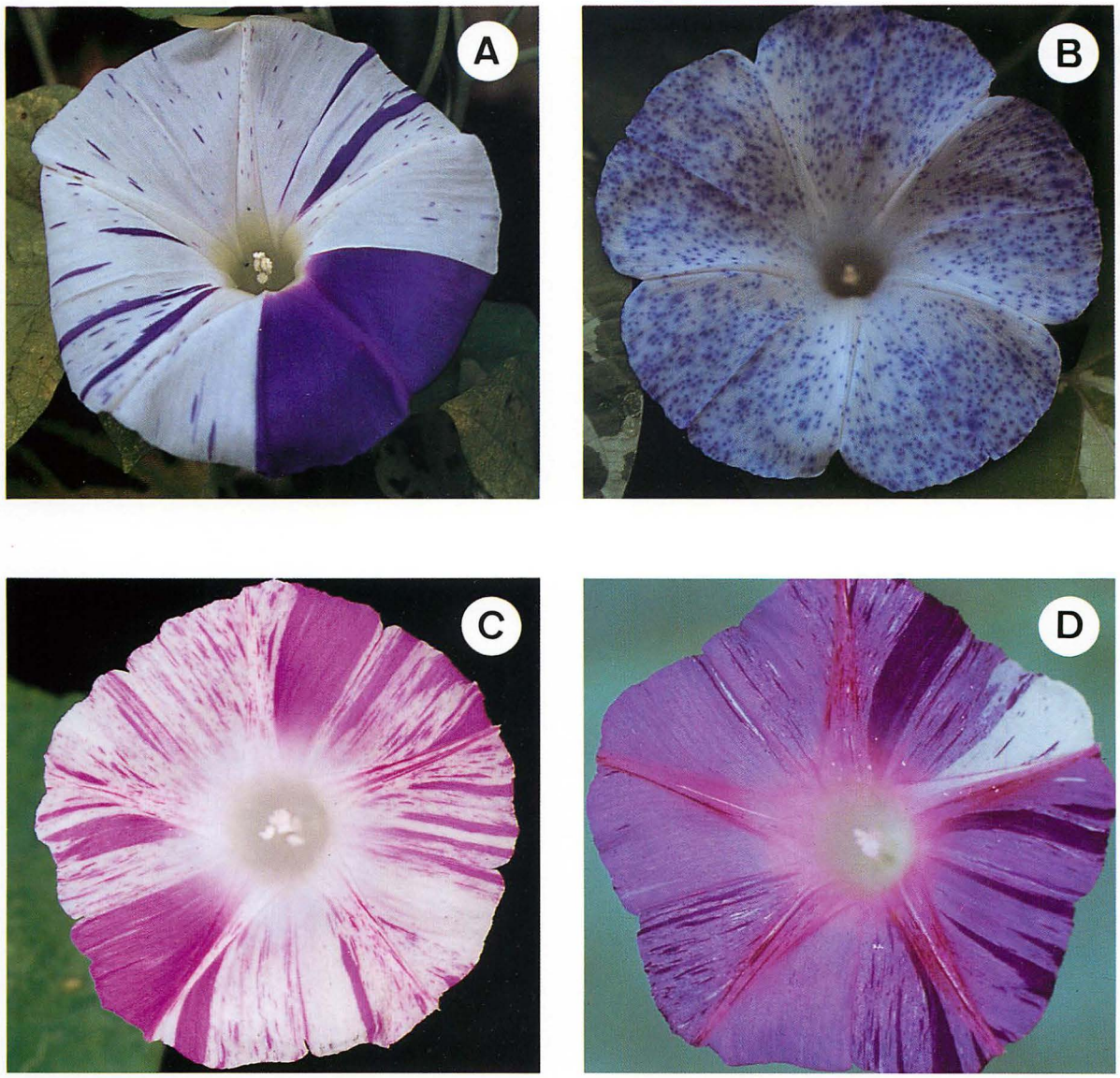

Fig.1 Flower variegation.
(A) *a-3*^{flecked}; (B) *speckled*; (C) a^{flaked} [a^{flaked}/a^{flaked}]; (D) a^{flaked} [A/a^{flaked}]

III. Transposition of the maize *Ac/Ds* transposable elements in transgenic rice plants.

To understand the behavior of the transposable elements, we are characterizing transposition in heterologous system using transgenic plants. A transgenic rice carrying a non-autonomous *Ds* element was crossed with another transgenic rice containing the active *Ac* transposase (T. Izawa, et al., (1997) *Plant Mol. Biol.* **35**, 219-229), we are currently investigating somatic transposition of *Ds* and its target selectivity in the F1 plants.

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DIVISION OF GENE EXPRESSION AND REGULATION II

Professor:	*Takashi Horiuchi*
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	Ken-ichi Kodama
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Post Doctoral Fellow:	*Katufumi Ohsumi* [1]
Graduate Students:	*Keiko Taki*
	Hiroko Urawa
Technical Staff:	*Kohji Hayashi*
	Yasushi Takeuchi

Elucidation of dynamic molecular mechanism of replication, recombination and repair processes of genome in prokaryotes and eucaryotes requires extensive research. We focus on interactive processes between replication and recombination. In 1997, the following three interactive subjects were given detailed attention. (1) We analyzed molecular mechanisms of a recombinational hotspot in *E. coli*, named HotH and we found that collision between replication and rRNA gene transcription is responsible for activation of HotH. (2) We developed a new system, in which the SOS response was induced by replication fork blocking at the *Ter* site located on pUC derivative plasmids. (3) I (T. H) became head of Japan *E. coli* genome project in April 1995 and we began to define nucleotide sequences from 13 minutes on the *E. coli* circular linkage map, in the clockwise direction, together with eleven independent groups of investigators in japan. In January 1997, we reached the region at 69 minutes, sequence data from where to 13 min region being registered in databank. Thus, we expressed the entire *E. coli* genome in a single continuous nucleotide sequence.

