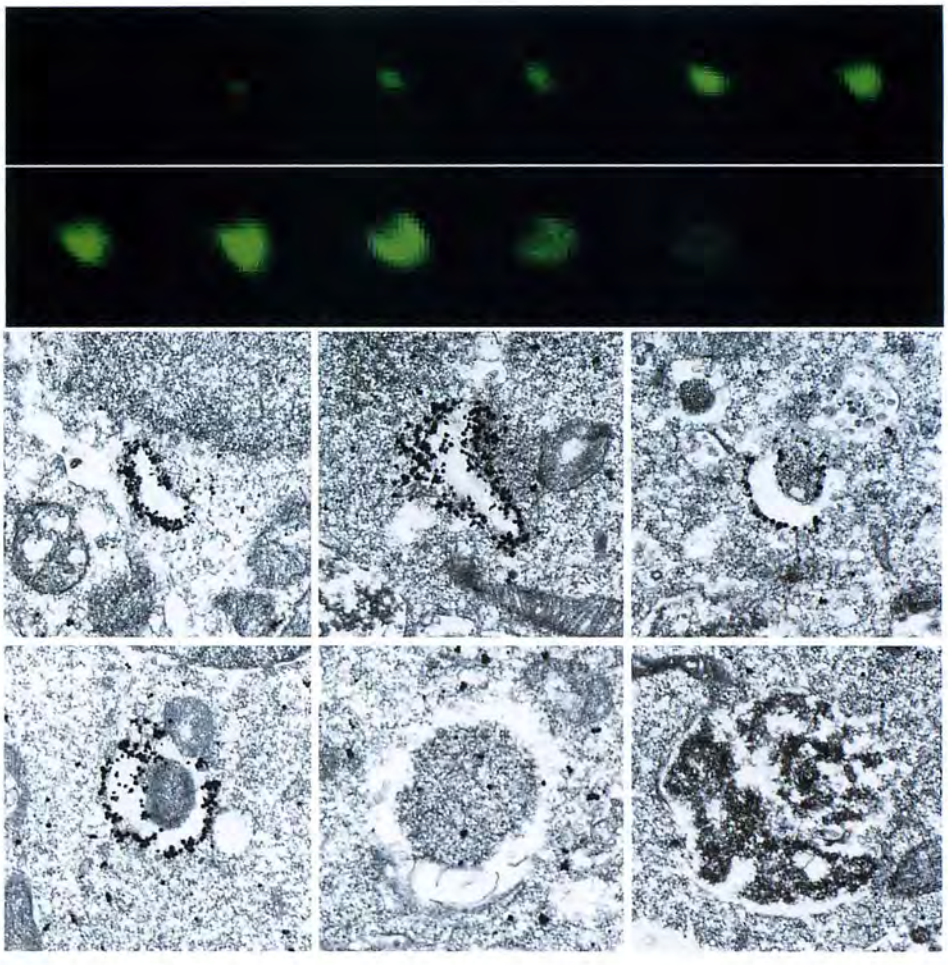
NATIONAL INSTITUTE FOR BASIC BIOLOGY

岡崎国立共同研究機構

基 礎 生 物 学 研 究 所



ANNUAL REPORT

2000

Development of isolation membrane into autophagosome traced with GFP-Apg5 on the membrane. The top panels show sequential images of autophagosome formation visualized with GFP-Apg5 by time-laps microscopy. The bottom panels show corresponding immunoelectron microscopic images using anti-GFP antibody.

Post Doctral Fellow
1 NIBB Research Fellow
2 JSPS Postdoctral Fellow
3 JSPS Research Associate
4 JST Fellow

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INTRODUCTION

The National Institute for Basic Biology (NIBB), a government-supported, basic research institute, was established in 1977. As a center of excellence, NIBB promotes the biological sciences by conducting first-rate research on site as well as in cooperation with other universities and research organizations. Researchers at NIBB investigate cell structure and function, reproduction and development, neuronal and environmental biology, gene expression and regulation, and molecular evolution of eukaryotic organisms.

In 2000, the Center for Integrative Bioscience (CIB) was established as a common center for interdisciplinary research among the NIBB and the other institutes in Okazaki. CIB consists of the Department of Development, Differentiation and Regeneration, the Department of Strategic Methodology, and the Department of Bio-environmental Science (formerly a part of NIBB). The Director-General of NIBB currently acts as the head of CIB. Two professors in the Department of Development, Differentiation and Regeneration have been nominated and will join the Center soon. To accommodate this new center together with the existing three common centers, i.e. the Research Center for Computational Science, the Center for Experimental Animals, and the Center for Radioisotope Facilities, a new campus space called the E-area was transferred from the Aichi University of Education to the Okazaki National Research Institutes. Construction is scheduled to be completed by the end of March, 2002.

In March, Professor Taisei Iguchi was appointed Professor of the Center for Bio-environmental Science. In April, all the members of the Center for Bio-environmental Science were reassigned to the Center for Integrative Bioscience. In July, Associate Professor Mitsuyasu Hasebe was promoted to Professor of the Division of Speciation Mechanisms II. Drs. H. Watanabe, M. Hayashi, Y. Sakai and T. Kiyosue were appointed as Associate Professors of the Center for Bio-environmental Science, the Division of Cell Mechanisms, the Adjunct Division of Cell Proliferation and the Adjunct Division of Biological Regulation, respectively. Adjunct Associate Professors Y. Ozeki and K. Owaribe departed NIBB.

We would like to congratulate Associate Professors Kaoru Itoh and Hiroshi Shibuya on their promotions to Professor at Nagoya University and Tokyo Medical and Dental University, respectively. Three research associates (equivalent to assistant professors) were promoted to associate professors of other universities. In addition, we replaced 2 research associates, 8 institute research fellows and 3 technicians with 2 research associates, 11 institute research fellows and 1 technician. The total number of personnels working at NIBB including graduate students and post doctoral fellows has been kept at 300 for several years.

As an international center for biological research, NIBB is responsible for conducting research projects in cooperation with various research groups. As a part of such cooperative activities, NIBB hosts International Conferences. In March the 43rd NIBB International Conference was sponsored by the Ministry of Education, Science, Sports and Culture and entitled "Mechanisms of Neural Network Formation" (Professors T. Yamamori, H. Fujisawa and F. Murakami, organizers). The 44th NIBB International Conference entitled "Evolution and Development. Generality and Diversity of Development in Animals and Plants" (Professors M. Hasebe and K. Agata and Dr. H. Tsukaya, organizers) was also held in March. In addition, NIBB continues to sponsor interdisciplinary symposia and study meetings on current topics by inviting leading scientists from around the world to the Institute. NIBB also provides a training course in biological sciences for young investigators. To assess our continuing improvement, the activities and future plans of two professors who have spent 10 years at NIBB were subjected to peer review by international scholars in related fields. We always welcome any suggestions concerning the research activities of NIBB.

Finally, I would like to congratulate Professor N. Murata for being awarded the Terry Galliard Medal from the International Association of Plant Lipid Research. Mr. T. Kirisako received the Nagakura Award. He is the fourth graduate student from NIBB to win the Award.

Hideo Mohri, D.Sc.
Director-General

ORGANIZATION OF THE INSTITUTE

The National Institute for Basic Biology (NIBB), is a one of the Okazaki National Research Institutes (ONRI) located on a hill overlooking the old town of Okazaki. NIBB was established in 1977 and its activities are supported by Monbukagaku-sho (the Ministry of Education, Science, Sports and Culture) of Japan. ONRI is composed of three independent organizations, NIBB, the National Institute for Physiological Sciences (NIPS) and the Institute for Molecular Science (IMS). In 2000, the Center for Integrative Bioscience was established as a common facility of the ONRI. The Center for Bio-environmental Science, began by NIBB in 1999, joined to this new Center.

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, comprised of distinguished scholars representing various fields of science and culture, advises the Director-General on basic policy. The Steering Council, comprised of professors within the Institute and an equal number of leading biologists outside NIBB, advises the Director-General on the scientific activities of the Institute. The Council, also makes recommendations on faculty appointments, the Institute's annual budget and future prospects.

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 subdivided 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 adjunct and headed by professors who hold joint appointments with other universities. Adjunct divisions have a resident research associate. This arrangement facilitates exchange in research activities in Japan. The Technical Department manages the activities of research technicians and helps to promote research activities of each division and to maintain the common research resources of the Institute. The Department also undertakes the technical education of staff.

Several members of the Center for Integrative Bioscience jointly work with the NIBB.

Research Support Facilities

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

Campus

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

GRADUATE PROGRAMS

The NIBB sponsors two graduate programs.

1. Graduate University for Advanced Studies

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 or equivalent at any university 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 enorolled in other universities and institutions are eligible to conduct research for fixed periods of time under the supervision of NIBB professors.

OFFICE OF DIRECTOR

Director-General: *Hideo Mohri*
Associate Professors: *Ryuji Kodama*
Kohji Ueno
Research Associates: *Kaoru Ohno*
Hiroki Kokubo (on leave)

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).

Studies using another lepidopteran species, *Orgyia recens approximans*, provided by Drs. Y. Arita and K. Yamada (Meijo University) is underway. In this species, the wing is normally formed until the beginning of the pupal period, but becomes conspicuously degenerated only in the female adult. 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.1. The tracheoles (fine threads) and the primary trachea (thick tube in the center) at the late stage of the pre-pupa

Another collaborative work with the laboratory of Dr. K. Watanabe (Hiroshima University) concerns mostly on the development of trachea and tracheole pattern in the swallow tail butterflies. Trachea and trcheoles are both important in delivering air into the wing and their pattern coincide with that of the boundary of degeneration and differentiation zones at the distal end of the wing. According to the observations, the pattern formation of wing epithelium is often dependent on tracheal and tracheole patterns. Basic research on the development of tracheal pattern formation is being done through the scanning electron microscopy and the bright field light microscopy of the fixed or fresh specimens to describe the exact pathway and the time course of the formation of elaborate pattern of trachea and tracheoles and to establish the cytological and developmental relationship between the formation of tracheal pattern and epithelial cell pattern, such as scale cell pattern.

The figure depicts how the tracheoles protrude from the primary trachea at the pre-pupa stage. These fine threads are arranged with even spaces and may closely related with the scale cell pattern formation (Fig. 1).

Protein palmitoylation and developmental mechanism at embryogenesis

Kohji Ueno

We have studied the molecular mechanisms of the development of cells and organs in the silkworm *Bombyx mori* and have found that a high molecular weight protein (p260/270) was expressed in abdominal leg cells during early embryonic stages. p260/270 was identified to be a protein palmitoylase which transfers palmitate to cysteine residues of proteins. Almost of small GTP-binding, heterotrimeric G, and G-protein-linked receptor proteins are known to be modified with palmitate through thioester linkages. These dynamic modifications are thought to be important in regulation of signal transduction.

To understand the molecular mechanism how the modification of protein palmitoylation regulates the development of cells and organs, a search for a homolog of p260/270 in vertebrate was undertaken. Homology search of an ESTdb (Expressed Sequence Tags data base) with the amino acid sequences of p260 and p270 identified mouse embryonic cDNA clones which were highly homologous to the amino acid sequences of p260 and p270. Since analysis of these clones revealed that the cDNA contained a long open reading frame encoding 2504 amino acids which showed 94% homology to rat fatty acid synthase (FAS), it was concluded that a homologue of *Bombyx* p260/270, i.e. FAS, is expressed during mouse embryogenesis.

In situ hybridization of mouse embryos revealed that the transcripts are detected mainly in the central and peripheral nervous system in mouse embryos from embryonic day 11.5. Immunocytochemical analyses of cultured mouse primary embryonic brain cells were performed to identify which cells express mouse FAS. This analysis revealed that mouse FAS was expressed specifically in neural cells in which growth-associated protein (GAP)-43 was expressed. GAP-43, by protein palmitoylation, regulates G_o signal transduction and neural axonal growth. In a cell-free system, purified FAS from mouse embryos transferred palmitate to GAP-43 through cysteine residues. Furthermore, cerulenin, an inhibitor of FAS, reduced axonal growth and *in vivo* palmitoylation of GAP-43 in cultured neurons. Figure 2 shows the effect of cerulenin on neuron morphology.

From these results, mouse FAS was speculated to be responsible for the palmitoylation of GAP-43 and subsequent regulation of axonal growth in mouse embryos. Since we also found that p260/270 was expressed in brain cells in insect embryos, a protein palmitoylase was speculated to regulate the axonal development during embryogenesis in invertebrate and vertebrate.

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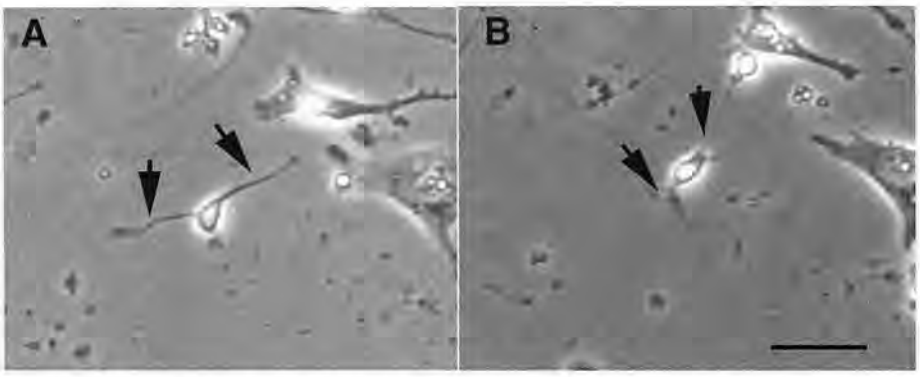

Fig.2. Effect of cerulenin on cultured primary neurons. Morphology was observed by phase-contrast microscopy after the addition of 400 μM cerulenin. (A); 0 min., (B) 120 min. Axonal outgrowths started to disintegrate and disappeared by 120 min. Arrows indicate axonal outgrowths. Bar represents 25 μm.

DEPARTMENT OF CELL BIOLOGY

Chairman: Mikio Nishimura

The department consists of two regular divisions and three adjunct divisions. The department conducts studies on molecular dynamics of the cell in higher plants and animals such as organelle differentiation, autophagy, cell motility, cytokinesis and neural development.

DIVISION OF CELL MECHANISMS

Professor:	*Mikio Nishimura*
Associate Professor:	*Makoto Hayashi (April 1-)*
Research Associates:	*Tomoo Shimada (-Feb. 28)*
	Shoji Mano
Technical Staffs:	*Maki Kondo*
	Katsushi Yamaguchi (-Sept. 1)
Post doctoral fellows:	*Yasuko Hayashi-Ishimaru*
	Kanae Shirahama
	Hiroshi Hayashi
	Kenji Yamada
	Naoto Mitsuhashi (Oct. 1-)
Graduate Students:	*Naoto Mitsuhashi (-Sept.30)*
	Kazumasa Nito
	Etsuko Watanabe
	Youichiro Fukao (April 1-)
JSPS Technical Staffs:	*Miwa Kuroyanagi*
	Chizuru Ueda
	Chihiro Nakamori
	Rie Takei (April 1-)
	Akiko Komori (May 23-)
Visiting Scientists:	*Yasuko Koumoto (-Feb. 28)*

Higher plant cells contain several distinct organelles that play vital roles in cellular physiology. During proliferation and differentiation of the cells, the organelles often undergo dynamic changes. The biogenesis of new organelles may occur, existing organelles may undergo a transformation of function, while other organelles may degenerate. Because the dynamic transformation of organellar function (differentiation of organelles) is responsible for flexibility of differentiation events in higher plant cells, the 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 greening of 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 b-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. The functional transformation between glyoxysomes and leaf peroxisomes is controlled by gene expression, alternative splicing, protein translocation and protein degradation.

To investigate the roles of microbody membrane proteins in the reversible conversion of glyoxysomes to leaf peroxisomes, we characterized several membrance proteins of glyoxysomes. One of them was identified as an ascorbate peroxidase (pAPX) that is localized on glyoxysomal membranes. Its cDNA was isolated by immunoscreening. The deduced amino acid sequence encoded by the cDNA insert does not have a peroxisomal targeting signal (PTS), suggesting that pAPX is imported by one or more PTS-independent pathways. Subcellular fractionation of 3- and 5-d-old cotyledons of pumpkin revealed that pAPX was localized not only in the glyoxysomal fraction, but also in the ER fraction. A magnesium shift experiment showed that the density of pAPX in the ER fraction did not increase in the presence of Mg^{2+}, indicating that pAPX is not localized in the rough ER. Immunocytochemical analysis using a transgenic Arabidopsis which expressed pumpkin pAPX showed that pAPX was localized on peroxisomal membranes, and also on a unknown membranous structure in green cotyledons. The overall results suggested that pAPX is transported to glyoxysomal membranes via this unknown membranous structure.

II. Microbody defective mutant of Arabidopsis.

It has been suggested that the functional conversion between glyoxysomes and leaf peroxisomes is controlled by gene expression, protein translocation, and protein degradation. A genetic approach is an effective strategy toward understanding the regulatory mechanism(s) of peroxisomal function at the level of gene expression, protein translocation, and protein degradation. We isolated and characterized 2,4-dichlorophenoxybutyric acid (2,4-DB)-resistant mutants. It has been demonstrated previously that 2,4-dichlorophenoxybutyric acid (2,4-DB) is metabolized to produce a herbicide, 2,4-D, by the action of peroxisomal fatty acid β-oxidation in higher plants. To isolate mutants that have defects in peroxisomal fatty acid β-oxidation, we screened mutant lines of Arabidopsis seedlings for growth in the presence of toxic levels of 2,4-DB. Genetic analysis revealed that these mutants can be classified as carrying alleles at three independent loci, which we designated ped1, ped2, and ped3, (where ped stands for peroxisome defective). The *ped1* mutant lacks the thiolase protein, an enzyme involved in fatty acid β-oxidation during germination and subsequent seedling growth. *Ped2* gene was identified by positional cloning and complementation analysis. The amino acid sequence of the predicted protein product is similar to that of human Pex14p, which is a key component of the peroxisomal protein import machinery. Therefore, we decided to call it *At*Pex14p. Analyses of the *ped2* mutant revealed that *At* Pex14p controls intracellular transport of both peroxisome targeting signal (PTS)1- and PTS2-containing proteins into three different types of microbodies, namely glyoxysomes, leaf peroxisomes and unspecialized peroxisomes. Mutation in the *PED2* gene results in reduction of enzymes in all of these functionally differentiated microbodies. The reduction in these enzymes induces pleiotropic defects, such as fatty acid degradation, photorespiration and the morphology of peroxisomes (Fig. 1). These data suggest that the *AT* Pex14p has a common role in maintaining

Figure 1. Reduced activity of photorespiration in *ped* 2 mutant.