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

In *E. coli* RNase H defective (*rnh*$^-$) mutants, we identified 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; a strikingly enhanced excisional recombination between the repeats occurs. We obtained 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 the HotA, B and C revealed that blocking of the replication fork at the *Ter* (replication terminus) site 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, called Chi.

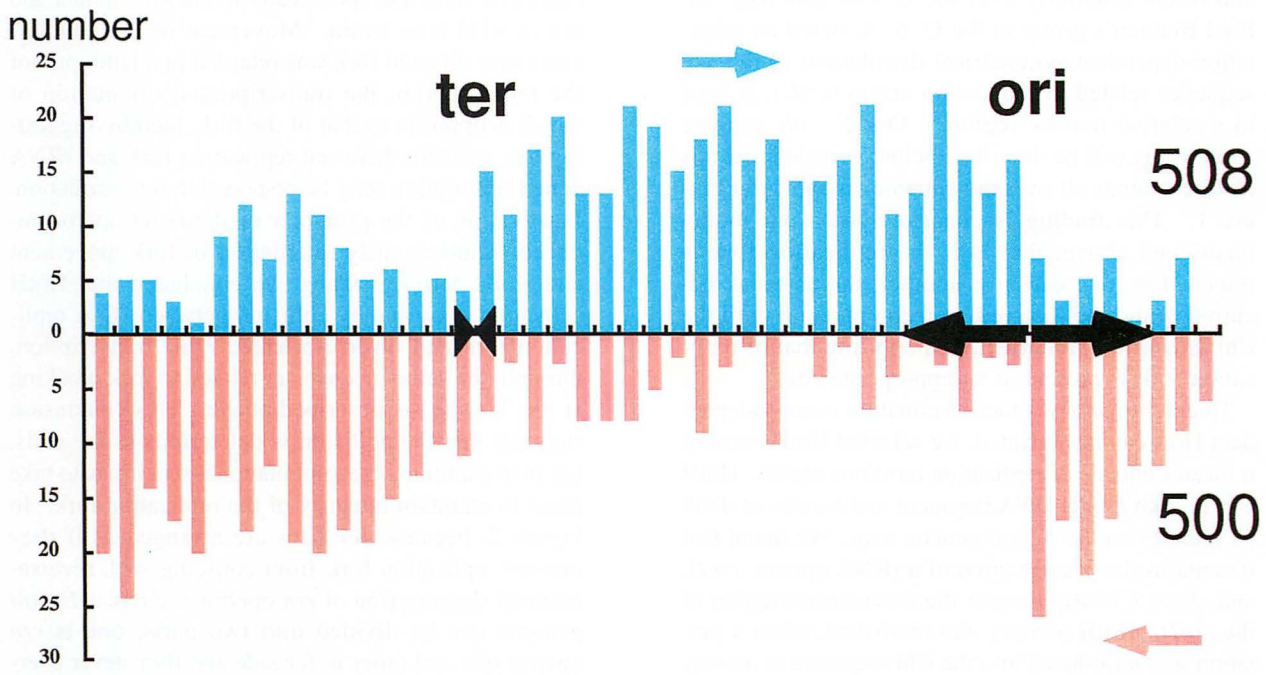

Figure 1. Orientation-dependent distribution of Chi sequence on *E. coli* genome

Blue and purple bars indicate the number of the Chi site in clock- and counter-clock-wise orientations in a each 100 kb interval of the genome, respectively. The total number of each oriented Chi site is almost the same. However, number of one oriented Chi are apparently dominant over that of the other in one of two half *ori-ter* sections and *vise versa*. Black arrow indicates direction of replication fork initiated from replication origin (ori) and an oriented fork is efficiently repaired by recombination using reverse-oriented Chi sequence when it is broken down.

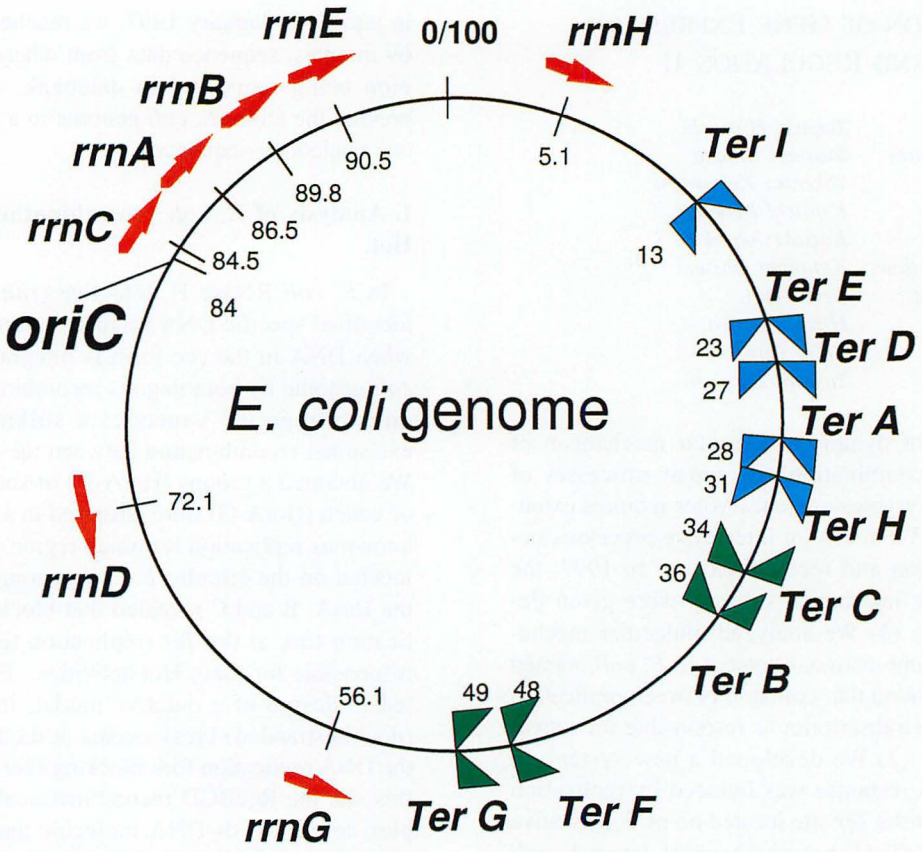

Figure 2. Arrangement of *rrn* operons and *Ter* sites on the *E. coli* genome.