Effect of CO2 on the growth of *ped*2 mutant. Wild-type *Arabidopsis* (WT/air) and *ped*2 mutant (*ped*2/air) were grown for 8 weeks in a normal atmosphere (36 Pa CO2) under constant illumination (100 μ E/m^2/s). The *ped*2 mutant was also grown for 8 weeks in an atmosphere containing 1000 Pa CO2 (*ped*2/CO2) under constant illumination (50 μ E/m^2/s).

physiological functions of each of these three kinds of plant microbodies by determining peroxisomal protein targeting.

III. Transport of storage proteins to protein storage vacuoles is mediated by large PAC (precursor-accumulating) vesicles.

Novel vesicles that accumulate large amounts of proprotein precursors of storage proteins were purified from maturing pumpkin seeds. These vesicles were designated precursor-accumulating (PAC) vesicles and have diameters of 200 to 400 nm. We characterized them to answer the question of how seed protein precursors are accumulated in the vesicles to be delivered to protein storage vacuoles. They contain an electron-dense core of storage proteins surrounded by an electron-translucent layer, and some vesicles also contained small vesicle-like structures. An immunocytochemical analysis revealed numerous electron-dense aggregates of storage proteins within the endoplasmic reticulum. It is likely that these aggregates develop into the electron-dense cores of the PAC vesicles and then leave the endoplasmic reticulum. Immunocytochemical analysis also showed that complex glycans are associated with the peripheral region of PAC vesicles but not the electron-dense cores, indicating that Golgi-derived glycoproteins are incorporated into the PAC vesicles. These results suggest that the unique PAC vesicles might mediate a transport pathway for insoluble aggregates of storage proteins directly to protein storage vacuoles.

In order to investigate the mechanism of the PAC vesicle formation, we constructed chimeric genes that encode fusion proteins consisting to both various lengths of polypeptides derived from pumpkin 2S albumin and a selectable marker enzyme, phosphinothricin acetyltransferase and expressed in Arabidopsis. A fusion protein expressed by one of the chimeric genes is accumulated as a proprotein-precursor form, and localized in novel vesicles of vegetative cells, that show distinct features that well much to the PAC vesicles. Despite of the accumulation of the fusion protein, the transgenic Arabidopsis is still sensitive to phosphinothricin. Phosphinothricin acetyltransferase contained in the fusion protein is obviously compartmentalized in the PAC vesicles that do not permit the detoxification of this herbicide. These results indicate that PAC vesicle can be induced in vegetative cells by ectopic expression of the protein that is destined to be compartmentalized into the PAC vesicles. Arabidopsis mutants that defect vesicular transport of the fusion protein are screened and characterized by using the transgenic plants.

In order to characterize the organelles in the vacuolar-sorting pathway, we constructed chimeric genes that encode various GFP fusion proteins and expressed in Tobacco BY-2 cells. The organelles in the vacuolar-sorting pathway were able to be visualized in vital conditions and were used for the characterization of the vacuolar-sorting pathway.

IV. Vacuolar processing enzymes in protein-storage vacuoles and lytic vacuoles.

Vacuolar processing enzyme (VPE) has been shown to be responsible for maturation of various seed proteins in plant vacuoles. *Arabidopsis* has three VPE homologues; βVPE is specific to seeds and αVPE and γVPE are specific to vegetative organs. We expressed the γVPE in a *pep4* strain of the yeast *Saccharomyces cerevisiae* and found that γVPE has the ability to cleave the peptide bond at the carbonyl side of asparagine residues. An immunocytochemical analysis revealed the specific localization of the γVPE in the lytic vacuoles of *Arabidopsis* leaves. These findings indicate that γVPE functions in the lytic vacuoles as the βVPE does in the protein-storage vacuoles. The βVPE promoter was found to direct the expression of the β-glucuronidase reporter gene in seeds of transgenic *Arabidopsis* plants. On the other hand, both the *αVPE* and *γVPE* promoters directed the expression in senescent tissues, but not in

young intact tissues. The mRNA levels of both *αVPE* and *γVPE* were increased in the primary leaves during senescence in parallel with the increase of the mRNA level of a senescence-associated gene (*SAG2*). Treatment with wounding, ethylene and salicylic acid up-regulated the expression of *αVPE* and *γVPE*. Our results suggest that vegetative VPE might regulate the activation of some functional vacuolar proteins that are known to respond to these treatments.

In order to clarify function of VPEs in vivo, their anti-sence Arabidopsis were generated and the characterization is under experiments.

V. Role of molecular chaperones in organelle differentiation.

Molecular chaperones are cellular proteins that function in the folding and assembly of certain other polypeptides into oligomeric structures but that are not, themselves, components of the final oligomeric structure. To clarify the roles of molecular chaperones on organelle differentiation, we have purified and characterized chaperonin and Hsp70s and analyzed their roles in the translocation of proteins into chloroplasts. In addition to mitochondrial chaperonin10 homologues, we isolated cDNAs for chloroplastic chaperonin 10 homologues from Arabidopsis thaliana. One of the cDNA insert was 958 bp long and encoded a polypeptide of 253 amino acids. The other cDNA insert was 603 bp and encoded a polypeptide of 139 amino acids. The former was comprised of two distinct Gro-ES domains whereas the latter had one Gro-ES domain. Functional analyses of these chaperonin homologues in differentiation of plastids are in progress.

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DIVISION OF BIOENERGETICS

Graduate University for Advanced Studies Professor: *Yoshinori Ohsumi*
Associate Professor: *Tamotsu Yoshimori*
Research Associates: *Yoshiaki Kamada*, *Takeshi Noda*
Technical Staff: *Yukiko Kabeya*
Post Doctoral Fellows: *Noboru Mizushima*[1], *Takahiro Shintani*, *Naotada Ishihara*[2], *Akio Kihara*[3]
Graduate Students: *Takayoshi Kirisako*[4], *Yoshinobu Ichimura*[4], *Kuninori Suzuki*[4], *Yoshinori Kobayashi*[4], *Atsuki Nara*[4], *Hideki Hanaoka*[4], *Akiko Kuma*[4,5], *Maho Hamasaki*[4,5], *Shisako Shoji*[4,6]
Visiting Scientists: *Satsuki Okamoto*

[1] *PRESTO, JST, Oct 1999~.*
[2] *til March 2000*
[3] *til Dec 2000*
[4] *Graduate University for Advanced Studies.*
[5] *from April 2000.*
[6] *from Oct.2000*

This division aims to understand the autophagy in respects to its molecular mechanism and its physiological role in higher eukaryotes. Cells execute degradation processes of their constituents together with biosynthetic processes. These two processes are well coordinated to regulate the biological activities. In other word, we must shed light on degradation process to fully understand the cell, because the study on the degradation has been retarded compared to the bio-synthetic process. Autophagy is well conserved in eukaryotes and is a major route for bulk degradation of cytoplasmic constituents and organelles in a lytic compartment, lysosome/vacuole.

I. Background

Upon nutrient starvation, autophagic process starts as building up a membrane structure, an autophagosome, in the cytoplasm. The autophagosome sequesters cytosol and organelles nonselectively. Then it is delivered to the vacuole/lysosome, and the cytoplasmic materials inside are degraded by vacuolar/ lysosomal proteases. We had discovered autophagy in a simple eukaryotic model organism, *Saccharomyces cerevisiae* and morphologically defined the whole process. We have isolated a set of autophagy-deficient mutants (*apg*), and have cloned most of the *APG* genes essential for autophagy. We are now characterizing these gene products.

II. Apg1-Apg13 protein kinase complex mediates induction of autophagy in response to starvation signal

We previously reported that Tor protein represses induction of autophagy during growing condition, since we observed that rapamycin, a Tor-specific inhibitor, induced autophagy even under such a condition. Next question is how inhibition of Tor function leads autophagy induction. Among 15 Apg proteins Apg1p and Apg13p show close relations with Tor. Apg1p is a protein kinase, and Apg1 kinase activity is enhanced by nutrient starvation or rapamycin-treatment. This activation requires *APG13*. Apg13p is highly phosphorylated in a Tor-dependent manner. In starved or rapamycin-treated cell, Apg13p is immediately dephosphorylated. And defect of autophagy by deletion of *APG13* is rescued by overexpression of *APG1*. These suggest that Apg13p plays a key role in signal transduction from Tor to Apg1p. We found that only dephosphorylated form (but not hyper-phosphorylated form) of Apg13p associates to Apg1p under starved condition. And we demonstrated that this association confers Apg1 activation which is required for the induction of autophagy. We also identified a couple of Apg1-binding proteins Apg17p and Cvt9p suggesting that Apg1 makes a large protein complex.

III. Two Distinct Phosphatidylinositol 3-Kinase Complexes Function in Autophagy and Carboxypeptidase Y Sorting

Vps30p/Apg6p is required for both autophagy and sorting of carboxypeptidase Y (CPY). Although Vps30p is known to interact with Apg14p, its precise role has remained unclear. We found that two proteins copurify with Vps30p. By mass spectrometry they were identified to be Vps38p and Vps34p, a phosphatidylinositol (PtdIns) 3-kinase. Vps34p, Vps38p, Apg14p, and Vps15p, an activator of Vps34p, were coimmunoprecipitated with Vps30p. These results indicate that Vps30p functions as a subunit of a Vps34 PtdIns 3-kinase complex. Phenotypic analyses indicated that

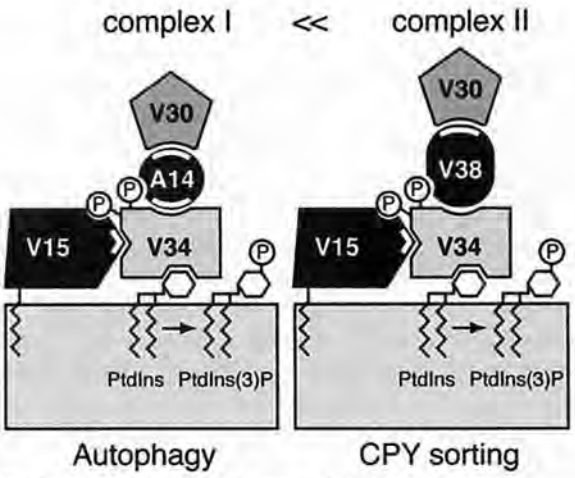

Fig.1 PI3 kinase complex is required for autophagy.
PI3 kinase(vps34) and its activator (vps15) form at least two complex. Complex I (Vps34, Vps15, Apg14, and Vps30) is essential for autophagy, and Complex II (Vps34, Vps15, Vps38, and Vps30) functions for vacuolar enzyme CPY sorting.

Apg14p and Vps38p are required for autophagy and CPY sorting, respectively, whereas Vps30p, Vps34p, and Vps15p are required for both processes. Coimmunoprecipitation using anti-Apg14p and anti-Vps38p antibodies and pull-down experiments showed that two distinct Vps34 PtdIns 3-kinase complexes exist: one, containing Vps15p, Vps30p, and Apg14p, functions in autophagy and the other containing Vps15p, Vps30p, and Vps38p functions in CPY sorting. The *vps34* and *vps15* mutants displayed additional phenotypes such as defects in transport of proteinase A and proteinase B, implying the existence of another PtdIns 3-kinase complex(es). We propose that multiple Vps34p-Vps15p complexes associated with specific regulatory proteins might fulfil their membrane trafficking events at different sites (Fig. 1).

VI. Discovery of novel lipidation reaction mediated by ubiquitin-like system

Apg8/Aut7, which plays an important role in the formation of autophagosome, tends to bind to membranes in spite of its hydrophilic nature. We showed that the mode of the association of Apg8 with membranes changes depending on a series of modifications of the protein itself. First, the carboxy-terminal Arg of newly synthesized Apg8 is removed by Apg4/Aut2, a novel cysteine protease, and a Gly residue becomes the carboxy-terminal residue of the protein that is now designated Apg8FG. Subsequently, Apg8FG forms a conjugate with an unidentified molecule "X" and thereby binds tightly to membranes. This modification requires the carboxy-terminal Gly residue of Apg8FG and Apg7, a ubiquitin E1-like enzyme. Finally, the adduct Apg8FG-X is reversed to soluble or loosely membrane-bound Apg8FG by cleavage by Apg4. The mode of action of Apg4, which cleaves both newly synthesized Apg8 and Apg8FG-X, resembles that of deubiquitinating enzymes.

We then succeeded to identify molecule X and discovred a novel mode of protein lipidation. Apg8 is covalently conjugated to phosphatidylethanolamine (PE) through an amide bond between the C-terminal glycine and the amino group of PE. This lipidation is mediated by a ubiquitination-like system (Fig. 2). Apg8 is a ubiquitin-like protein that is activated by an E1 protein, Apg7, and is transferred subsequently to an E2 enzyme Apg3/Aut1. Apg7 activates two different ubiquitin-like proteins, Apg12 and Apg8, and assigns them to specific E2 enzymes, Apg10 and Apg3, respectively. This reversible lipidation of Apg8 appears to be coupled to the membrane dynamics of autophagy and the Cvt pathway.

V. Mammalian Apg proteins; localization and function

Autophagy was first described in mammalian cells in the 1960s and its morphology and regulation have been extensively investigated. However, molecular mechanism underlying its process is poorly understood. Recent studies on yeast Apg proteins revealed that

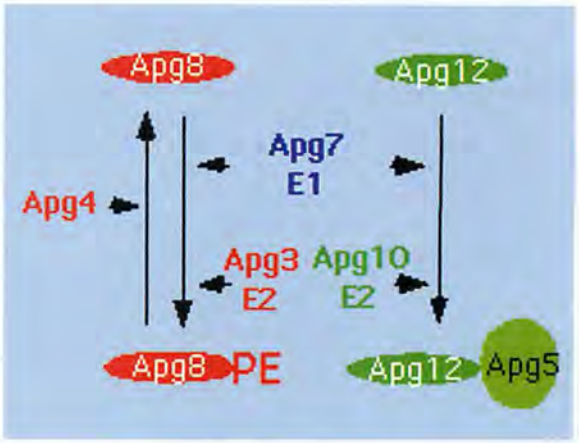

Fig.2 Two Ubiquitin-like systems essential for autophagy
Apg12 and Apg8 are activated by common E1 enzyme, Apg7. Then they are assigned to different conjugating enzymes, Apg10 and Apg3, respectively. Finally Apg12 forms a conjugate with a single target molecule, Apg5. Apg8 conjugate with a membrane phospholipid, phosphatidylethanolamine (PE). Apg8-PE is reversed to Apg8 by Apg4.

molecular machinery of autophagy seems highly conserved among eukaryotes. We are analyzing the mammalian orthologues of Apg proteins.

Apg8 has at least three mammalian homologues, one of which, LC3, was identified to be involved in autophagy. Newly synthesized rat LC3, a 142 amino acid protein, was immediately processed to remove the C-terminal 22 amino acids to generate the LC3-I form, which was further converted to the LC3-II form. While LC3-I was cytosolic, LC3-II was membrane bound and enriched in the autophagic vacuole fraction (Figure 3A and B). LC3-II may correspond to yeast Apg8-PE (the PE-conjugated form of Apg8). Immunoelectron microscopy revealed LC3 was present both inside and outside of autophagosomes in addition to cytoplasm. LC3 was less detected on autolysosome, suggesting that some LC3 dissociated from the membrane after autophagosome formation (Figure 3B). Consistent with such localization, the amount of LC3-II was correlated with the extent of the autophagosome formation. Although LC3 was originally identified as microtubule-associated protein 1 light chain 3, involvement of microtuble in LC3 function and autophagy has remained to be clarified. LC3-II is the first mammalian protein identified that specifically associates with the autophagosome membranes.

The Apg12-Apg5 protein conjugation system is another well conserved machinery. Most mammalian Apg5 was present in the Apg12-conjugated form in the cytoplasm. However, a small fraction of the Apg12-Apg5 conjugate localized to the isolation membranes when autophagy proceeds (Figure 3A and B). Using GFP-tagged Apg5, we revealed that the cup-shaped isolation membrane is developed from a small crescent-shaped compartment. These small structures have never been recognized unless labeled with Apg5, but they are

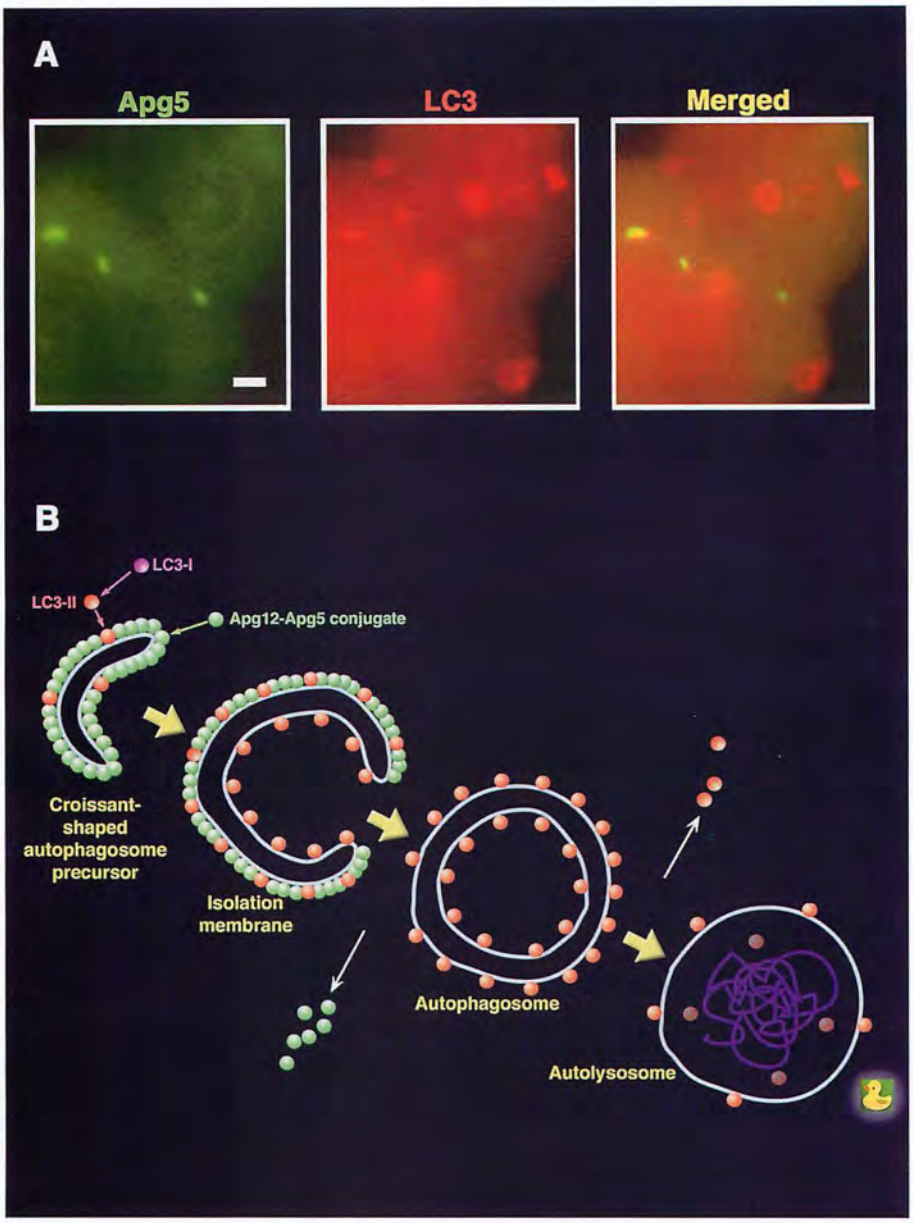

Fig.3.
A. Distribution of Apg5 and LC3 in mouse embryonic stem cells.
YFP-LC3 (middle panel) localizes to spherical autophagosomes and semi-spherical isolation membranes, whereas CFP-Apg5 (left panel) localizes to only isolation membranes. Bar, 2 μm.