Reds arrows indicate locations and transcriptional direction of *rrn* operons. ⟨ means DNA replication terminus (*Ter*) site which can block replication fork approaching from the right.

The Chi sequence had been considered to distribute and orient randomly over the *E. coli* genome. Dr. Fred Blattner's group in the U. S. A. noted an orientation-dependent symmetrical distribution of the Chi sequence related to replication origin (*oriC*), at least in a relative narrow region. Our *E. coli* genome analysis, as will be described below, revealed that this feature extends all over the genome, as shown in Figure 1. This finding is consistent with our model mentioned above; that is if the replication fork is impeded by one cause or another, the ds-break occurred cannot be repaired by recombination until the Chi sequence in an orientation opposite that of replication fork is present at an appropriate site.

To determine how other termination event-independent Hots can be activated, we selected HotH because it locates 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 a rRNA operon, *rrnD*, and also a Chi sequence at the downstream region of the *rrnD*. HotH activity was abolished, when a mutation was introduced into the Chi sequence to destroy its activity, which means that the Chi is probably required for HotH activity. To investigate events near the HotH site, we asked whether progress in the DNA replication fork is blocked or retarded in or around of HotH region, and for this we used 2 dimensional (2D) agarose gel electrophoresis. We detected the replication fork passing through this region, in the counterclockwise direction specifically in the *rnh*⁻ mutant and not in wild type strain. Movement of the counterclockwise directed fork was retarded in a latter part of the *rrnD* operon, the transcriptional orientation of which is opposite to that of the fork, thereby suggesting that collision between replication fork and rRNA gene transcription may be responsible for retardation. Inactivation of the promoter of the *rrnD* operon reduced simultaneously retardation of fork movement and HotH activity. Thus, we concluded that HotH activation is caused by collision between DNA replication fork and reverse-oriented *rrnD* transcription, through the same mechanism related to fork blocking at the *Ter* site, as described above. This conclusion suggests that the collision is deleterious to the cells, because continuous recombinational repair has to take place to maintain integrity of the replication fork. In Figure 2, because *Ter* sites are arranged as if they prevent replication fork from colliding with reverse-oriented transcription of *rrn* operons; circular *E. coli* genome can be divided into two parts, one is *rrn* operon side and other is *Ter* side and they never overlap, which strongly suggests that prevention of collision between fork and transcription may be the physiological role of the *Ter* site.

II. SOS inducibility by replication fork blocking at *Ter* site on plasmid and its dependency on distance from *ori* to *Ter* sites.

We developed a new system, in which the SOS response was induced by replication fork blocking at the *Ter* site located on pUC derivative plasmids. In the system, production of *Ter* binding protein, known as Tus protein, can be controlled by placing the structural gene, *tus*, under the *araC* promoter and SOS induction is fully controlled by omitting or adding arabinose. The extent of SOS response was controlled by measuring the activity of β-galactosidase, the expression of which is under control of the *sfiA* gene, a typical SOS responsive one, the product of which inhibits cell division. SOS inducibility dependent on *recA*$^+$ and *lexA*$^+$ genes. Using this system, we found that plasmids at a long distance from plasmid *ori* to *Ter* were SOS positive but ones with a short distance were SOS negative. Analyses revealed that up to 2.5 kb, the SOS response is hardly induced, but in a more extended range to at least 4.8 kb, inducibility showed good linear correlation to the distance. Replication intermediate molecules of the SOS$^+$ and SOS$^-$ plasmids were purified through CsCl density gradient centrifugation and tertiary structures were analyzed using 2D gel electrophoresis and electron-microscopy. In particular we examined the region of single stranded (ss) DNA found at the Y-junction at the *ori* or *Ter* site, because ssDNA functions as a cofactor for *in vitro* activation of RecA protein; none of differences between them found. While the cause of *ori-Ter* length dependent SOS induction needs more study, one possibility is that a ds-break occurs at the arrested fork and degradation product from the ds-end may trigger SOS induction. In such case, a longer DNA to be degraded may more efficiently induce the SOS response. Another possibility is that some specific tertiary structure of the θ type DNA molecule may be responsible for SOS induction, and to form the structure, a longer *ori-Ter* DNA may be needed.

III. The *E. coli* genome project

E. coli genome project in Japan was begun in 1989, the objective being to analyze, in an independently living organism, nucleotide sequences from 0 min in a clockwise direction. In April 1995, DNA sequences of the 0-12.7 min region in the genetic map were determined, published or registered. At that time, I (T. H.) was appointed leader of the Japan *E. coli* genome project and a group of researchers was re-organized to analyzed DNA sequences of the region from 12.7 min to 70 min, using Kohara lambda clones and the 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 data (69.0-100 min) of Dr. Fred Blattner and colleagues in the USA, the entire *E. coli* genome was expressed in a single, continuous nucleotide sequence, in which about 3400 putative open reading frames were identified. At this time, Blattner's group independently determined the entire *E. coli* DNA sequence. Figures 1 and 2 show arrangements of Chi sequences and *Ter* sites on whole genome, respectively; these are some results of the genomic sequence analysis. Our sequence data, registered in the DNA Data Bank in Japan (DDBJ) are also available through Web sites (http://mil.geges.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*
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Yuriko Komine
Akiya Watakabe
Institute Research Fellow: Takashi Kitsukawa
Postdoctoral Fellow: *Rejan Vigot* [2]
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Yoshinori Shirai
Graduate student: *Shiro Tochitani*
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Akishi Onishi (Kyoto University)
Shuzo Sakata (Kyoto University)
Nobuaki Tanaka (Kyoto University)
Hiroshi Fujita (Kyoto 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. In order to prove this hypothesis, we are currently working to identify new members of the family which are specifically expressed 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 and 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 causes 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 identifying several molecules which are induced in Purkinje cells 3 hours after the conjunction in collaboration with Dr. Ryoji Yano's group (Brain Research Institute, RIKEN). One of these genes is curently further being characterized. We are also examining the expression of the genes under cerebellar or hippocampal learning paradigm.