B. Model of dynamic association of Apg12-Apg5 and LC3 with autophagic membranes.
Apg12-Apg5 conjugate localizes to the crescent-shaped autophagosome precursors. While these structures elongate and maturate into cup-shaped isolation membranes, cytosolic LC3-I is recruited to the membrane in the Apg12-Apg5-dependent manner and accumulated as the LC3-II form. Apg5 biases its localization to the outer side of the membrane. Apg5 plays an essential role in this membrane development. Immediately before or after the completion of autophagosome formation, Apg5 detaches from the membrane. Some LC3 also dissociate from the autophagosomal membrane thereafter.

likely to be direct precursors of the autophagosome. Apg5 localized on the isolation membrane throughout its elongation process. Apg5 was preferentially distributed in the outer side of the membrane and detaches from it immediately before or after autophagosome formation is completed (Figure 3B). To examine the role of Apg5, we generated Apg5-deficient ES cells. *APG5* $^{-/-}$ cells are viable but bulk protein degradation was significantly reduced. Morphological analysis revealed that autophagosome formation was impaired in *APG5* $^{-/-}$ cells. We also showed that the covalent modification of Apg5 with Apg12 was not required for its membrane targeting but is essential for involvement of Apg5 in elongation of the isolation membranes. Intriguingly, Apg12-Apg5 was required for targeting of LC3 to the isolation membranes and LC3-II generation.

Therefore, the Apg12-Apg5 conjugate plays essential roles in isolation membrane development in co-operation with LC3. In addition, our studies provided good molecular markers, LC3 and Apg12-Apg5, for autophagic membrane at all stages and isolation membranes, respectively, which so far have been defined only by morphology.

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DIVISION OF CELL PROLIFERATION (ADJUNCT)

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JST Research Staff: *Takeshi Awasaki (~ Sep. 30, 2000 CREST, Oct. 1, 2000~ PRESTO)*
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Nobuaki Tanaka (Graduate Univ. Advanced Studies)
Hideo Otsuna (Nagoya University)

The aim of this adjunct division, started in June 1998, is to understand the basic rules by which elaborate neural circuits develop and function. With less than 10^5 neurones, and subject to powerful molecular and genetic techniques, the brain of the fruit fly *Drosophila melanogaster* is a good model system for investigating the whole of an easily accessible nervous system that shares certain of the architectural and functional features of the more complex vertebrate brains. Third year of the five-year term, we continued a large-scale screening to find strains useful for this purpose.

I. Comprehensive identification of cells in the adult brain

A comprehensive and detailed anatomical knowledge of the brain is a prerequisite for 1) analysing the phenotypes of nervous system-related mutants, 2) identifying the cells that express cloned genes, 3) understanding the way information is processed in the brain, and 4) devising computer models that simulate brain functions. In spite of the hundred years of efforts using Golgi and other anatomical techniques, however, the circuit structure of higher order associative regions of the brain is still essentially unresolved. Moreover, traditional neuroanatomy tends to focus only on the mature adult brain, leaving the developmental processes largely uninvestigated. Since many nervous system-related mutants show structural defects, however, understanding the role of the responsible genes requires detailed basic knowledge about when and how the brain structure is formed. Thus, "developmental neuro-anatomy" becomes all the more important in the age of molecular cloning.

The GAL4 enhancer-trap system, which is widely used for mutagenesis and gene cloning of *Drosophila*, is also a powerful tool for obtaining a vast array of transformant strains that label specific subsets of brain cells. We screen such lines from a stock of 4500 GAL4 strains made by the "NP consortium", a joint venture of eight Japanese *Drosophila* laboratories organised by us. Our screening consists of two stages. In the first step, all the lines are crossed with the flies carrying the UAS-*GFP* transgene, which fluoresces only in the cells where GAL4 expression is active. The patterns of the GAL4-expressing cells are recorded from freshly dissected, unfixed adult brain tissue using a high-speed confocal microscope. Photographs of between 20 and 100 optical sections are taken for each line. As of December 2000, ca. 108,000 photographs depicting 3,400 of the total 4,500 strains have been accumulated in a computer database. In the second step, useful lines are selected from the database, and fixed and clealised brain specimens at various developmental stages are subjected to confocal serial sectioning with a conventional confocal microscope and to three-dimensional reconstruction with a UNIX workstation.

Although the long-term aim of this project is to identify as many neurones and glial cells as possible to get the comprehensive overview of the fly brain structure, at the initial stage a few brain regions are chosen for intensive study. The first target is to identify projection interneurons that connect lower-level sensory neuropile and higher-order associative regions. These fibres convey olfactory, gustatory, auditory and visual sensory information.

For visual pathways, we identified in total 30 types – among which 22 were novel – of projection neurones (in total ca. 500 cells) that connect lower-level sensory neuropile in the optic lobe to the higher order regions in the central brain (Fig. 1). For olfactory pathways, we identified 12 types of projection neurones (in total ca. 75 cells), that connect glomeruli in the first-order sensory neuropile of olfaction (olfactory lobe, antennal lobe) to the second-order processing cites (mushroom body and lateral horn). Further completion of such connection map would give us important insights about how sensory information is conveyed and integrated before association with signals of other sensory modalities.

II. Mapping of neurotransmitters and receptors in the adult brain

To understand the neural network of the brain, it is also important to reveal what kind of synapses is used in each identified neurone. For this purpose, information about the types of neurotransmitters and receptors is indispensable. Previously, such information is obtained by staining brain tissue with various antibodies. This approach, however, has the limitation due to the availability of good andibodies. Antibodies raised against transmitters or receptors of certain animal species do not always label corresponding molecule in other species.

Taking the advantage of the completion of *Drosophila* genome project, we thus employed a novel approach. Using homology and other information, we first search genes that code receptors or enzymes associated to transmitter synthesis. Cells that express those genes are labelled by using in-situ RNA hybridisation. This technique can visualise only the cell bodies of the labelled cells. We then screen GAL4 enhancer-trap strains that label cells in the corresponding area of the cortex, and perform double labelling to certify the colocalisation of GAL4

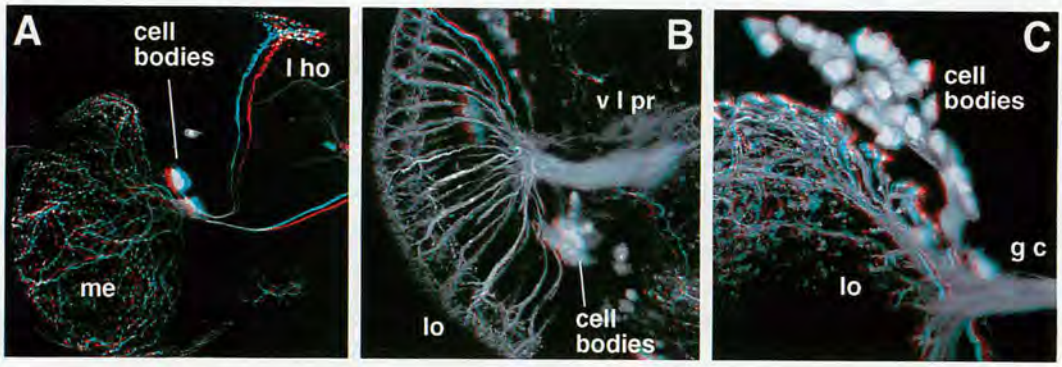

Fig. 1 Neurones that connect optic lobe and the central brain Three-dimensional reconstruction of about 200 confocal sections. Red-Cyan stereograph, posterior view of the whole-mount brain. To get 3-D image, use red filter for the left eye and green (or blue) filter for the right eye. A: Neurones that connect the outer layer of medulla (me) to lateral horn (l ho). A subset of fibres project to contralateral medulla. B: Columnar array of neurones that connect lobula (lo) and ventrolateral protocerebrum (v l pr). Note that arborisation occurs only at defined layers in the outer region of the lobula neuropile. C: Another type of array neurons that connect both sides of lobula via great commissure (g c). Arborisation occurs only in the inner region of lobula.

expression and in-situ label. This way, we can map the position and morphology of cells that use certain transmitters and receptors. First year of this project, we established a reliable and efficient protocol for in-situ staining of the dissected whole brain, and successfully visualised the expression pattern of three receptors and three enzymes associated with transmitter synthesis.

III. Analysis of the cell lineage-dependent modular structures in the brain

Combining the flippase-FRT recombination induction system and GAL4-UAS expression activation system, we have previously developed a novel technique – the "FRT-GAL4 system" – with which one can label a small number of neural stem cells at any desired developmental stage and reveal the projection patterns of their progeny at a later period.

The central brain of *Drosophila melanogaster* is produced by an average of 85 stem cells (neuroblasts) per hemisphere. We found that the majority of clones keep their cell bodies in tightly packed clusters. In 30 out of the 32 clone types identified so far, the neurites fasciculate to form a single bundle that runs from a cell body cluster, and innervate a limited number of neuropile regions in a stereotypic manner, forming clearly defined units of neural circuits. These suggest that in many cases the progeny of a single stem cell forms a lineage-dependent circuit structure unit, which we named a "clonal unit."

The clustering of clonal cell bodies and the fasciculation of neurites are already apparent in the developing larval brain. In larvae, glial cells form the border of clonal cell body clusters.

IV. Development of an improved red fluorescent protein (RFP) reporter system suitable for double labelling and birth order analysis of neural fibres

The green fluorescent protein (GFP) and its enhanced mutants are widely used to monitor protein localisation and gene expression of various organisms. Recently, red fluorescent protein, such as DsRed, was isolated from corals. Its significantly red-shifted emission and absorption maxima provide good complementation to GFP. Its use, however, is still restricted by its rather low quantum yield and parasitic green fluorescence peak that cross-talks with GFP emission. To overcome this problem, we developed a new variant of DsRed (DsRed S203Y), which is significantly brighter than the wild-type and free from green fluorescence peak, making it an ideal reporter for double labelling with GFP.

DsRed requires significantly longer fluorescence maturation time than GFP. By co-expressing GFP and DsRed under the control of the same promoter, time after the onset of gene expression can be monitored by its colour change from green (GFP only) to yellow (green by GFP + red by mature DsRed) over time. By using a GAL4 enhancer-trap strain that drives expression shortly after cells are born, it is possible to study birth order of the labelled cells. Though birth order analysis has been possible by pulse-chase labelling of DNA-replicating cells with ^{3}H-thymidine or bromodeoxyuridine (BrdU), the label was limited to cell bodies. Since GFP and DsRed spread even into fine cellular-protrusions, the GFP/DsRed system makes it possible to reveal birth order of fibre processes. We applied this to the developing mushroom body neurons of *Drosophila* brain and visualised, for the first time, the formation order of fibres within a neural fiber bundle (Fig. 2).

(This work is performed under collaboration with Drs. Vladislav V. Verkhusha, Hiroki Oda and Shoichiro Tsukita of ERATO Tsukita Cell Axis Project, Kyoto.)

V. Contribution to the science community

As a joint venture with German and US research groups, we maintain *Flybrain*, a web-based image database of the *Drosophila* nervous system (http://flybrain.nibb.ac.jp). Over 2000 images has

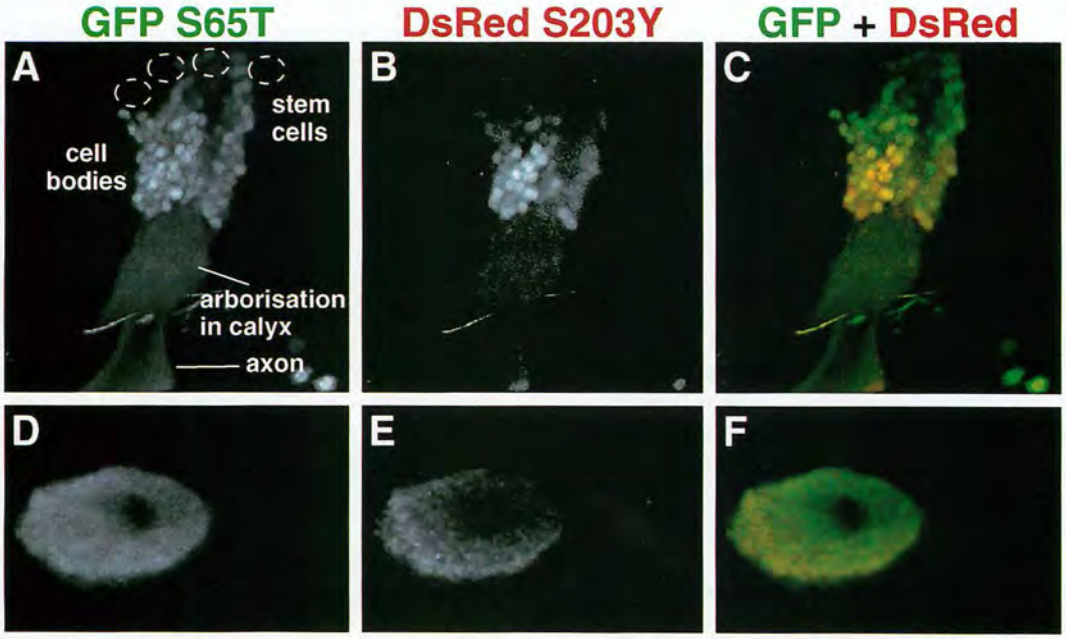

Fig. 2 Birth order analysis of neural fibres in the mushroom body Late third-instar larval brain of the GAL4 enhancer-trap strain *201y* expressing UAS-GFP S65T and UAS-DsRed S203Y. A-C: Reconstruction of horizontal optical sections. D-F: Vertical section of the axon bundle of pedunculus. GFP labels a majority of cell bodies in the larval mushroom body (A). Arborisation and axons are also labelled. Accordingly, most fibres are labelled in the section of the axon bundle (D). DsRed, on the other hand, labells only cell bodies that are far from the stem cells, which are older than those near the stem cells (B). In the section, Only fibres near the periphery of the bundle are labelled (E). Comparison of GFP and DsRed labelling (C, F) reveals that older fibres run in the periphery. Thus, it is likely that newly elongating fibres run into the core, rather than along the outer surface, of the fibre bundle.

already been stored and served worldwide. Another database maintained here is *Jfly*, which is intended to help the exchange of information among Japanese-speaking *Drosophila* researchers (http://jfly.nibb.ac.jp). Archives of research-related discussions, images, movies and experimental protocols, as well as meetings and job announcements, are provided.

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DIVISION OF CELLULAR COMMUNICATION

(ADJUNCT)

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Associate Professor:	*Katsushi Owaribe (- Aug. 15)*
Research Associate:	*Takako Kato-Minoura*
Postdoctoral Fellows:	*Yoshiaki Hirako*
	Itsushi Minoura
Graduate student:	*Ken-ichi Wakabayashi (University of Tokyo)*

The research in this laboratory is aimed at an understanding of the molecular mechanisms that regulate the assembly and function of cytoskeletal proteins. Specifically, we are currently studying the functional properties of axonemal dynein and actin in *Chlamydomonas*, an organism ideally suited for genetic and molecular biological studies.

I. Function of Multiple Axonemal Dyneins

It is well established that the beating of cilia and flagella is based on sliding movements of outer-doublet microtubules driven by motor proteins dyneins, but how the sliding is converted into axonemal oscillatory bending movement has not been made clear. Recently, various lines of evidence have suggested that dynein is crucially important also in the sliding-bending conversion mechanism. Thus our research effort is now focused on the properties of various dyneins.

Biochemical studies by us and other laboratories have established that a single flagellar axoneme contains at least eleven kinds of dynein heavy chains in inner and outer arms. The question is how different dynein heavy chains differ in function. To answer this question, we have been isolating and characterizing mutants that lack different kinds of axonemal dyneins. During the last ten years, we have isolated as many as 15 genetically different mutants lacking various subsets of dyneins. The isolation of these mutants greatly advanced our understanding of the function and organization of various dyneins within the axoneme, because only three mutants had been known to lack dynein heavy chains before we started mutant isolation.

The motility phenotypes of the isolated mutants have indicated that different dynein species differ in function in a fundamental manner. For example, the outer-arm heavy chains are important for flagellar beating at high frequency, whereas the inner-arm heavy chains are important for producing proper waveforms. Indirect evidence also suggests that the force generation properties differ greatly among different heavy chains. Interestingly, the axoneme can beat without some of these heavy chains, but cannot beat if certain combinations of heavy chains are lost. It appears that simultaneous presence of dyneins with different properties is necessary for the axonemal beating. Thus, it should be important to understand the mechano-chemical property of each dynein. To this end, we are currently trying to directly measure the force production in wild-type and mutant axonemes that lack various combinations of dyneins; we have constructed an experimental device to measure minute axonemal force with a fine glass needle. Preliminary results indicate that the force produced in the mutant axoneme lacking the outer arm or part of the inner arm is reduced to about 1/3 of that in the wild-type axoneme.