Publication List

Fujiwara, T., Yamamori, T., Yamaguchi, K., and Akagawa, K. (1997) Interaction of HPC-1/syntaxin 1A with the cytoskeletal protein, tubulin. *Biochem. Biophy. Res. Comm.* **231**, 352-355.

Matsuzawa, M., Muramatsu, T., Yamamori, T., Knoll, W. and Yano, R.(in press) Novel neuronal activities of midkine were examined on embryonic cerebellar neurons using a difined culture system. Cellul. and Molec. Neurobiol.

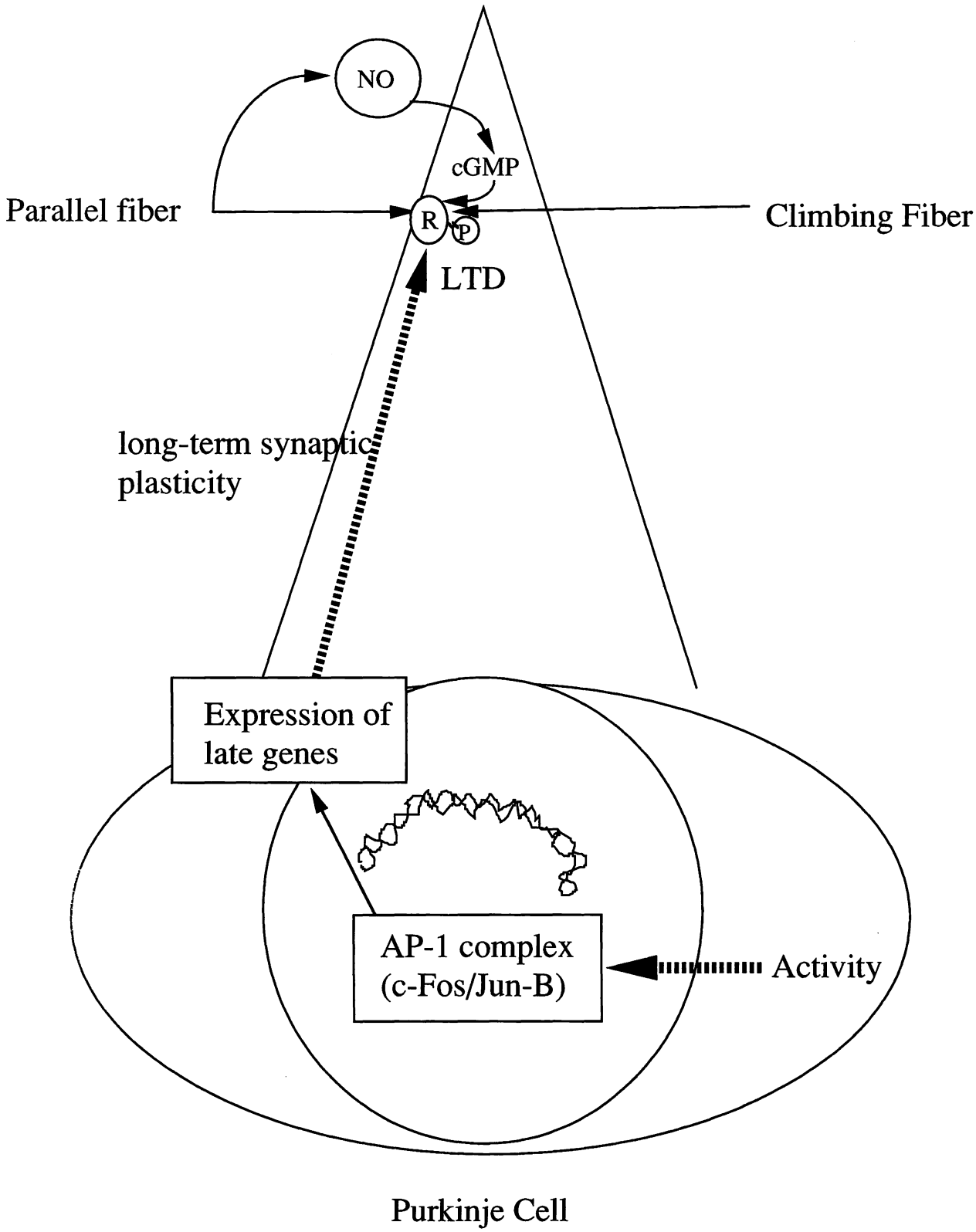
NO
cGMP
Parallel fiber
R
P
Climbing Fiber
LTD
long-term synaptic
plasticity
Expression of
late genes
AP-1 complex
(c-Fos/Jun-B)
Activity
Purkinje Cell

Fig. 1

DIVISION OF SPECIATION MECHANISMS II

Professor: *Ken-ichirou Morohashi*
Associate Professor: *Mitsuyasu Hasebe*
Research Associate: *Satoru Ishihara*
Technical Staff: *Sanae Oka*
Post Doctral Fellow: *Naoe Kotomura* [4]
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Ken Kawabe (Kyushu Univ.)
Tokuo Mukai (Kyushu Univ.)
Mikihiko Kato (Nagoya Univ.)
Hirofumi Mizusaki (Kumamoto Univ.)
Tomoaki Nishiyama (Univ. of Tokyo)
Keiko Sakakibara (Univ. of Tokyo)
Ryosuke Sano (Chiba Univ.)
Tatsuji Shikayama (Kyushu Univ.)
Kazuyoshi Someya (Univ. of Tokyo)
Hayato Yokoi (Nagoya Univ.)
Visiting Fellow: *Rumiko Kofuji*
Yoshiyuki Kojima
Assistant: *Hisae Tsuboi*

This division was established recently 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 differentiation 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 identified a steroidogenic tissue specific transcription factor designated Ad4BP/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 these distinct tissues. 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, 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 these issues, functions and regulation of nuclear transcription factors, WT1, SRY, SOX9, and DAX1 as well as Ad4BP/SF-1, all of which are critically implicated in gonadal and adrenocortical differentiation, have been 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 land plants 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 homologue 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 their functions are in progress. Comparisons of the gene functions and swapping experiments of genes among the organisms will make possible to infer what kinds of changes of developmental genes caused morphological diversity of oragnisms.

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RESEARCH SUPPORT

TECHNOLOGY DEPARTMENT

Head: Hiroyuki Hattori

Common Facility Group
Chief: Kazuhiko Furukawa

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Mamoru Kubota (Subunit Chief)
Chieko Nanba
Toshiki Ohkawa
Kaoru Sawada
Tomoki Miwa
Makiko Itoh (Technical Assistant)
Keiko Suzuki (Technical Assistant)
Misayo Masuda (Technical Assistant)

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Yousuke Kato (Subunit Chief)
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Takayo Itoh (Technical Assistant)

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Akio Murakami (Unit Chief)
Sonoko Ohsawa
Yumiko Makino
Takeshi Mizutani
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Masayo Iwaki
(Kazuhiko Furukawa)
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Maki Kondo(Subunit Chief)
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Developmental Biology Group
Hiroko Kobayashi(Unit Chief)
Chiyo Takagi
Naomi Sumikawa

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

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

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 enligtement 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 insititutes 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*
Associate Professor: *Masakatsu Watanabe*
Research Associates: *Yoshio Hamada, (Tissue and Cell Culture)*
Atsushi Ogiwara (Computer)
Post Doctral Fellow: *Mineo Iseki [1] (Large Spectrograph)*
Technical Staffs: *Masayo Iwaki (-August 31, 1997)*
Mamoru Kubota
Chieko Nanba
Toshiki Ohkawa
Kaoru Sawada
Tomoki Miwa
Makiko Ito (April 14, 1997-)
Misayo Masuda (September 1, 1997-)

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 30kW Xenon arc lamp and has a compound grating composed of 36 smaller individual gratings. It projects a spectrum of a wavelength range from 250nm (ultraviolet) to 1,000nm (infrared) onto its focal curve of 10m 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 tropical 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, 20W output), two CW dye lasers (Coherent, CR599-01) (420-930nm, 250-1000mW output), A/O modulators (up to 40MHz) to chop the laser beam, a beam expander, and a tracking microbeam irradiator (up to 200mm s^{-1} in tracking speed, down to 2mm 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, many kind of computers are equipped: UNIX servers and engineering workstations (Sun Ultra Enterprise server, SPARC stations, IRIS machines, NEWS machines, etc.), and some personal computers (Macintosh's and Windows machines). All of these machines, as well as almost every PC in each laboratory, are connected each other with local area networks, which are also linked to the high performance multimedia backbone network of Okazaki National Research Institutes.

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, database retrievals, homology search services, and so on. Providing network communication services is also an important task of this laboratory. We maintain the World-Wide Web server that contains the NIBB home pages (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 under extraordinary environments such as strong light intensity, low or high temperatures.

5. *Experimental Farm*

This laboratory consists of two 20 m^2 glass-houses with precise temperature and humidity control, three green houses (each 6 m^2) at the P1 physical containment level, a small farm, two greenhouses (45 and 88 m^2) with automatic sprinklers, two open aquariums (30 and 50t) 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 cleanbenches, 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 (30km 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 transduction 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: The genome sequencing project of an important Gram-positive bacterium *Bacillus subtilis* had been completed in 1997. This project was carried out under the international collaboration, and seven research teams participated in the sequencing from Japan. We took part in this project for assembly and analysis of the sequence data of Japan. The genome contains about 4,200 ORFs, and about a half of them seemed to constitute paralogous clusters. According to the comprehensive sequence analysis, some protein families like transporters, regulators, and sensors showed a tendency to the paralog. A database system called BSORF has been constructed in collaboration with Nara Institute for Science and Technology and the Human Genome Center of the University of Tokyo (http://bacillus.genome.ad.jp). Since the *Bacillus* genome project has been shifted to the functional analysis, we are also trying to integrate the functional information into the database.

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).

Publication List:

I. Faculty

Furuhashi, K., Tada, Y., Okamoto, K., Sugai, M., Kubota, M. and Watanabe, M. (1997). Phytochrome participation in induction of haustoria in *Cuscuta japonica*, a holoparasitic flowering plant. *Plant Cell Physiol.* **38**, 935-940

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Ogiwara,A., Ogasawara,N., Watanabe,M., and Takagi, T. (1997) Comprehensive Sequence Analysis of *B. subtilis* Genome Using the BSORF Database. *Proc. Genome Informatics 1997*, 320 - 321.

II. Cooperative Research Program for the Okazaki Large Spectrograph

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RADIOISOTOPE FACILITY (Managed by NIBB)

Head: *Masaharu Noda*
Associate Professor: *Kazuo Ogawa*
Technical Staff: *Kazuhiko Furukawa (Radiation Protection Supervisor), Yosuke Kato (Radiation Protection Supervisor), Yoshimi Matsuda (Radiation Protection Supervisor)*
Supporting Staff: *Takayo Ito, Risako Shirai*

This Facility consists of a central experimental station which is placed in the Common Building I and three smaller substations which are placed in National Institute for Basic Biology (NIBB) Building, Laboratories of Gene Expression and Regulation (LGER) Building which opened in May 1, 1997, and National Institute for Physiological Sciences (NIPS) Building. These spaces are controlled areas and only the persons who are permitted by the Radiation Protection Supervisor can enter. The members of the Radioisotope Facility have a variety of businesses such as monitoring and recording the going in and out the controlled areas of the persons, purchasing the radioisotopes for the users from the Japan Radioisotope Association (JRA), and passing the radioisotope wastes to the JRA. They also serve as lecturers of the training course required for the registration of beginners and for annual registrations of users. At every opportunity, the Radiation Protection Supervisors are providing them appropriate guidance for radioisotope handling and the law of interest.