As a by-product of these experiments, we have recently succeeded in detecting elasticity between the outer-doublet microtubules. Our results confirmed that there is an elastic component that connects adjacent outer-doublet microtubules, as has been postulated by theoretical studies of cilia and flagella. Such an elastic component has been considered crucial for axonemal beating, since it is regarded as responsible for restricting the amplitude of microtubule sliding and for generating oscillatory movements.

II. Function of Actin and an Actin-related Protein in *Chlamydomonas*

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 motility systems - should somehow cooperate in the inner arm dynein although the function of actin in dynein arms 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 been known to have only a single gene of conventional actin, and the mutant *ida5* was found to express no conventional actin at all. On close inspection, the cytoplasm and axonemes of this mutant were found to contain a novel actin-like protein (NAP) which displays exceptionally low homology (64%) to conventional actin. The mutant *ida5* is deficient in the formation of the fertilization tubule and thus has a low mating efficiency. However, it displays normal cell division and grows as rapidly as wild type, possibly because NAP can substitute for actin in important cellular functions. Thus conventional actin and NAP may overlap in some, but not all, cellular functions. It is interesting to note that NAP is expressed in significant amount only in the mutant lacking actin; i.e., the expression of NAP appears to depend on the presence of actin. We are currently investigating how such regulation takes place.

We have recently succeeded in transforming the mutant *ida5* with cloned actin gene and found that inner dynein arms become restored upon transformation. Transformation with NAP gene is underway. Studies with artificially mutated actin gene will enable us to determine what functions are carried out by actin and NAP and, in particular, whether actin or NAP is really essential for cytokinesis, assembly and function of inner dynein arms, or other fundamental processes in *Chlamydomonas*.

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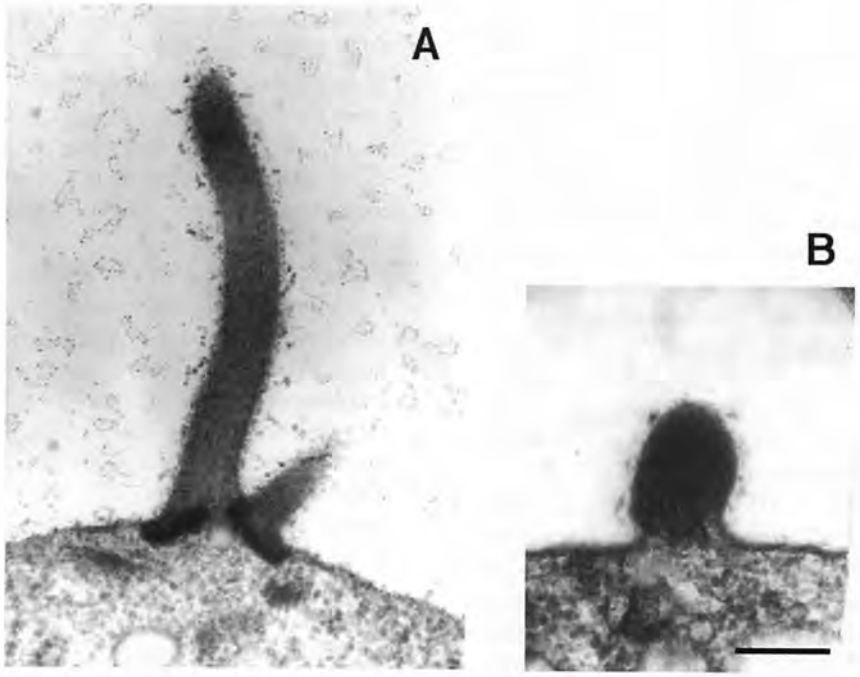

Fig. 1. Fertilization tubules in wild-type (A) and *ida5* (B) mt+ gametes produced in response to a 1 hour exposure to 10 mM dibutyryl-cAMP and 1 mM IBMX. Bar, 0.3 μm. Wild-type fertilization tubules have been shown to contain F-actin bundles.

DIVISION OF CELL FUSION (ADJUNCT)

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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*.

S. pombe is an excellent system to investigate the changes in the actin cytoskeleton during cell cycle since F-actin patches, F-actin cables and F-actin ring are only visible structures in the cell. The F-actin ring is considered to correspond to the contractile ring in animal cells. It is formed during anaphase in this organism.

S. pombe cells have two II-type myosin heavy chains called Myo2 and Myo3/Myp2. Recently, we studied how myosin accumulates at the division site. First, we showed that Cdc4, an EF-hand protein, appears to be a common myosin light chain associated with both Myo2 and Myo3. Loss of function of both Myo2 and Myo3 caused defect in the F-actin ring (contractile ring) assembly at the division site, like the phenotype of *cdc4* null cells. It is suggested that Myo2, Myo3 and Cdc4 function in a cooperative manner in the formation of the F-actin ring during mitosis.

Next, we investigated dynamics of myosin-II during mitosis in *S. pombe* cells. In early mitosis, Myo2 was detected primarily as dots widely located in the medial cortex. Myo2 fibers also became visible following the appearance of the dots. The Myo2 dots and fibers then fuse with each other to form a medial cortical network. Finally the network is packed into a thin contractile ring. In mutant cells that cannot form the F-actin ring such as *cdc3*, *cdc8* and *cdc12*, Myo2 is able to accumulate as the dots in the medial cortex, whereas no accumulation of the Myo2 dots was detected in *cdc4* cells. Moreover, F-actin did not seem to be required for the accumulation of the Myo2 dots.

A truncated Myo2 which lacks putative Cdc4-binding sites (Myo2ΔIQs) was able to rescue *myo2* null cells, *myo3* null cells, *cdc4* mutant cells and *cdc4* null cells. The Myo2ΔIQs could assemble into a normal-shaped ring in these cells. Thus, its assembly at the division site does not require function of either Cdc4 or Myo3.

On the other hand, we studied reorganization of actin-myosin cytoskeleton at the growing ends of the cleavage furrow of *Xenopus* eggs. At the the furrow formation, a cortical movement towards the division plane occurs at the growing ends of the furrow. Immunofluorescence microscopy demonstrated that myosin II assembles at the growing end as spots probably as a result of the cortical movement. Actin filaments assemble a little later after the formation of the myosin spots as small clusters which we call "F-actin patches", at the same positions as the myosin spots. The F-actin patches seemed to be formed and grow through new actin polymerization rather than assembly of pre-existing cortical F-actin. This was substantiated by microinjection of rhodamine-G-actin near the growing end: the microinjected G-actin was rapidly incorporated in the F-actin patches. The F-actin patches then align tandemly to form short F-actin bundles, and then the short bundles form long F-actin bundles which compose the contractile ring. The myosin spots are aligned on the long F-actin bundles and fused each other to show fibrous appearance.

We also concentrate our study on function of actin-regulatory proteins, including ADF/cofilin family proteins, during cytokinesis using *Xenopus* eggs and embryos. ADF/cofilin family proteins exist in all animals and plants examined and have been shown to be essential. We found that ADF/cofilin family proteins are essential for cytokinesis (Abe, Obinata, Minamide, and Bamburg, J. Cell Biol. 132: 871-875, 1996). Recent studies revealed that ADF/cofilin accelerates turnover of actin filaments both in vitro and in vivo. Most recently, we found a novel actin-regulatory protein which induces disassembly of actin filaments cooperatively with ADF/cofilin. cDNA analysis revealed that this protein is a *Xenopus* homologue of yeast actin interacting protein 1 (AIP1). Thus, we designated this protein as *Xenopus* AIP1 (XAIP1). Purified XAIP1 itself binds to pure actin filaments to some extent, but it induces a rapid, drastic disassembly of actin filaments associated with cofilin. Microinjection of this protein into *Xenopus* embryos arrested development by the resulting actin cytoskeletal disorder. XAIP1 represents the first case of a protein cooperatively disassembling actin filaments with ADF/cofilin family proteins.

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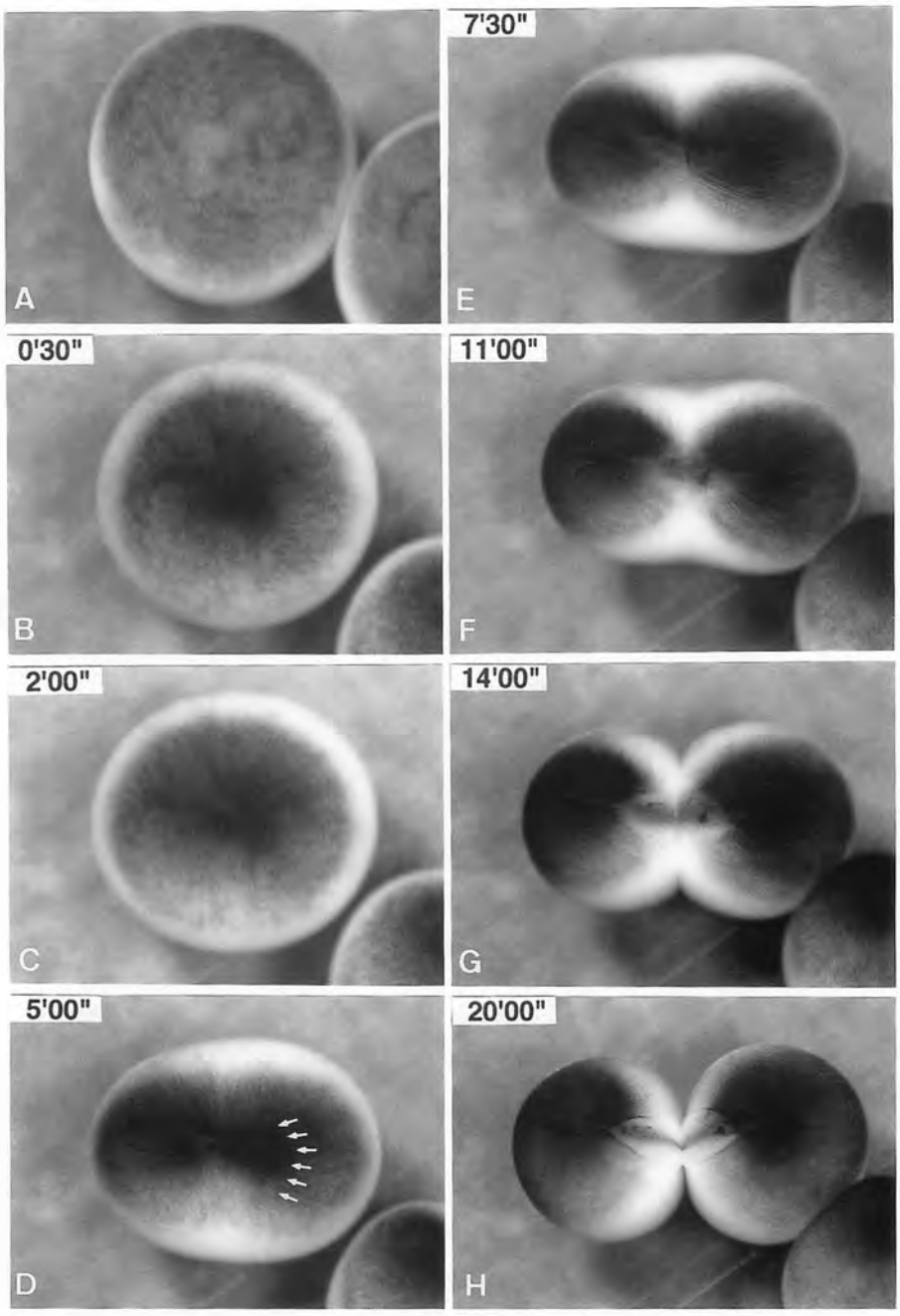

Fig. 1 First cleavage of a *Xenopus* egg.

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) gene regulation in cell differentiation and growth, and (3) molecular basis of body plans.

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	Chika Matsuda

The pituitary-gonadal axis plays an important role in regulating gametogenesis in vertebrates. Gonadotropins typically act through the biosynthesis of gonadal steroid hormones which in turn mediate various stages of gametogenesis. Their effects are particularly profound in teleost fishes which provide several excellent models for investigating the basic hormonal mechanisms regulating gonadal sex differentiation and gametogenesis (spermatogenesis, sperm maturation, oocyte growth and oocyte maturation). Our research focuses on (1) the identification of steroidal mediators involved in gonadal sex differentiation and gametogenesis, and (2) the mechanisms of synthesis and action of these mediators.

I. Endocrine regulation of gonadal sex differentiation

Sex determination and gonadal development vary considerably in fish. In addition to gonochorism, several types of hermaphroditism (protandry, protogyny and synchronous hermaphroditism) are found in fish. Nile tilapia, *Oreochromis niloticus*, is an excellent example of the precise nature of steroidogenic actions during gonadal sex differentiation. In this fish, all genetic female (XX) or male (XY) broods can be obtained by artificial fertilization of normal eggs (XX) and sex-reversed, pseudo male sperm (XX), or normal eggs (XX) and super male sperm (YY), respectively. Fertilized eggs hatch after 4 days at 26℃. On the day of hatching, primordial germ cells (PGCs), which are morphologically distinguishable from somatic cells, are located in the outer layer of the lateral plate mesoderm around the hind gut. At 3 days post-hatching, PGCs are located in the gonadal anlagen after the formation of the coelomic cavity in the lateral plate mesoderm rather than through active migration.

The gene *vasa* encodes a DEAD (Asp-Glu-Ala-Asp) family of putative RNA helicase and is present in the germ line of several animal species. *Vas* (a *Drosophila vasa* homologue) gene expression pattern in germ cells during oogenesis and spermatogenesis has bee examined using all genetic females and males of tilapia. In the ovary, *vas* is expressed strongly in oogonia to diplotene oocytes and becomes localized as patches in auxocytes and then strong signals are uniformly distributed in the cytoplasm of previtellogenic oocytes, follwed by a decrease from vitellogenic to postvitellogenic oocytes. In the testis, *vas* signals are strong in spermatogonia and decrease in early primary spermatocytes. No *vas* RNA expression is evident in either diplotene primary spermatocytes, secondary spermatocytes, spermatids or spermatozoa. The observed differences in *vas* RNA expression suggest a differential function of *vas* in the regulation of meiotic progression of female and male germ cells. We have also generated medaka (*Oryzias latipes*) transgenic lines with green fluorescent protein (GFP) fluorescence controlled by the regulatory regions of the *olvas* gene in the germ cells (Fig. 1). The intensity of GFP fluorescence increases dramatically in PGCs located in the ventrolateral region of the posterior intestine around stage 25 (the onset of brood circulation). Whole-mount *in situ* hybridization and monitoring of ectopically located cells by GFP fluorescence suggest that 1) the increase in zygotic *olvas* expression occurs after PGC specification and 2) PGCs can maintain their cell characteristics ectopically after stages 20-25. The GFP expression persists throughout the later stages in the mature ovary and testis.

In tilapia, mitosis of germ cells begins around 10 days post-hatching in genetic females, but can not be

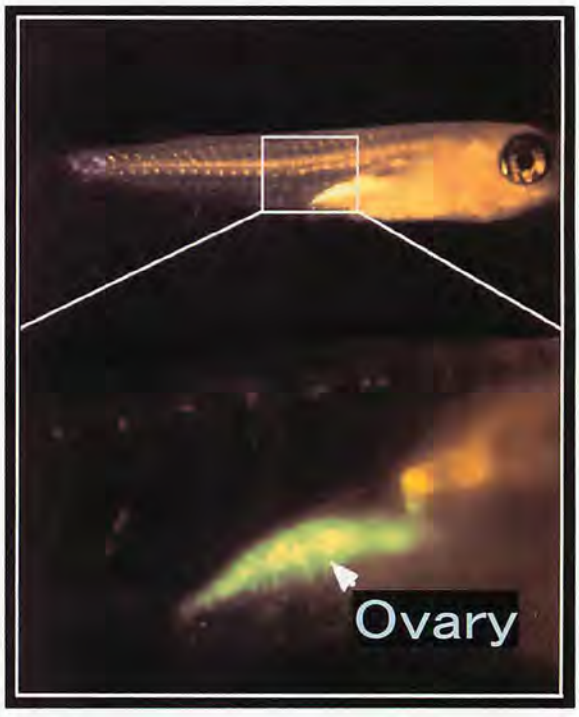

Fig. 1 GFP fluorescent germ cells in the ovary of female medaka, *Oryzias latipes*. The ovary contains numerous oocytes which can be seen as a green fluorescence.

confirmed until after sex differentiation in testes of genetic males. During the course of morphological sex differentiation, the behavior of somatic cells in the gonad is often sex-specific. In these cases, the sex of the gonad is easily distinguishable. In tilapia, gonadal sex is morphologically distinct at 20-25 days post-hatching. Ovarian differentiation is initially marked by storomal elongation of the gonad for the formation of the ovarian cavity. Testicular differentiation is characterized by the appearance of a narrow space in the storomal tissue representing the formation of the efferent duct. Steroid-producing cells in ovaries, but not testes, at the undifferentiated and differentiating stages express all of the steroidogenic enzymes required for estradiol-17β biosynthesis from cholesterol. These results strongly suggest that endogenous estrogens act as the natural inducers of ovarian differentiation in tilapia. This hypothesis is further supported by evidence of masculinization of genetic female tilapia by inhibition of estrogen synthesis using an inhibitor of cytochrome P450 aromatase. In contrast, the ability of steroid-producing cells to synthesize steroid hormones in the testes only appears at the time of testicular differentiation. Transcripts of estrogen receptors (ER) α and β first appear in both female and male gonads of fry 10-15 days post-hatching. ERα and ERβ exhibit different expression patterns suggesting differential roles of ERα and ERβ in estrogen action on gonadal sex differentiation. We have isolated two DM (*Doublesex/Mab-3* DNA-binding mitif)-domain cDNAs from tilapia testis and ovary, named *DMRT1* and *DMO*, respectively. *DMRT1* is expressed only in Sertoli cells and *DMO* is detected only in oocytes by *in situ* hybridization. The correlation between expression of *DMRT1* and testicular differentiation of both normal XY-male and sex reversed XX-males suggest that *DMRT1* is a candidate testis determining gene in tilapia. In contrast, abundant *DMO* expression in pre- and early vitellogenic oocytes in XX- and sex reversed XY-females indicates a relationship between *DMO* and oocyte growth.