The total number of users in and out the controlled areas from April to December, 1997 was 8,922. As shown in Figure 1A, the number of users in the LGER substation was comparable to that in the central station. The total radioisotopes purchased in this period for each controlled area and nuclide are shown in Figure 1. Although a variety of nuclides can be used in the central station, only ^{125}I, ^{35}S, ^{32}P, ^{14}C, ^{33}P, and ^{3}H were purchased within this period. From April, 1997, we have been allowed to use ^{33}P in all the controlled areas.

Figure 1A

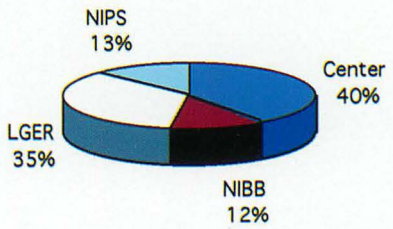
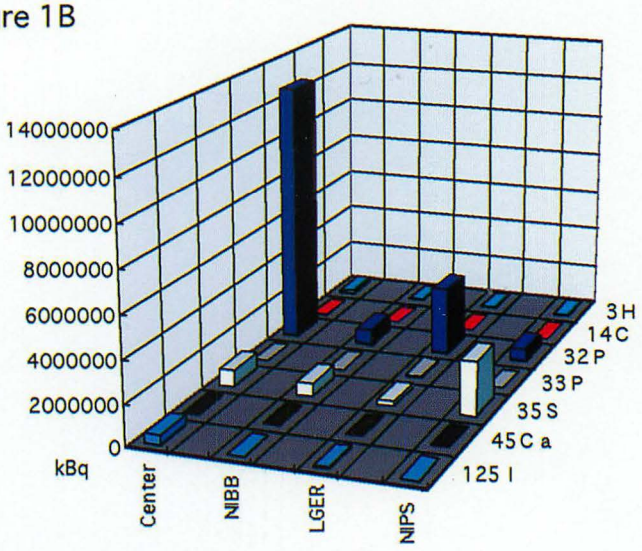


Figure 1B



The teaching staff of the Radioisotope Facility is 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 2 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).

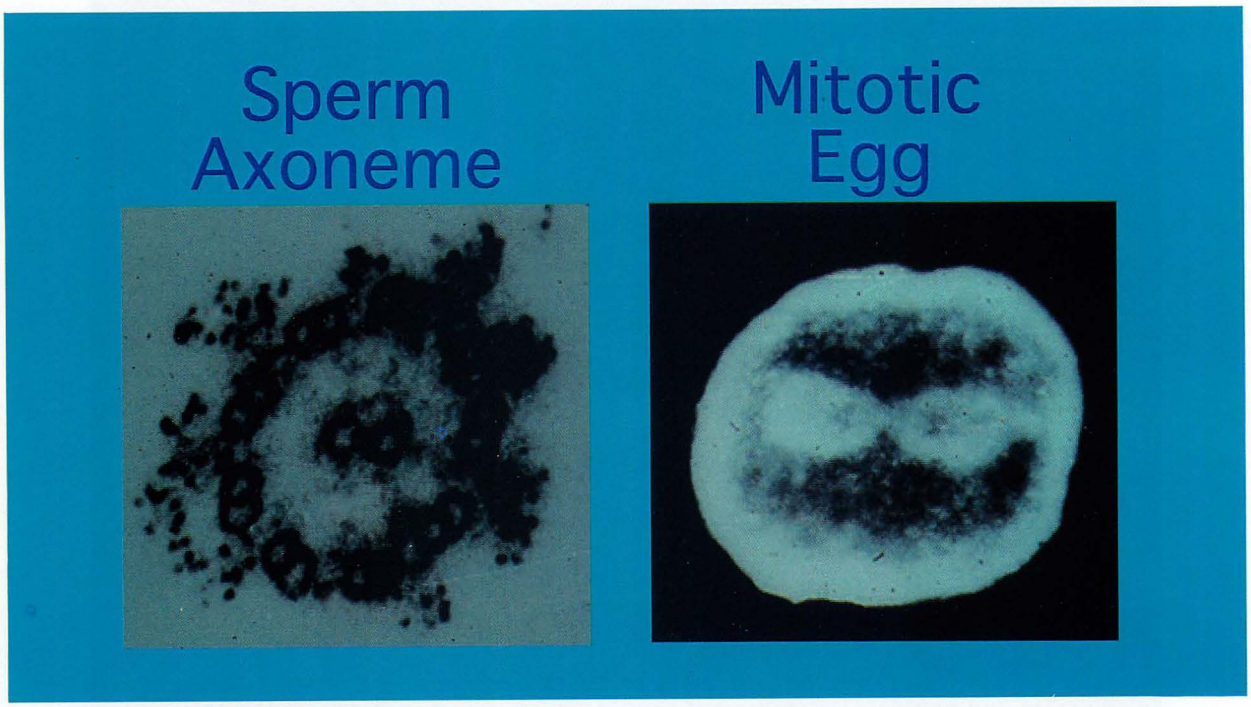


Fig. 2

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 dis-

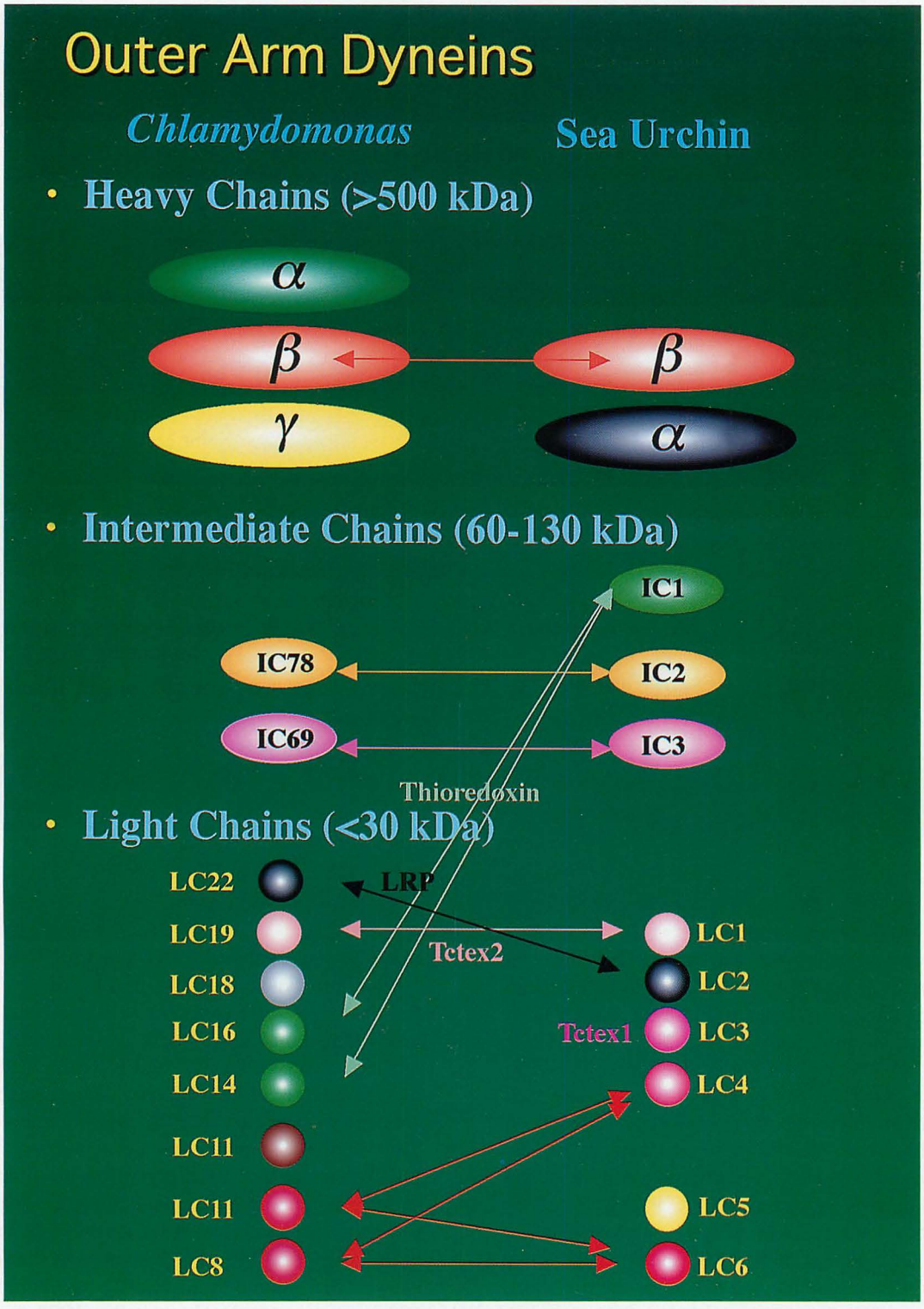


Fig . 3. Substructures of outer arm dyneins from sea urchin sperm flagella and *Chlamydomonas* flagella.

tinct 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 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. Alternative 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. They propose here that the three intermediate chains are the basic core units of sperm outer arm dynein because of their ubiquitous existence.

They are now isolating cDNA clones that encode LCs of sea urchin outer arm dynein as part of an effort to understand why dynein is so very large and complex. The outer arm dynein of sea urchin sperm flagella contains six light chains with molecular masses of 23.2, 20.8, 12.3, 11.5, 10.4, and 9.3 kDa, respectively. They have cloned the cDNA for the 23.2 kDa (LC1) and the 12.3 kDa (LC3) polypeptides and found that they are highly homologous to mouse Tctex2 and Tctex1, respectively. These mouse proteins are encoded by the t complex region that is involved in transmission ratio distortion (TRD), male sterility and the development of the germ cells. Their finding raises the possibility that axonemal dyneins are involved in this phenomenon. TRD may be caused by the dysfunction of multiple axonemal dyneins.

Publication List:

Kazuo Ogawa, and Hideo Mohri (1997) Establishment of a dynein motor superfamily. In Recent Advances in Marine Biotechnology. Vol I. Endocrinology and Reproduction (Edited by M. Fingerman, R. Nagabhushanam, and M.-F. Thompson), pp 249-281. Oxford & IBH Publishing Co. New Delhi.

THE CENTER FOR ANALYTICAL INSTRUMENTS
(Managed by NIBB)