II. Endocrine regulation of spermatogenesis

Spermatogenesis is an extended process of differentiation and maturation of germ cells resulting in haploid spermatozoa. The principal stimuli for vertebrate spermatogenesis are thought to be pituitary gonadotropins and androgens. However, the mechanisms of action of these hormones remain unresolved. Using an organ culture system for eel testes consisting of spermatogonia and inactive somatic cells, 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 the production of activin B. 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. cDNAs encoding two androgen receptors (ARα and ARβ) have been cloned, for the first time in any vertebrates, from eel and tilapia testes. *In situ* hybridization reveals that although both AR mRNAs are present in eel testes prior to HCG injection, only ARα transcripts increase during HCG-induced spermatogenesis suggesting that ARα and ARβ play different roles in spermatogenesis. Activin B binds to activin type I and II receptors on spermatogonia to stimulate *de novo* synthesis of G1/S cyclins and CDKs leading to the initiation of mitosis. Interestingly, cyclin A1 transcripts are first detected in primary spermatocytes during HCG-induced spermatogenesiss in eel testes suggesting an important role for cyclin A1 in the progression to meiosis of male germ cells. Overexpression of GFP-labeled cyclins A and E in type A spermatogonia induces spermatogonial proliferation, followed by meiosis

III. Endocrine regulation of oocyte maturation

Meiotic maturation of fish oocytes is induced by the action of maturation-inducing hormone (MIH). 17α,20β-dihydroxy-4-pregnen-3-one (17α,20β-DP) has been identified as the MIHs of several fish species. The interaction of two ovarian follilce cell layers, the thecal and granulosa cell layers, is required for the synthesis of 17α,20β-DP. The theal layer produces 17α-hydroxyprogesterone that is converted to 17α,20β-DP in granulosa cells by the action of 20β-hydroxysteroid dehydrogenase (20β-HSD). The preovulatory surge of LH-like gonadotropin is responsible for the rapid expression of 20β-HSD mRNA transcripts in granulosa cells during oocyte maturation. Two types of 20β-HSD cDNAs are expressed in ovarian follicles of rainbow trout (*Oncorhynchus mykiss*). Recombinant proteins produced by expression in *E. coli in vitro* show that one (type A) has 20β-HSD activity but that the other (type B) does not. Among the three distinct residues between the protein products encoded by the two cDNAs, two residues (positions 15 and 27) are located in the N-terminal Rossmann fold, the coenzyme binding site. We have then generated mutants by site-directed mutagenesis at the following positions: MutA/I15T, MutB/T15I, and MutB/Q27K. Enzyme activity of wild-type A is abolished by substitution of Ile-15 by Thr (MutA/I15T). Conversely, enzyme activity is acquired by the replacement of Thr-15 with Ile in type B (MutB/T15I). MutB/T15I mutant shows properties similar to the wild-type A in every aspect tested. Mutation MutB/Q27K has only partial enzyme activity, indicating that Ile-15 plays an important role in enzyme binding of cofactor NADPH.

17α,20β-DP induces oocyte maturation by acting on a pertussis toxin-sensitive G-protein-coupled membrane receptor. The early steps of 17α,20β-DP action involve the formation of downstream mediator of this steroid, the maturation-promoting factor or metaphase-promoting factor (MPF) consisting of cdc2 kinase and cyclin B. 17α,20β-DP induces oocytes to synthesize cyclin B which activates a preexisting 35-kDa cdc2 kinase via phosphorylation of its threonine 161 by a threonine kinase (MO15), thus producing the 34 kDa

active cdc2. The function of the MOS/MAPK pathway during 17α,20β-DP-induced oocyte matration has been investigated using goldfish oocytes. Mos is absent in immature oocytes. It appears before the onset of germinal vesicle breakdown (GVBD), increases to a maximum in mature oocytes arrested at MII and disappears after fertilization. MAPK is activated after Mos synthesis but before MPF activation, and its activity reaches maximum at MII. Injection of either *Xenopus* or goldfish *c-mos* mRNA into one blastomere of 2-cell-stage *Xenopus* and goldfish embryos induces metaphase arrest, suggesting that goldfish Mos has a cytostatic factor (CSF) activity. Injection of constitutively active *Xenopus c-mos* mRNA into immature goldfish oocytes induced MAPK activation, but neither MPF activation nor GVBD occurs. Conversely, the injection of goldfish *c-mos* antisense RNA inhibits both Mos synthesis and MAPK activation in the 17α,20β-DP-treated oocytes, but these oocytes undergo GVBD. These results indicate that the Mos/MAPK pathway is not essential for initiating goldfish oocyte maturation despite its general function as a CSF.

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Cell and tissue differentiation proceeds systematically based on a number of gene expressions that commence successively along with the passage of time. As the consequence, a fertilised egg develops into a variety of tissues and organs comprising specialised cells in terms of their structures and functions. Accordingly, it is no doubt that investigation of the mechanisms underlying the cell and tissue-specific gene expression at a molecular base is essential for obtaining a proper understanding of the process of tissue differentiation. In our division of Cell Differentiation, two distinct but closely correlated studies at their central concepts have proceeded. One of them is the study for comprehensive understanding of sex differentiation at the levels from the gonad function to reproductive behavior, and the other is the study focussing on head formation through characterization of the function of head organizer.

I. Gene regulatory cascade in the steroidogenic tissue differentiation

When a differentiation process of a tissue is considered, it is reasonable to assume a tissue-specific gene regulatory cascade in which certain genes encoding transcription factors are involved as the critical components. In the cascades required for adrenal and gonadal differentiation, Ad4BP/SF-1 is locates upstream of tissue-specific genes, including the steroidogenic *CYP* genes, and locates downstream of other transcription factors regulating the *Ad4BP/SF-1* gene. Considering that the cascade flows from upstream to downstream through out the tissue differentiation and Ad4BP/SF-1 is an essential transcription factor in the gonadal cascade, identification of the components consisting the cascade as well as their genetical relationship is essential for fully understanding the mechanisms of the tissue differentiation.

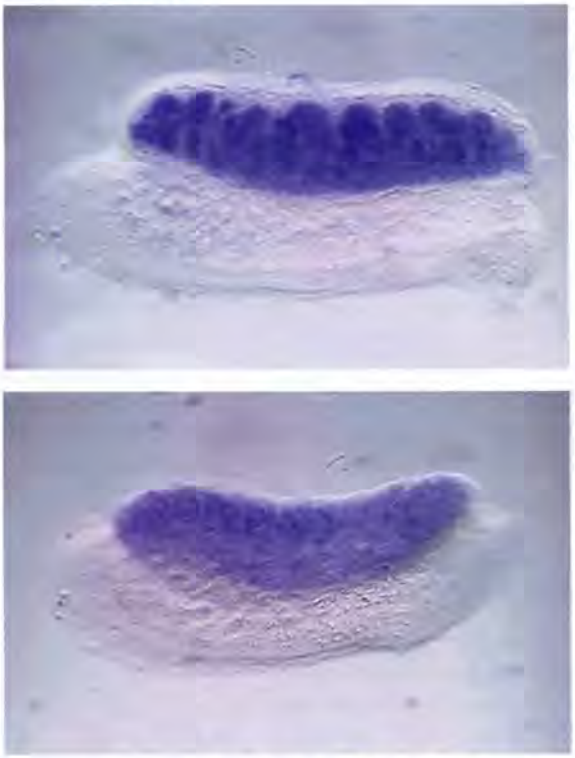
Based on the aspect above, the regulatory region of the *Ad4BP/SF-1* gene has been analysed *in vivo* by making transgenic mice. However, our *in vivo* study in a recent few years has not yet been successful, probably because the regulatory region locates far upstream or far downstream from the structural gene of *Ad4BP/SF-1*. On the contrary, our in vitro study with cultured cells provided a novel mechanism regulating Ad4BP/SF-1 gene, in which an activating signal from one of growth factors, Wnt, is implicated. Although the fine mechanism has been under investigation, the study from this aspect will give us a novel insight into gonad differentiation.

Dax-1 is another transcription factor of our interest, which is also implicated in the steroidogenic tissue differentiation. Our previous study revealed that the factor acts as a suppressor of Ad4BP/SF-1. However, regulation of the suppressive effect has remained to be clarified at the molecular level. We recently uncovered the function of the amino terminal half of Dax-1 containing a unique repeated sequence. When Dax-1 functions as the suppressor, a certain amino acid sequence in the repeats is essential for interaction with target nuclear receptors, and thereby the transcription mediated by the nuclear receptors are largely inhibited. Although it remains unclear how the inhibition activity is regulated in a variety of physiological conditions, this interaction gave us a cue to address it.

In addition to these transcription factors, Sox-9, Wt-1, Emx-2, and GATA-4 are known to be implicated in the gonad development. In order to isolate novel factors interacting with all these transcription factors above, yeast two-hybrid screening has been performed using a cDNA library constructed with an mRNA prepared from mouse fetal gonads. Extensive screening resulted in isolation of molecules including coactivators and other type of transcription factors. Some of them are novel factors and have interesting structures making us to anticipate novel regulations of the transcription. Distributions and functions of these interacting molecules have been examined.

II. Sex-differentiation observed in adrenal cortex

Our previous study indicated that Ad4BP/SF-1 is expressed in all three zones of the adrenal cortex while Dax-1 is expressed in only outer zone, the zona glomerulosa, but not in inner zones, the zona fasciculata and reticularis. However, this distribution revealed by immunohistochemistry was quite distinct from that obtained with *in situ* hybridization. To explain the



Expression of Factor X, which was isolated by two-hybrid screening as a molecule interacting with Ad4BP/SF-1. Factor X is expressed in the developing gonads (E12.5 testis (upper) and ovary (lower)).

discrepancy between the two methods, close examination was carried out with a series of adrenal cortex of both sexes at several developing stages from fetal to adult. Although the distribution of Dax-1 was identical between the two sexes before puberty, distinct distribution was clearly observed after sexual maturation. This sexually dimorphic expression disappeared by castration and emerged again after testosterone replacement. Injection of testosterone into female mice make the expression profile altered into that of male. Taken together, our in vivo studies suggested that androgen and its receptor downregulate Dax-1 gene transcription, which is interestingly inconsistent with a common understanding that androgen receptor activates target gene transcription in a ligand dependent manner. The mechanism of suppression of the Dax-1 expression by androgen receptor and its ligand is further investigating at a molecular level.

III. Molecular mechanism for head formation

It has been clarified that the anterior visceral endoderm (AVE) and mesendoderm (AME) plays critical roles for head formation by the induction of specific gene expressions in anterior neuroectoderm. Lim1, a LIM homeodomain-containing transcription factor, is expressed at the AVE and primitive streak-derived cells during gastrulation. Since a striking function of Lim1 was revealed by making gene disrupted mice which displayed headless phenotype, molecular events after commencing the *Lim1* expression have been investigated through identifying a set of downstream genes of the transcription factor. For identification, differential screen was performed using subtraction between cDNA pools generated from a small number of cells corresponding to the AVE of wild and KO individuals. A number of candidate genes showing a loss of expression in the KO and a close correlation with Lim1 in terms of their expression profiles have been isolated so far. The functions of these genes are investigating extensively by gene disruption studies.

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The complex morphogenesis of organisms is achieved by consecutive cell-to-cell interactions of germ layers as well as tissues during development. Recent studies suggest that polypeptide growth factors (PGFs) are essential component controlling such intercellular communications in a variety of organisms. These cell communications via PGFs are regulated by a number of processes including secretion, activation, diffusion, reception by specific receptors and intracellualr signaling. In addtion to secretory factors, transcription factors which act cell-autonomously have critical roles in the determination of cell fates. Our main interest is to know how pattern formation in morphogenesis is regulated by PGFs and transcription factors during development. We address this problem using several model animals, including frog, fly, acidians and nematode with the view of extracellular and intracellular signaling, employing embryology, genetics, molecular and cellular biology and biochemistry. In addition, we are currently introducing micro/macroarray technology to understand precise genetic program controlling early development.

I. Role of Xmsx-1 as a negative regulator of head formation

Embryos are patterned along dorso-ventral (DV) axis by the action of PGFs. Signaling triggered by PGFs leads to the activation of their target genes. Several homeobox genes are induced in response to PGFs in early *Xenopus* development. In particular, Xmsx-1, an amphibian homologue of vertebrate Msx-1, is well characterized as a target gene of bone morphogenetic protein (BMP). In order to clarify molecular basis for ventalization by BMP and in vivo significance of Msx-1, we examined whether Xmsx-1 activity is required in the endogenous ventralizing pathway, using a dominant-negative form of Xmsx-1 (VP-Xmsx-1). The VP-Xmsx-1 induced a secondary body axis, complete with muscle and neural tissues, when the fusion protein was overexpressed in ventral blastomeres. Interestingly, a more potent dominat negative Xmsx-1 induced ectopic head with eyes and cement gland. These results suggest that Xmsx-1 activity is necessary for both mesoderm and ectoderm to be ventralized and that head formation may represent a default state of BMP/Msx1 activity. We are currently investigating the molecular machanism of head repression in ventral side of embryo.

II. Genetic dissection of TGF-β/BMP signaling utilizing *Drosophila* model system

Fruit fly (*Drosophila melanogaster*) is one of the ideal model organisms to elucidate certain intracellular signal transduction pathways by genetic methods. A Drosophila BMP homolog, Decapentaplegic (Dpp) plays crucial roles in a number of developmental events including patterning of the adult wing. We have established two transgenic lines that express activated Dpp/BMP receptors. These mutants exhibit stable Dpp-gain-of-function phenotypes in the wing. One of the transgenic lines, *tkv-CA::71B*, expressed constitutively activated Tkv (Dpp type-I receptor) shows ectopic wing vein formation in the entire wing blade (Fig. 1A). Another line, *ALK2::69B*, expresses activated vertebrate BMP type-I receptor and shows abnormal wing outgrowth toward anterior and posterior direction (Fig. 1B). These phenotypes are sensitive to the gene dosage of the Dpp signal component such as Mad (Fig. 1C). Combinatorial screening has been done utilizing a Deficiency-kit (minimum set of the deficiency mutant lines that covers about 70 % of the Drosophila genome) and P-element insertion lethal stocks from public stock centers. We isolated 19 dominant suppressers to either or both *tkv-CA::71B* and *ALK2::69B*. We named these mutants *Suppresser of constitutively activated Dpp signaling* (*Scad*). Alleles of punt, Mad, shn and dCrebA were found in the isolated Scad mutants. It has been reported that the function of these genes are involved in the Dpp signaling. We focused to further analyze two novel loci, Scad67 and *Scad78* at a molecular level. Candidate genes for *Scad67* and *Scad78* were isolated. *Scad67* encodes a novel putative nuclear protein of a putative Zn-finger motif with a weak homology to the PIAS family transcriptional regulators. *Scad78* encodes a homolog of vertebrate putative transcription co-factor TRAP240. Mosaic analyses of *Scad67* and *Scad78* mutants suggest that these putative transcriptional regulators are involved not only in the Dpp signal transduction but also in other signal transduction cascades.

We have also been interested in the function of a MAPKKK class protein kinase, TGF-β activated

kinase1 (TAK1). TAK1 was originally isolated as a downstream signal component of TGF-β and BMP receptors. TAK1 function was extensively studied in cultured cells, however, the in vivo function of TAK1 has not yet been fully understood. To dissect in vivo function of TAK1, we generated transgenic flies which express an activated form and a dominant negative form of TAK1s (Drosophila and mouse TAK1s) in the fly visual system. Ectopic activation of TAK1 signaling led to ectopic apoptosis and resulted in small eye phenotype. Genetic and biochemical analyses indicated that the JNK signaling pathway is specifically activated by TAK1. Loss-of-function analysis using dominant negative TAK1s suggested that TAK1 participates in cell movement, cell shape control and apoptosis in *Drosophila*. Recently candidates of *dTAK1* mutants were isolated. Phenotypic analysis of these mutants is ongoing.

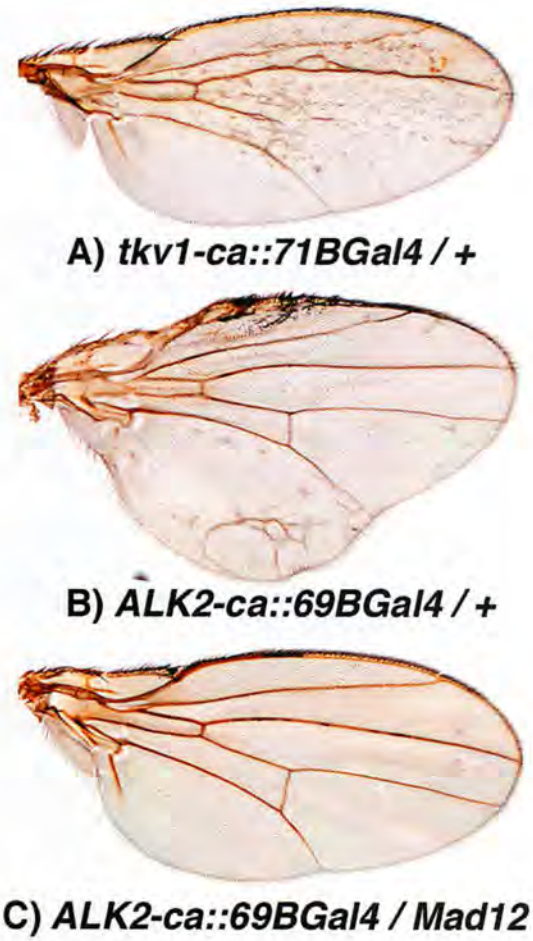

Figure 1. The wing phenotypes genetic interaction utilized for the screening. Expression of activated Dpp/BMP type-I receptors are resulted in the stable wing phenotypes (A, B). These phenotype is sensitive to the dosage of Dpp signal comonent *Mad* (C).

III. Brachyury downstream notochord differentiation in the ascidian embryo

Ascidians, urochordates, are one of the three chordate groups, and the ascidian tadpole is thought to represent the most simplified and primitive chordate body plan. It contains a notochord, which is a defining characteristic of chordate embryo composed of only 40 cells. To understand the morphogenesis in this simple system, we have focused on a gene, *Brachyury*, which is known to play an important role in the notochord development. In ascidian, *Brachyury* is expressed exclusively in the notochord and the misexpression of the *Brachyury* gene (*Ci-Bra*) of *Ciona intestinalis* is sufficient to transform endoderm into notochord. This gene encodes a sequence-specific activator that contains a T-box DNA-binding domain, and in vertebrates, it is initially expressed throughout the presumptive mesoderm and gradually restricted to the developing notochord and tailbud. The phenotype of the *Brachyury* mutants in mice and zebrafish revealed that this gene is essential for notochord differentiation. Our goal is to elucidate the down stream pathway of this important gene in ascidian in order to set the stage for understanding not only the formation and function of the notochord but how this important structure has evolved. We conducted the subtractive hybridization screens to identify potential *Brachyury* target genes that are induced upon *Ci-Bra* overexpression. Out of 501 independent cDNA clones that were induced cDNAs, 38 were specifically expressed in notochord cells (Fig. 2). We characterized 20 of them by determining the complete nucleotide sequences and *in situ* hybridization analyses which show the spatial and temporal expression patterns of the cDNAs. These potential *Ci-Bra* downstream genes appear to encode a broad spectrum of divergent proteins associated with notochord formation and function.

IV. TGF-β family in nematode

We have previously shown that CET-1/DBL-1, a member of TGF-β superfamily regulates body length in *C. elegans*. To understand molecular mechanism of body length regulation by the secreted factor, we aimed to identify target genes regulated by CET-1/DBL-1 signaling using a cDNA-based array analysis followed by functional analysis with double stranded RNAi. We identified yk298h6 to be one of genes suppressed in CET-1/DBL-1 overexpressing worm with a shortened body length. Disruption of yk298h6 resulted in long worm, suggesting that it encodes a negative regulator of body length.

yk298h was then mapped to, and shown to be identical with, *lon-1*, a known gene that affects body length. LON-1 encodes a protein with a motif sequence that is conserved from plants to human. Expression studies confirm that LON-1 is repressed CET-1/DBL-1 suggesting that LON-1 is a novel downstream component of the *C. elegans* TGF-β.

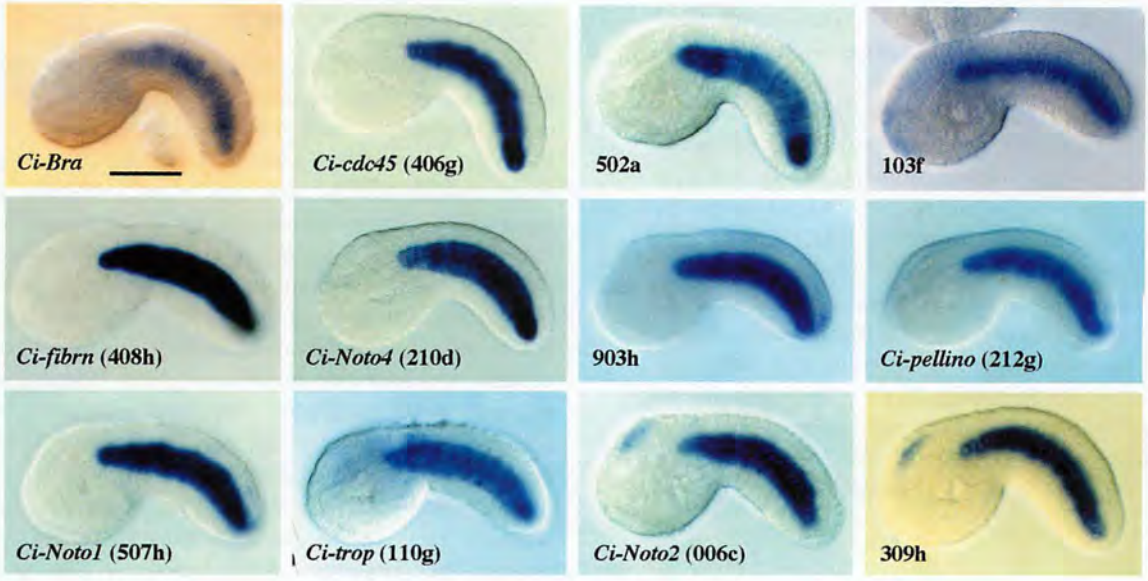

Figure 2. Expression of *Ci-Bra* and its downstream notochord genes in *C. intestinalis* tailbud embryos revealed by whole-mount *in situ* hybridization. Scale bar, 100 μm.

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DIVISION OF DEVELOPMENTAL BIOLOGY (ADJUNCT)

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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 pattern of gene expression in various parts of the plant body. Our research attention is focused to elucidate the mechanisms involved in the regulation of gene expression in response to sugars and the role of such regulation in the growth and development of plants.

During the growth of plants, new organs develop as carbohydrate sink, and many vegetative organs show sink-to-source transition after their maturation. Sugar affects many aspects of these organ developments in plants. To obtain insights into the role of sugar-regulated gene expression in the growth and development of plants, we took genetic approach using *Arabidopsis thaliana*. To aid this purpose, we established more than 7,000 independent lines of Arabidopsis plants transformed with T-DNA containing multiple copies of the enhancer sequence. Among these lines, we screened for mutants that not only showed defects in the development of leaves or anomalies in the flowering time but also showed the altered patterns of the sugar-inducible expression of a gene for β-amylase (*Atβ-Amy*). More than 20 such mutants, designated as *uns* (*unusual sugar response*), were isolated. Similar to *lba1* and *hba1* mutants of Arabidopsis isolated previously [Mita, S., Murano, M., Akaike, M. and Nakamura, K., *Plant J.* **11**: 841-851 (1997); Mita, S., Hirano, H. and Nakamura, K. *Plant Physiol.* **114**: 575-582 (1997)], the sugar-inducible expression of *Atβ-Amy* in *uns* mutants was either significantly reduced or enhanced compared to that in the wild-type plants depending on the mutant line.

In several of *uns* mutants, we could identify mutated genes responsible for the mutant phenotypes. One of them, *UNS6*, codes for a protein that shows amino acid sequence similarity to a gene of *Saccharomyces cerevisiae*, which is essential in yeast and has been suggested to play a role in the assembly of the transcription preinitiation complex. The *uns6* mutant plants show enhanced sugar-inducible expression of *Atβ-Amy* and several other genes and defects in leaf development. However, despite *UNS6* is a single-copy gene in Arabidopsis, mutant plants do not show severe developmental defects other than leaf development. Furthermore, the sugar-responsible expression of several other genes is not affected in the mutant plants. Thus, *UNS6* may have some unique function other than a general role in transciption initiation in Arabidopsis.

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DEPARTMENT OF REGULATION BIOLOGY

Chairman: Masaharu Noda

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

DIVISION OF MOLECULAR NEUROBIOLOGY

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	Shigemi Takami (~ Jun. 23, 2000)
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	Hiroyuki Kawachi[4] *(~ Aug. 31, 2000)*
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	Takeshi Ohkawara
	Hiroo Takahashi (Apr. 1, 2000 ~)
	Chika Saegusa (~ Sept. 30, 2000)
	Takuji Yoshitomi (Apr. 1 ~ Oct. 31, 2000)
Visiting Scientist:	*Ikuko Watakabe (Sept. 18, 2000 ~)*
JST Technical Staff:	*Masae Mizoguchi*
	Megumi Goto
	Minako Ishida (~ Dec. 31, 2000)

We have been studying the molecular and cellular mechanisms underlying the development and functioning of the vertebrate central nervous system. We are currently searching for and analyzing the functions of molecules involved in various cellular events in brain morphogenesis and brain function, such as generation of neuroblasts, migration to form the laminar structure and various nuclei, elongation and path-finding of neural processes, the formation and refinement of specific connections between neurons, and also synaptic plasticity. We have been using various techniques including molecular biology, biochemistry, immunological methods, neuroanatomy, cell and organotypic culture, embryo manipulation (gene transfer and gene targeting), and behavioral analysis.

I. Molecular mechanism of the retinotectal projection

Topographic maps are a fundamental feature of neural networks in the nervous system. Understanding the molecular mechanisms by which topographically ordered neuronal connections are established during development has long been a major challenge in developmental neurobiology. The retinotectal projection of lower vertebrates including birds has been used as a readily accessible model system. In this projection, the temporal (posterior) retina is connected to the rostral (anterior) part of the contralateral optic tectum, the nasal (anterior) retina to the caudal (posterior) tectum, and likewise the dorsal and ventral retina to the ventral and dorsal tectum, respectively. Thus, images received by the retina are precisely projected onto the tectum in a reversed manner. In 1963, 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, and Eph families of receptor tyrosine kinases and their ligands were recently identified as candidates for such positional labels, the molecular mechanism of retinotectal map formation remains to be elucidated.

Since 1993, we have been devoting our efforts to searching for topographic molecules which show asymmetrical distribution in the embryonic chick retina. In the first-round screening using a cDNA subtractive hybridization technique, we identified two winged-helix transcriptional regulators, CBF-1 and CBF-2, expressed in the nasal and temporal retina, respectively. Furthermore, our misexpression experiments using a retroviral vector showed that these two transcription factors determine the regional specificity of the retinal ganglion cells, namely, the directed axonal projections to the appropriate tectal targets along the anteroposterior axis. To further search for topographic molecules in the embryonic retina, we next performed a large-scale screening using a new cDNA display system called Restriction Landmark cDNA Scanning (RLCS). With the assistance of a computer image-processing software, we successfully identified 33 molecules along the nasotemporal axis and 20 molecules along the dorsoventral axis, with various asymmetrical expression patterns in the developing retina. We have elucidated the primary structures of all these cDNA clones and examined their expression patterns during development. These included many novel molecules together with the known molecules: transcription factors (CBF-2, COUP-TFII, etc.), receptor proteins (EphA3, EphB3, etc.), secretory factors, intracellular proteins, and so on.

Among them, we recently identified a novel retinoic acid (RA)-generating enzyme, RALDH-3, which is specifically expressed in the ventral region of the retina, together with a dorsal-specific enzyme RALDH-1. In chick and mouse embryos, the expression of *Raldh-3* is observed first in the surface ectoderm overlying the ventral portion of the prospective eye region earlier than that of *Raldh-1* in the dorsal retina, and then the *Raldh-3* expression shifts to the ventral retina (Fig. 1). Furthermore, we found that *Raldh-3* is a downstream target of *Pax6* which is known to be the master gene for the eye development in many species. It is well known that RA is essential for the eye development. These results suggest that RALDH-3 and RALDH-1 are the key enzymes for the dorsoventral patterning at the early stage of the retinal development.

Currently, with respect to the identified topographic molecules, we are conducting over- and misexpression experiments using viral vectors and *in ovo* electroporation to elucidate their molecular functions. We expect that our studies will lead to elucidation of the molecular mechanism underlying the retinal patterning and topographic retinotectal projection, and ultimately to uncovering the basic principles for establishing complicated

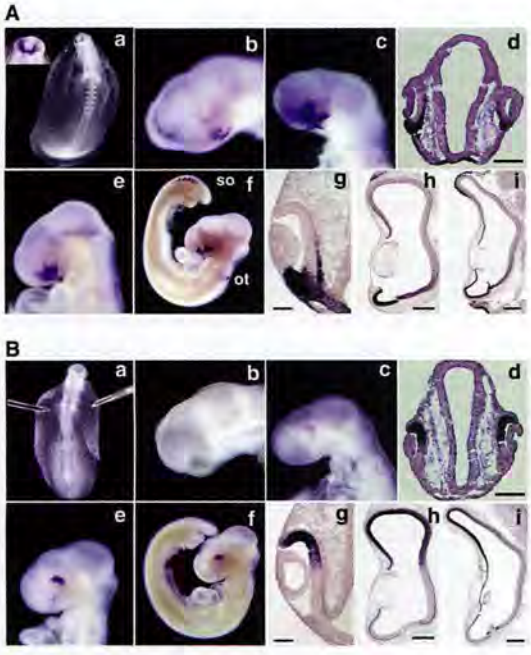

Fig.1 Expression patterns of *Raldh-3* (A) and *Raldh-1* (B) during chick development
All embryos were hybridized with DIG-labeled antisense riboprobes. Developmental stages of embryos are stage 10 (A, a), stage 11 (B, a), stage 12 (A and B, b), stage 14 (A and B, c, d), stage 16 (A and B, e), stage 20 (A and B, f, g), E6 (A and B, h), and E8 (A and B, i), respectively. The expression of *Raldh-3* is observed first in the surface ectoderm overlying the ventral portion of the prospective eye region earlier than that of *Raldh-1* in the dorsal retina. Coronal sections are oriented in dorsal up and ventral down. so, somites; ot, otic vesicle. Scale bars: 100 μm (A and B, d, g, h); 200 μm (A and B, i).

but extremely precise neural networks.

II. Functional roles of protein tyrosine phosphatase ζ

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. Many types of receptor-type protein tyrosine phosphatases (RPTP) have been cloned and characterized. In 1994, we found that PTPζ/RPTPβ, a nervous system-rich 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. PTPζ is expressed from the early developmental stage to the adulthood. This suggests that this molecule plays variegated roles in the brain development and brain functions.

In an attempt to reveal the signal transduction mechanism of PTPζ, we first tried to identify the ligand molecules of this receptor. We found that PTPζ binds pleiotrophin/HB-GAM and midkine, closely related heparin-binding growth factors which share many biological activities. The chondroitin sulfate portion of PTPζ is essential for the high affinity binding (Kd = ~0.25 nM) to these growth factors, and removal of chondroitin sulfate chains results in a marked decrease of binding affinity (Kd = ~13 nM). We further revealed that chondroitin sulfate interacts with Arg^{78} in Cluster I, one of the two heparin-binding sites in the C-terminal half domain of midkine. This is the first demonstration that chondroitin sulfate plays an important regulatory role in growth factor signaling.

Next, we examined the roles of pleiotrophin/midkine-PTPζ interaction in neuronal migration using the glass fiber assay and Boyden chamber cell migration assay. Pleiotrophin and midkine on the substratum stimulated migration of neurons in these assays. 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 to the culture medium strongly suppressed this migration. Experiments using various midkine mutants with various affinities for PTPζ indicated that the strength of binding affinities and the neuronal migration-inducing activities are highly correlated. These results suggest that PTPζ is involved in migration as a neuronal receptor for pleiotrophin and midkine.

In order to reveal the intracellular signaling mechanism of PTPζ, we performed yeast two-hybrid screening using the intracellular region of PTPζ as bait. We found that PTPζ interacts with PSD-95/SAP90 family members, SAP102, PSD-95/SAP90 and SAP97/hDlg, which are concentrated in the central synapses mediating protein-protein interactions to form large synaptic macromolecular complexes. Here, the C-terminus of PTPζ binds to PSD-95/SAP90 proteins through the second PDZ domain. This suggests that PTPζ is involved in the regulation of synaptic function. However, PSD-95/SAP90 family members are not likely to be the substrate for PTPζ because this family members are not tyrosine-phosphorylated.

To identify the substrate molecules of PTPζ, we have recently developed the yeast substrate-trapping system. This system is based on the yeast two-hybrid system with two essential modifications: conditional expression of v-src to tyrosine-phosphorylate the prey proteins and screening using a substrate-trap mutant of PTPζ as bait (Fig. 2). Using this system, we identified GIT1/Cat-1 as a PTPζ substrate. PTPζ and GIT1/Cat-1 were colocalized in the growth cones of mossy fibers from pontine explants and in the ruffling membranes and processes of B103 neuroblastoma cells (Fig. 2). Moreover, pleiotrophin increased tyrosine phosphorylation of GIT1/Cat-1 concomitantly with its recruitment to the stimulated PTPζ in B103 cells. It is known that Cat-1 regulates Pak, a serine threonine kinase which serves as a target for the small GTP-binding proteins, Cdc42 and Rac, and is implicated in a wide range of cellular events

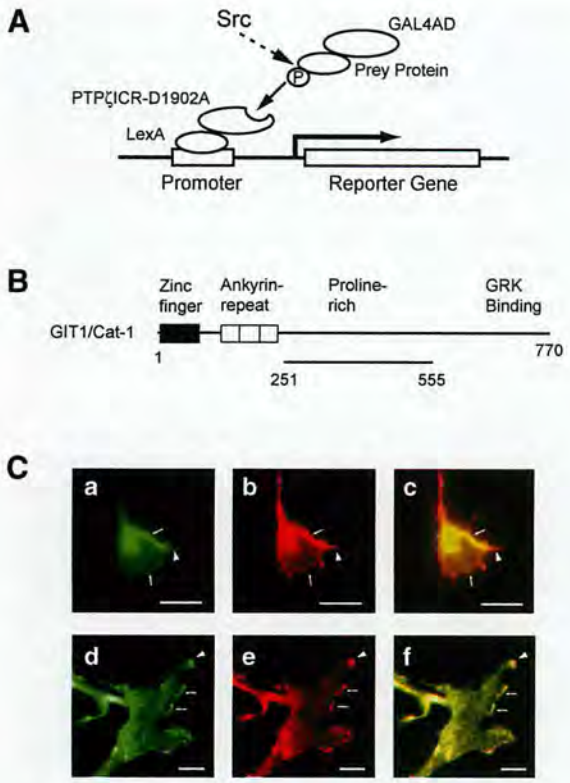

Fig. 2 The yeast substrate-trapping system and GIT1/Cat-1
(A) In the presence of 1 mM methionine when v-src is not induced, only the standard two-hybrid bindings occur. In the absence of methionine, prey proteins (substrates for PTPζ) could be phosphorylated by the induced v-src, and trapped by the bait consisting of the whole intracellular domain with an Asp1902Ala mutation (PTPζ ICR-D1902A). This complex formation leads to activation of transcription of the reporter genes, *HIS3* and *LacZ*. We performed screening in two steps and selected the colonies which showed an increase in blue color development upon induction of v-src.
(B) GIT1/Cat-1 contains a zinc-finger region, three ankyrin-repeat regions, a potential SH3-binding site and a GRK binding site. The region encoded by the clone isolated by the yeast substrate-trapping system is shown with a solid line underneath.
(C) The mossy fibers extending from pontine explants were doubly immunostained with anti-GIT1/Cat-1 (a) and anti-6B4 PG (b) antibodies. The combined image is shown in (c). The colocalization of both proteins was evident at the peripheral regions (arrows) and filopodial processes (arrowhead) of the growth cones. B103 cells were doubly immunostained with anti-GIT1/Cat-1 (d) and anti-6B4 PG (e) antibodies. The combined image is shown in (f). GIT1/Cat-1 and PTPζ were colocalized in the processes (arrowhead) and ruffling membranes (arrows) of the cells. Scale bars: 5 μm (a-c), 10 μm (d-f).

including the cell adhesion and cell morphological change. Pleiotrophin, PTPζ and GIT1/Cat-1 might regulate the neuronal migration and neurite extension by controlling the Pak signaling pathway.