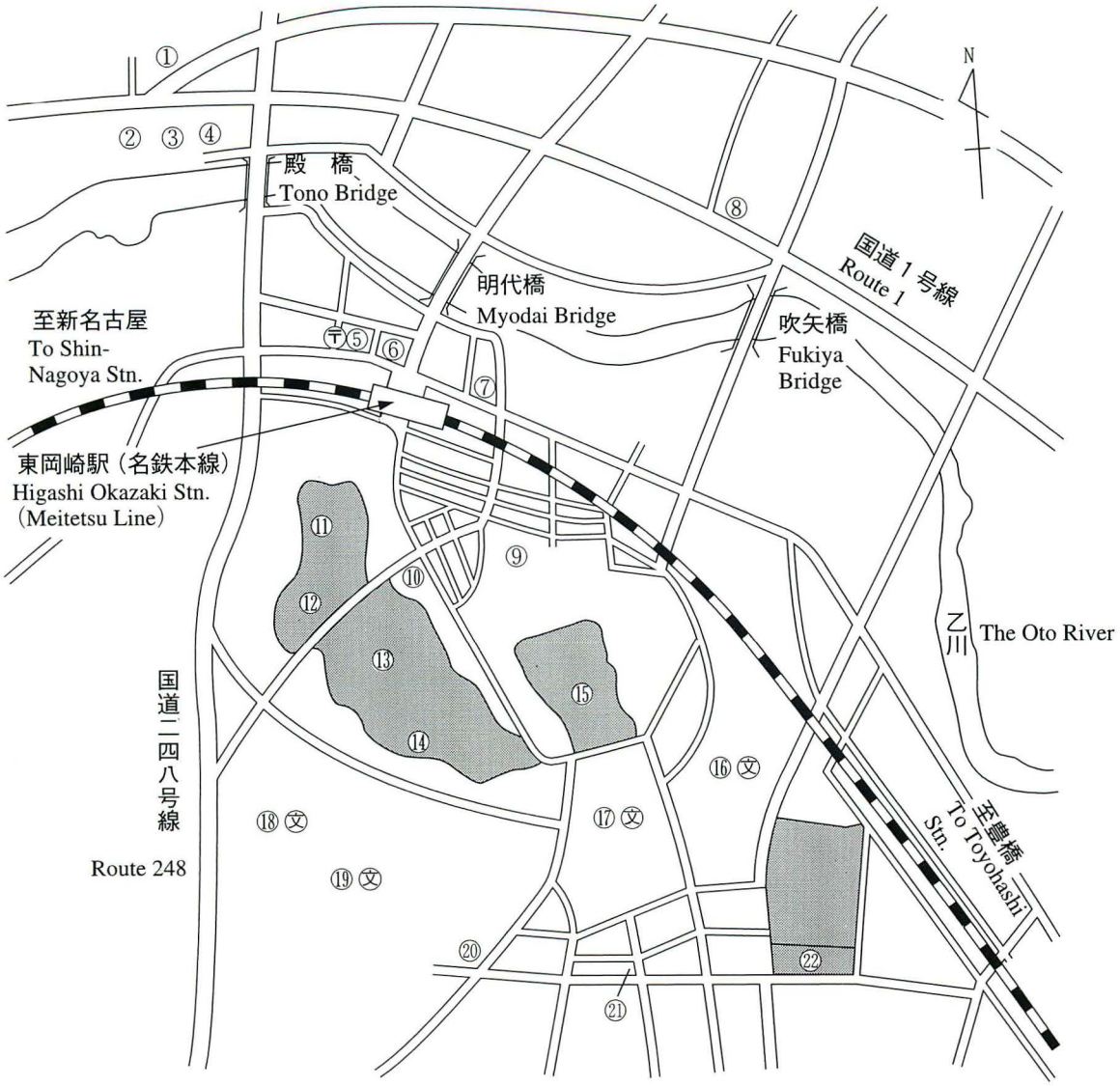
Head of Facility: *Tetsuo Yamamori*
Technical Staffs: *Akio Murakami*
Sonoko Ohsawa
Yumiko Makino
Takeshi Mizutani
Hatsumi Moribe (Apr. 21, 1997 -)

The Center, equipped with various types of instruments, serves for general use and supports biochemical structural analyses of biomolecules of proteins, nucleic acids and lipids. Amino acid sequence analysis, amino acid analysis, and chemical syntheses of peptides and DNA/RNA are carried out to support researchers in NIBB and NIPS. In April 1997, the Center started new services; site-specific modification of synthetic peptides (biotinylation and fluorophore labeling) and mass spectrometric analysis of peptides and proteins. Newly installed instruments in 1997 are Biomolecular Interaction Analysis System, Flow Cytometer, FT-IR Spectrophotometer and Microplate Luminometer. Some of instruments of the Center are opened to researchers outside the Institute.

Representative instruments are listed below.

Protein Sequencers (ABI 492, ABI 473A)
Amino Acid Analyzer (Hitachi L8500A)
Peptide Synthesizers (ABI 433A, ABI 432A)
Plasmid Isolation System (Kurabo PI-100)
DNA Sequencers (ABI 373S, ABI 310)
DNA/RNA Synthesizers (ABI 394, ABI 392)
Thermal Cyclers (Perkin Elmer PJ-1000, Takara TP-300)
Integrated Thermal Cycler (ABI CATALYST Turbo 800)
Gas Chromatograph (Shimadzu GC-14APF-SC)
Glycoprotein Analysis System (Takara Glyco-Tag)
High Performance Liquid Chromatographs (Shimadzu LC-10AD, Shimadzu LC-6AD)
Integrated Micropurification System (Pharmacia SMART)
Flow Cytometer (Coulter EPICS XL)
NMR Spectrometer (Bruker AMX-360wb)
EPR Spectrometer (Bruker ER-200D)
GC/Mass Spectrometer (JEOL DX-300)
Inductively Coupled Plasma Atomic Emission Spectrometer (Seiko SPS1200A)
Spectrofluorometers (Hitachi 850, Simadzu RF-5000)
Spectrophotometers (Hitachi 330, Hitachi 557, Varian Cary 5G, Perkin Elmer Lambda-Bio)
Microplate Luminometer (Berthold MicroLumat LB 96P)
Time-resolved Fluorescence Microplate Reader (Pharmacia DELFIA Research)
Microplate Readers (Corona MTP-120, MTP-100F)
Spectropolarimeter (JASCO J-40S)
FT-IR Spectrophotometer (Horiba FT-730)
Laser Raman Spectrophotometer (JASCO R-800)
Biomolecular Interaction Analysis Systems (Pharmacia BIAcore 2000, Affinity Sensors IAsys)
Bio Imaging Analyzer (Fujifilm BAS2000)
Electrophoresis Imaging Systems (PDI Discovery Series, BIOIMAGE)
Microscopes (Carl Zeiss Axiophot, Axiovert)
Microscope Photometer (Carl Zeiss MPM 03-FL)

Figure 1. Amino acid sequence analysis of proteins by an automated protein sequencer.



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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

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Super Market "Uny"

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