To further study the physiological function of PTPζ *in* vivo, we generated PTPζ-deficient mice in which the PTPζ gene was replaced with the LacZ gene in 1997. By investigating the expression of LacZ in heterozygous mutant mice, we demonstrated that neurons as well as astrocytes express PTPζ in the central nervous system. We are currently studying the phenotype of PTPζ-deficient mice biochemically, anatomically, physiologically and ethologically, and have already found abnormalities in behavior, learning and memory, etc.

III. Physiological roles of Na_v2 ion channel

Voltage-gated sodium channels (NaChs) are responsible for generating action potentials in excitable cells and play many important physiological roles. Cloning of NaChs revealed marked conservation in the primary structures that underlies their functional similarity. Thus, all cloned NaChs had been grouped into a single gene family. However, recently, novel NaChs, human $Na_v2.1$, mouse $Na_v2.3$ and rat NaG/SCL11, were cloned from inexcitable cells such as glial cells. These molecules closely resemble each other but are divergent from the previously cloned sodium channels including the regions involved in activation, inactivation and ion selectivity. Thus, these molecules should be grouped into a new subfamily of NaChs (Na_v2). To date, Na_v2 channels have not been expressed in a functionally active form using *in vitro* expression systems, and therefore the functional properties of these NaChs are not yet clear.

To clarify the physiological function and the cells expressing Na_v2 channels *in vivo*, we generated knockout mice in which Na_v2 channel gene was replaced with the *LacZ* or *neo* gene by gene targeting. In this study, we found that mouse $Na_v2.3$ and rat NaG/SCL11 genes are species counterparts. Analysis of the targeted mice allowed us to identify Na_v2-producing cells by examining the *lacZ* expression. Besides in the lung, heart, dorsal root ganglia and Schwann cells in the peripheral nervous system, *Na_v2* was expressed in neurons and ependymal cells in restricted areas of the central nervous system, particularly in the circumventricular organs that are involved in body-fluid homeostasis (see Fig. 1A in the part of Center for Transgenic Animals and Plants). Under water-depleted conditions, the mutant mice showed markedly elevated *c-fos* expression in neurons in the subfornical organ and organum vasculosum laminae terminalis compared with wild-type animals. This suggests that these neurons are in a hyperactive state in the *Na_v2*-null mice. Moreover, the null mutants showed abnormal intakes of hypertonic saline under both water- and salt-depleted conditions (Fig. 1B, *ibid.*). These findings suggest that the Na_v2 channel plays an important role in the central sensing of the body-fluid sodium level, and in regulation of salt intake behavior. We are currently examining the electrophysiological property of the cells derived from Na_v2-null mutant mice and wild-type mice to gain insight into the channel property of Na_v2.

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DIVISION OF CELLULAR REGULATION

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The research efforts of this division are devoted to developing a full understanding of the molecular mechanisms that allow plants to acclimate to and tolerate various kinds of stress that arise from changes in environmental conditions, with particular emphasis on extreme temperatures and high salinity. In 2000, significant progress was made in the following areas as a result of studies of higher plants and cyanobacteria.

I. A sensor for low-temperature signals

Low temperature is an important environmental factor that affects the growth and behavior of all living organisms. Many organisms are able to acclimate to low temperatures by regulating the expression of various genes. However, mechanisms for the perception and transduction of low-temperature signals remain to be characterized. In the cyanobacterium *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis*), expression of the genes for fatty acid desaturases is enhanced by low temperature. Moreover, the decrease in membrane fluidity that occurs upon a downward shift in temperature appears to be a primary signal for induction of the expression of the genes for these desaturases.

In various bacteria, yeast and plants, physical and chemical stimuli are perceived by a group of proteins that includes histidine kinases. We attempted to disrupt individually all the putative genes for histidine kinase in *Synechocystis* and then we examined the subsequent response to low temperature of the promoter of the *desB* gene, which encodes the ω3 fatty acid desaturase, by monitoring the activity of a reporter gene for luciferase. Among 41 mutant lines with disrupted genes for histidine kinase, we identified two mutants, in which, respectively, the *hik19* gene and the *hik33* gene had been inactivated. Mutation of these two genes also reduced the extent of induction at low temperature of other genes whose expression is induced by low temperature, such as the *desD* gene for the Δ6 desaturase and the *crh* gene for RNA helicase. Hik33 has two membrane-spanning domains at its amino terminus and it is likely that this enzyme is localized on the cell membrane. By contrast, it is likely that Hik19 is a soluble protein. Therefore, Hik33 might be a primary sensor and Hik19 might be a signal transducer. This is, to our knowledge, the first discovery of a cold-sensing protein in any type of cell.

II. Analysis of the regulation of gene expression using a DNA microarray

The *Synechocystis* DNA microarray, CyanoCHIP™, covers 97% of all the open reading frames (ORFs) found in the genome of this cyanobacterium. Analysis of temperature-dependent gene expression with this DNA microarray revealed that genes whose expression is enhanced by low temperature encode proteins that are, for the most part, subunits of the transcriptional and translational machinery, such as the α subunit of the RNA polymerase, a σ factor, and some protein subunits of the ribosome. These proteins might be essential for reversal of suppression of protein-synthesizing activity by low temperature. By contrast, most cold-repressible genes were found to encode components of the photosynthetic machinery, such as subunits of photosystem I and of phycobilisomes. These changes in gene expression might represent the acclimative responses of photosynthetic organisms that allow maintenance of photosynthetic activity at a certain level regardless of temperature. To our surprise, we found that the expression of a number of genes for proteins of unknown function was also enhanced or repressed by low temperature.

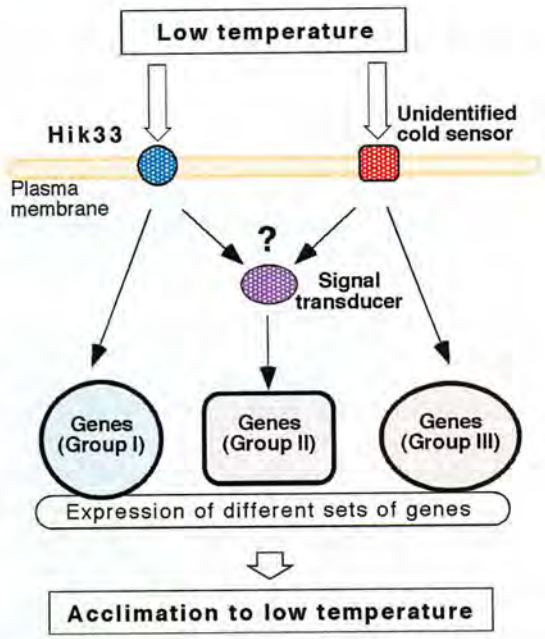

Figure 1. A hypothetical scheme for transduction of low-temperature signal.

Our examination of the cold-dependent regulation of gene expression with the DNA microarray also revealed that the cold-regulated genes could be divided into three groups by reference to the effect of inactivation by mutation of Hik33. In the first group, regulation of gene expression by low temperature was totally abolished upon inactivation of Hik33; in the second group, the extent of such regulation was reduced by half; and, in the third group, such regulation was totally unaffected. These observations indicate that Hik33 might regulate the expression of certain cold-regulated genes, while expression of those genes that were unaffected by inactivation of Hik33 might be regulated by another system for transduction of the low-temperature signal (Fig. 1; Suzuki, Kanesaki, Mikami, Kanehisa, and Murata *Mol. Microbiol.* in press).

III. The importance of membrane lipids in protection of the photosynthetic machinery against salt stress

High salinity is one of the main environmental factors that severely limit the growth and the productivity of plants and microorganisms. We showed previously that membrane lipids are intimately involved in the protection of both plants and microorganisms against low-temperature stress. To provide further insight into the importance of membrane lipids in stress tolerance, we examined the tolerance to salt stress of the photosynthetic machinery of *Synechococcus* sp. PCC 7942 in which we had genetically enhanced the unsaturation of fatty acids in the membrane lipids. Our results revealed that the unsaturation of fatty acids enhanced the salt tolerance of cells at least in terms of the activities of photosystem I, photosystem II and the Na^+/H^+ antiport systems. The enhanced tolerance was mediated, at least in part, by the increased tolerance of the photosynthetic machinery to salt-induced damage and by an increase in the cell's ability to repair the photosynthetic and Na^+/H^+ antiport systems. These results serve to emphasize the physiological importance of the repair of the photosynthetic machinery and the Na^+/H^+ antiport system in the protection of *Synechococcus* cells from salt-induced damage.

IV. Characterization of the Na^+/H^+ antiporters of *Synechocystis*

Control of membrane permeability to Na^+ ions and to counteracting K^+ ions is one of the most important aspects of the acclimation of cells to high-salt environments. Na^+/H^+ antiporters are membrane-bound proteins that play an important role in maintenance of the balance of intracellular concentrations of Na^+ and K^+ ions in plant, fungal and bacterial cells. We used a mutant of *Escherichia coli* that is deficient in Na^+/H^+ antiport activity as a host to examine functional complementation with genes for putative Na^+/H^+ antiporters from the genome of *Synechocystis*. We tested five genes and found that at least two genes encoded Na^+/H^+ antiporters with different respective affinities for Na^+ and Li^+ ions. To our knowledge, this is the first functional characterization of Na^+/H^+ antiporters from a cyanobacterium. The coexistence of high-affinity and low-affinity Na^+/H^+ antiporters in *Synechocystis* is consistent with the ability of this organism to acclimate to a wide range of extracellular concentrations of Na^+ ions (Inaba, Sakamoto, and Murata *J. Bacteriol.* in press).

V. The role of glycinebetaine in tolerance to freezing temperatures

Glycinebetaine (GB) is a zwitterionic quaternary amine that is found in a large variety of microorganisms, plants and animals. It belongs to a group of compounds known as compatible solutes, which act very efficiently to stabilize the structure and function of cellular macromolecules such as protein complexes and membranes. In a previous study, we cloned the *codA* gene for choline oxidase from *Arthrobacter globiformis.* Choline oxidase catalyzes the conversion of choline to GB. We introduced the gene for this enzyme into *Arabidopsis thaliana,* which does not synthesize GB. The resultant transformed plants not only accumulated high levels of GB but also exhibited significantly elevated tolerance to a broad spectrum of environmental stresses, such as high salt, low and high temperatures, and strong light.

In 2000, we found that transformation of *A. thaliana* with the *codA* gene also enhanced the freezing tolerance of transformed plants. The accumulation of GB in chloroplasts dramatically improved the survival of mature plants, stabilized membranes, and efficiently protected the photosynthetic machinery at freezing temperatures (Fig. 2). The observation that the

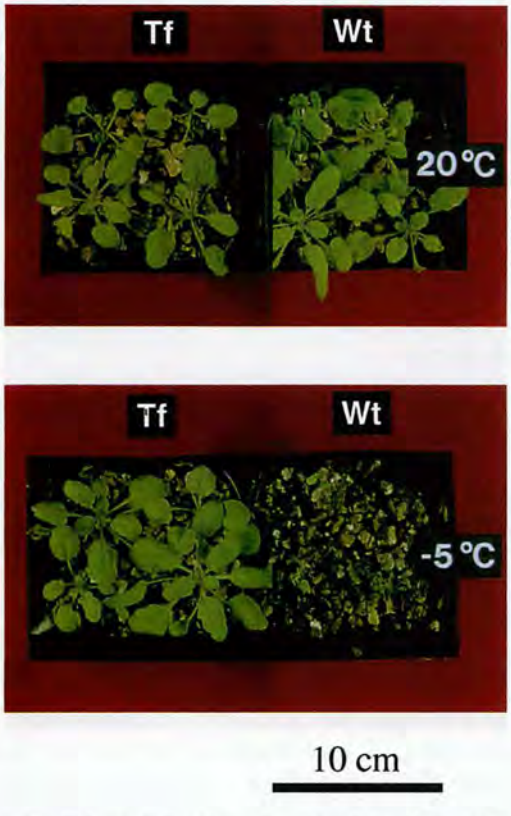

Figure 2. Enhanced freezing tolerance of *Arabidopsis* plants that have been transformed with the *codA* gene for choline oxidase.
Wild-type (Wt) and transformed (Tf) plants were grown at 22°C for 33 days. They were then incubated at 20°C or -5°C for 2 h and subsequently incubated at 22°C for seven days to examine survival. The transformed plants accumulated glycinebetain (GB) at 0.90 μmol g^{-1} fresh weight, whereas wild-type plants contained no GB. Adapted from Sakamoto *et al.*, *Plant J.*, **22**, 449-453 (2000).

transformed plants exhibited tolerance to various kinds of abiotic stress suggests that, in addition to its stabilizing effect on proteins and membranes, GB might, under stress conditions, contribute to the maintenance of cellular functions of fundamental importance, such as transcription and translation. We are now focusing our attention on the molecular mechanisms by which GB enhances the tolerance to various kinds of stress, with particular emphasis on possible protection of the transcriptional and translational apparatus from stress-induced damage.

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(1) Original articles

Allakhverdiev, S.I., Sakamoto, A., Nishiyama, Y., Inaba, M., and Murata, N. (2000) Ionic and osmotic effects of NaCl-induced inactivation of photosystems I and II in *Synechococcus* sp. *Plant Physiol.*, **123**, 1047-1056.

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DIVISION OF BIOLOGICAL REGULATION AND PHOTOBIOLOGY (ADJUNCT)

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Plants use light as an environmental factor which controls their development as well as their other physiological phenomena. Phytochrome and blue light receptors, such as cryptochrome and phototropin (nph1), are the main photoreceptors for plant photomorphogenesis. The goal of our research is to clarify the signal transduction pathways of photomorphogenesis. One of our major subjects is chloroplast photo-relocation movement which is thought to be one of the simplest phenomena in this field. We use the fern *Adiantum capillus-veneris* and the moss *Physcomitrella patens* as model plants for our cell biological approach not only because the gametophytes are very sensitive to light, but also because the organization of the cells is very simple. We also use *Arabidopsis* mutants to clarify the genes regulating chloroplast photo-relocation movement.

I. Cloning and characterization of blue-light photoreceptors

We have described many blue-light induced photomorphological responses in gametophytes of the fern *Adiantum capillus-veneris*. As the first step in understanding the molecular mechanisms of these blue-light responses, we are cloning and sequencing the genes of blue light receptors, and are studying intracellular distributions of the gene products and their function in *Adiantum* and *Physcomitrella*.

1-1 Cryptochromes

We identified two cryptochrome genes from *Physcomitrella patens*, designated *CRY1a* and *CRY1b* genes, and made single and double disrupants of these genes using gene targeting by means of homologous recombination. Using these disruptants, it was revealed that blue light signals via cryptochromes inhibited the transition of cell types of the moss protonema from chloronema to caulonema, but induced side-branching of the protonema. Gametophore induction and its growth and development were also regulated.

1-2 Phototropin

Phototropin is another blue light photoreceptor isolated recently in higher plants, and is a flavin binding protein with light sensitive protein kinase activity. A cDNA of *Adiantum NPL1*, a homologue of phototropin has been sequenced. The complete cDNA clone is 3492 bp in length and encodes a protein of 1092 amino acids.

II. Chloroplast relocation

2-1 *Arabidopsis*

Chloroplasts accumulate at the cell surface under weak light and escape from the cell surface to the anticlinal wall under strong light to optimize photosynthesis. The mechanism of chloroplast relocation, however, is not known. We screened several mutants from T-DNA tagging lines as well as EMS lines of *Arabidopsis*. Gene analysis of several mutants defective in chloroplast relocation movement showed that npl1 is the blue light receptor for the avoidance response under strong light. Both nph1 and npl1 are the blue light receptors for the chloroplast accumulation response under weak light. Gene analysis of mutants defective in accumulation response are also under way.

2-2 *Adiantum*

Adiantum phytochrome3 (PHY3) is a unique kimeric protein with a phytochrome structure in the N-terminal half and a phototropin structure in the C-terminal half. Heavy-ion-beam- or EMS-induced mutants of *Adiantum* which do not show phototropic response and chloroplast photorelocation movement under red light were revealed to lack the *PHY3* gene or to have a mutation in the *PHY3* gene. It was confirmed by gene silencing that phy3 is the photoreceptor mediating red light-induced tropic response and chloroplast photorelocation movement. However, since these phenomena induced by blue light are normal in these mutants, it is still unknown whether phy3 might or might not absorb blue light as a photoreceptor.

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Imaizumi, T., T. Kanegae and M. Wada (2000) Cryptochrome nucleocytoplasmic distribution and gene expression are regulated by light quality in the fern *Adiantum capillus-veneris*. *Plant Cell* **12**: 81-96.

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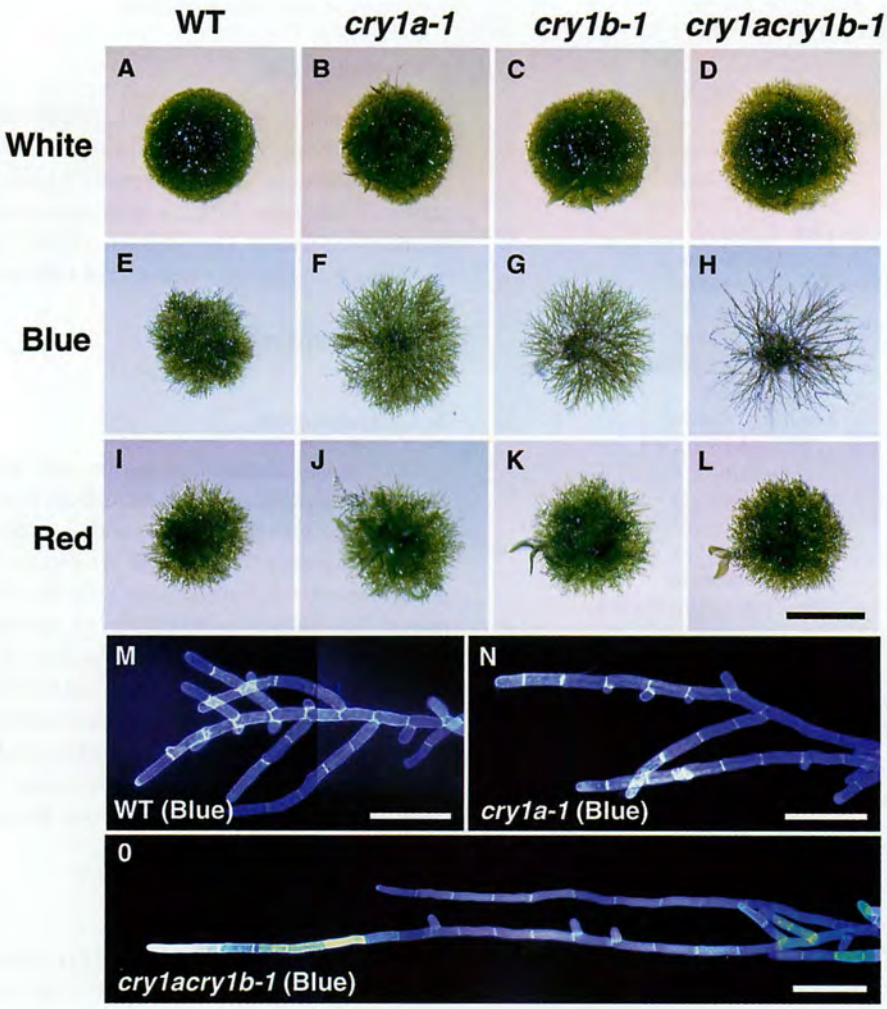

Figure. Protonemal Colonies of the Cryptochrome Disruptants under Different Light Conditions.

The protonemal colonies (ca. 1 mm in diameter) of wild type, *cry1a*, *cry1b*, and *cry1acry1b* strains were inoculated on the agar plates. These plates were incubated under continuous white, blue, or red light for 10 days. Representative colony images of one of each cryptochrome disruptant were shown. The colonies are the following strains: (A, E, and I) the wild type (WT); (B, F, and J) *cry1a-1*; (C, G, and K) *cry1b-1*; and (D, H, and L) *cry1acry1b-1*. The light conditions are given: (A-D) white light; (E-H) blue light; and (I-J) red light.

The blue-light grown protonemata are shown in M-O. These are (M) wild type; (N) *cry1a-1*; and (O) *cry1acry1b-1*. The protonema cell walls were stained with a drop of 0.1% (v/v) Calcofluor White to help distinguish each cell. The appearance of protonemata of *cry1b* strains was similar to that of *cry1a* strain protonemata.

The bar in L represents 2 mm for the panels A-L, and the bars in M-O represents 200 μm.

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The vertebrate nervous system contains a large number of neurons that are connected to each other by specific axonal projections. We are interested in how the complex but precise neuronal network, which is indispensable for functioning of the nervous system, is constructed during development. The goal of our research in this division is to elucidate the cellular and molecular mechanisms underlying the neuronal network formation in vertebrates, with current focuses on the mechanisms of axon guidance and neuronal migration.

I. Mechanism of Axonal Guidance

During development of the nervous system, growing axons are guided through specific pathways to correct targets. Our research interest focuses on the cellular and molecular mechanisms of axon guidance in the vertebrate nervous system. We are particularly interested in how guidance mechanisms operating in different phases of axonal growth are assembled to generate the complex but precise axonal wiring patterns in the brain. To address these issues, we are first trying to understand axonal wiring patterns during development with reference to the structural organization of the brain. Using whole-mounted preparations of embryonic rat brains, we will reveal whole axonal trajectories by labeling with neuronal tracers, such as DiI, or by immunohistochemical staining with antibodies against molecular markers expressed in a subset of neurons. We will next explore the axonal guidance mechanisms generating these wiring patterns. Following questions will be addressed. 1) What structures along the pathway have key roles in axonal guidance? 2) What guidance cues exist in these structures? Are these attractive cues or repulsive cues? Are these short-range cues or long-range cues? 3) What molecules are responsible for these cues? 4) How do multiple guidance mechanisms work in concert to generate specific wiring patterns? To answer these questions, we use neuroanatomical techniques, in vitro culture techniques including dissociated cell, explant and whole-mount cultures, and biochemical and molecular biological techniques.

II. Mechanism of Neuronal Migration

A variety of neurons migrate from their birthplace to the position where they finally settle. Neuronal migration in the vertebrate central nervous system occurs both along the radial axis and along the tangential axis of the neural tube. Another interest of our research focuses on the cellular and molecular mechanisms underlying the radial and tangential neuronal migration. In many regions of the central nervous system, neurons migrate radially from the ventricular zone where they are born toward the pial surface. We are investigating the mechanisms of radial migration, using neurons in the neocortex as a model system. In addition to the radial migration, some neurons in the brain, such as interneurons in the forebrain, move tangentially for a long distance. We are also investigating how tangentially migrating neurons are guided for a long distance through specific routes to the final positions, using neurons that migrate from the rhombic lip at the dorsal rim of the hindbrain, such as cerebellar granule cells and precerebellar neurons.

To address these issues, we developed in vitro culture systems that reconstruct migratory events occurring in vivo. We labeled migrating neurons with green fluorescent protein (GFP) by transplantation of small piece of explant taken from a transgenic rat expressing GFP or by introducing GFP cDNA into limited regions of the brain by electroporation. Slices of the brain or flat-mounted brain preparation are then cultured on permeable membrane filters. These culture systems enable us to analyze migration pattern of neurons in real time (Fig. 1). Using these in vitro culture systems, we are currently investigating the cellular and molecular mechanisms of neuronal migration.

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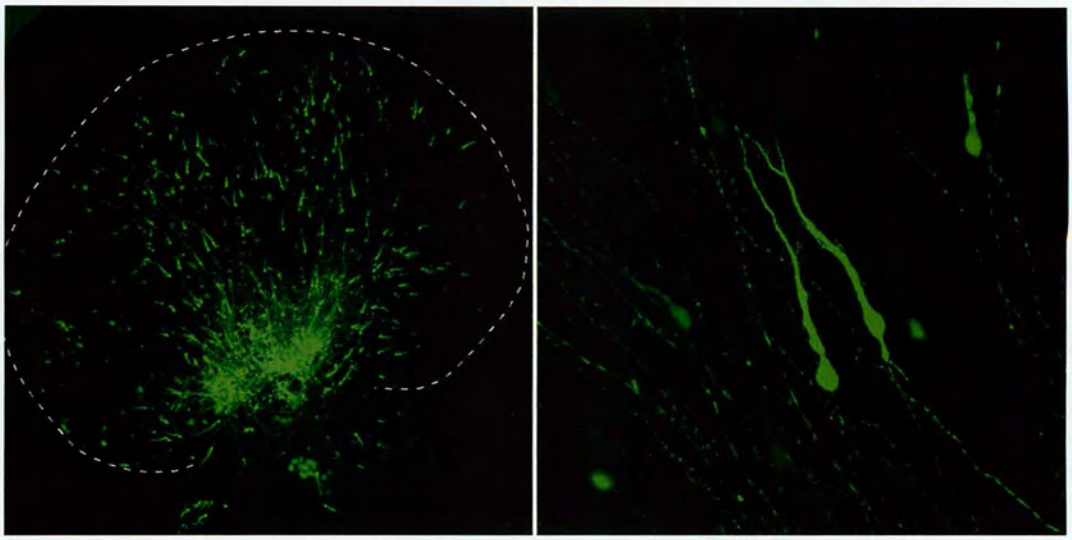

Figure 1. Migrating neocortical neurons visualized with green fluorescent protein (GFP) in vitro.
(Left) Slice culture of rat neocortex that was introduced with GFP cDNA into the ventricular zone by electroporation. A broken line shows the outer margin of the slice. GFP-positive neurons were moving radially from the ventricular zone (bottom) toward the pial surface (top).
(Right) High power view of labeled migrating neurons. Leading processes of neurons extended toward the pial surface.

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 microorganisms, plants and animals.

DIVISION OF GENE EXPRESSION AND REGULATION I

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	Laurel Caitlin Coberly[1)]

[1)]*from Duke University*

The main interest of the group is in understanding the biology of the dynamic genome, namely, genome organization and reorganization and its impact on gene expression and regulation. Although there are many elements affecting organization and reorganization of the genome, we are currently focused on mobile genetic elements in general and plant transposable controlling elements in particular.

I. Spontaneous mutants in the Japanese morning glory.

The Japanese morning glory (*Ipomoea nil* or *Pharbitis nil*), displaying blue flowers, is believed to be originated from southeast Asia and has an extensive history of genetic and physiological studies. The plant had been introduced into Japan from China in about 8th century as a medicinal herb, seeds of which were utilized as a laxative, and has become a traditional horticultural plant in Japan since around 17th century. A number of its spontaneous mutants related to the colors and shapes of the flowers and leaves have been isolated, and about 10% of these mutants carry mutable alleles conferring variegated phenotypes. Several lines of evidence indicate that an *En/Spm*-related transposable element *Tpn1* and its relatives, which we termed *Tpn1*-family elements, are major sources of these spontaneous mutations. Indeed, we have succeeded to identify two of these mutable alleles for flower pigmentation, *flecked* and *speckled*, which are caused by integration of *Tpn1*-related elements, *Tpn1* and *Tpn2*, respectively. Both *Tpn1* and *Tpn2* are non-autonomous elements and their transposition is mediated by a *Tpn1*-related autonomous element. Among selfed progeny of a mutable *flecked* line, a white variant displaying white flowers occasionally appeared. Some of the selfed progeny of the white variant bore only white flowers whereas others produced a few flecked flowers together with white flowers. In these white variant derivatives, the excision of *Tpn1* occurred rarely. We are speculating that appearance of the white variant is probably due to epigenetic inactivation of the autonomous element. In accordance with this notion, we also found that the apparent stable *r-1* allele conferring white flowers is caused by insertion of a non-autonomous *Tpn1*-family element, *Tpn3*, into the *CHS-D* gene encoding a chalcone synthase for anthocyanin biosynthesis.

II. A new procedure for isolation of a gene tagged by a transposable element belonging to the *Tpn1* family in the Japanese morning glory.

Transposable elements are regarded as a powerful mutagen and as an effective tool to isolate genes tagged by transposon insertions. The Japanese morning glory contains around 500-1000 copies of an *En/Spm*-related element *Tpn1* and its relatives, which act as major spontaneous mutagens. We have previously developed an amplified restriction fragment length polymorphism (AFLP)-based mRNA fingerprinting (AMF) procedure which is based on the systematic comparison of differently expressed transcripts in the same tissue in different lines, and succeeded in applying AMF for the identification of a new mutable allele caused by integration of a transposable element into an anthocyanin biosynthesis gene. Since transposon mutagenesis has become a powerful tool for the isolation of genes of interest, we have attempted to develop a new protocol for identifying tagged genes by insertion of *Tpn1*-related elements in the Japanese morning glory. Our transposon tagging method, named simplified transposon display (STD), was based on our AMF procedure and is simple and requires neither biotinylated oligonucleotides nor streptavidin-capturing which are essential in other transposon display methods published recently.

III. Identification and characterization of the *Purple* gene encoding a vacuolar Na^+/H^+ exchanger, InNHX1, for blue flower coloration in the Japanese morning glory.

We have applied STD for identification of a mutable allele, *purple-mutable* (*pr-m*), which confers purple flowers with blue sectors (Fig. 1). The flower variegation is regarded to be due to recurrent somatic mutation from the recessive purple to the blue revertant allele, *Purple-revertant* (*Pr-r*) and we assumed that the *pr-m* allele is caused by insertion of a *Tpn1*-family element. To characterize the *Purple* (*Pr*) gene, we chose pairs of siblings carrying either the *pr-m* or *Pr-r* allele homozygously. No alterations were detected in the anthocyanin pigment compositions between the *pr-m* and *Pr-r* lines. The *pr-m* mutant showed partial increase in the vacuolar pH during flower opening and its reddish-purple buds change into purple open flowers. The vacuolar pH in the purple open flowers of the mutant was significantly lower than that in the blue open flowers, indicating that the *pr* mutant fails to increase the vacuolar pH.

We have succeeded in identifying the *pr-m* mutation that is caused by integration of an *En/Spm*-related transposable element, *Tpn4*, into the *Pr* gene encoding a

vacuolar Na^+/H^+ exchanger, InNHX1 (Fig. 1). The *Pr* gene comprises 15 exons, and *Tpn4* is integrated into the first untranslated exon. The genome of *I. nil* carries one copy of the *Pr* (or *InNHX1*) gene and its pseudogene. The *Pr* gene is most abundantly expressed at around 12 h before flower opening in the petals, which must correlate with the increase in the vacuolar pH for the blue flower coloration. The isolated *Pr* gene is able to show functional complementation to a deletion mutation in the *NHX1* gene encoding a vacuolar Na^+/H^+ exchanger in yeast (*Saccharomyses cerevisiae*), indicating that the Pr gene product bears the NHX1 activity (Fig. 1). The NHX1 proteins are shown to be important for salt tolerance and intracellular protein trafficking in yeast and plants. We have thus added a new biological role for blue flower coloration in the Japanese morning glory by the vacuolar alkalization. The vacuolar pH has been regarded to play an important role in the blue flower coloration, and InNHX1 is the first major component characterized for increasing vacuolar pH responsible for blue flower coloration.

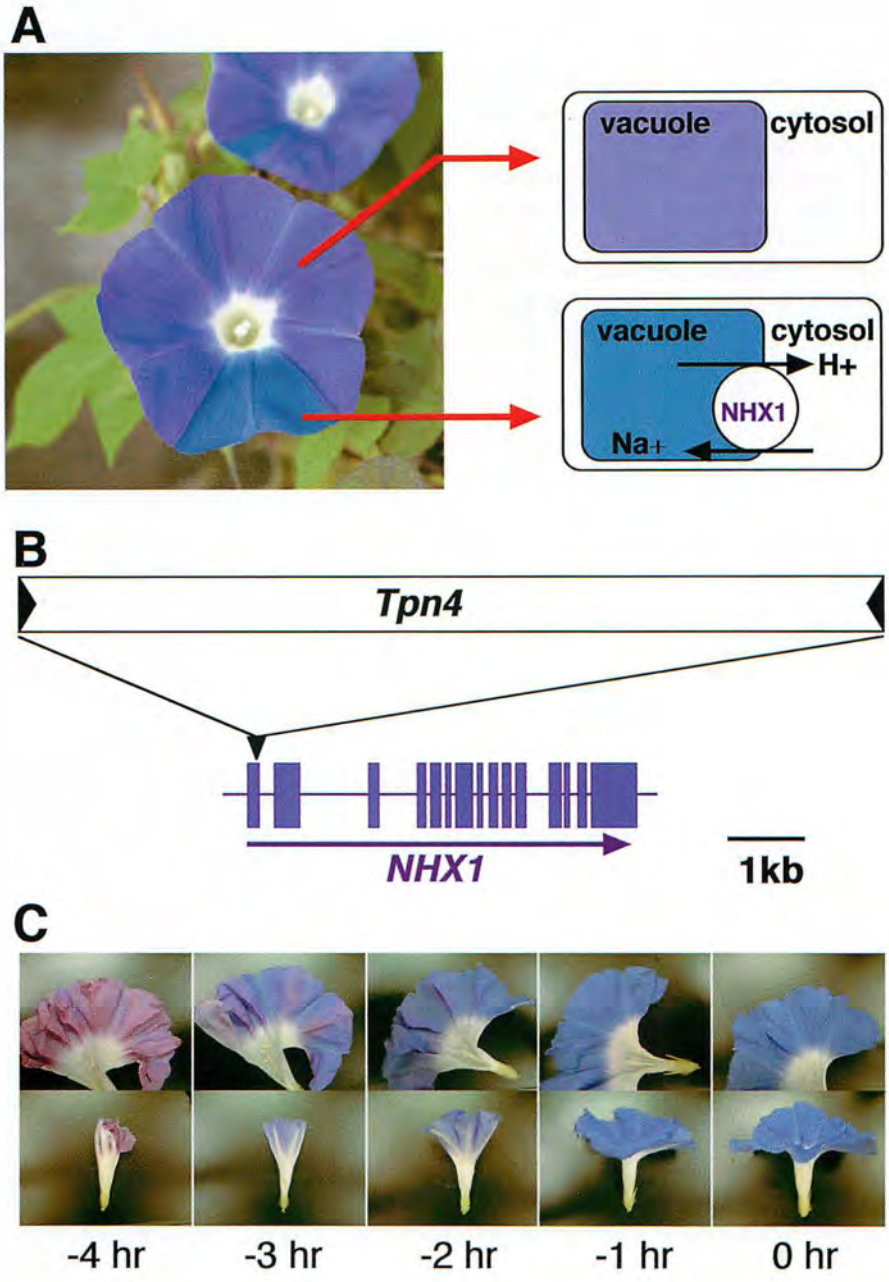

Figure 1. Blue flower coloration and the vacuolar pH in the Japanese morning glory.
A, Flower variegation and the *Pr* gene product, InNHX1, for blue flower coloration. B, The genomic structure of the mutable *pr-m* allele. C, Blue flower coloration during flower opening in the *Pr-r* plant. Petals in the upper line are the artificially opened petals of the buds in the lower line.

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DIVISION OF GENE EXPRESSION AND REGULATION II

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*** *She obtained her doctorate Sept. 2000.*

Homologous recombination, which may occur in all organisms, involves genetic exchange between two parent-derived homologous chromosomes in meiosis in addition to the repair of DNA damage induced by physical and chemical agents. As a result of our analyses of recombinational hotspots of *E. coli* and *S. cerevisiae*, in particular the activity related to DNA replication fork-blocking events, the physiological function of homologous recombination (especially in normally growing cells) is better understood. In 2000, work on the following three subjects has advanced our knowledge of the dynamics and function of the genome.

I. Identification of *cis*-essential sequences for amplification of the ribosomal RNA gene repeats in yeast.

We previously found that some recombinational hotspots in *E. coli* present in regions where progress of a DNA replication fork is severely impeded. Detailed analysis of this phenomenon revealed that when the fork is blocked, recombinational enzymes construct a new replication fork after breakdown of the arrested fork by introducing a double-stranded break at either of the sister chromatids. Consequently, this recombination reaction for renewal of the replication fork is a primary source of recombinational hotspots. Next, we showed that this phenomenon occurred not only in prokaryotes but also in eucaryotes. In most eucaryotes, the DNA replication fork blocking site, called *RFB* (replication fork barrier), is located in each unit of ribosomal multigenes (rDNA). It has been reported that in budding yeast (*S. cerevisiae*) when a non-transcriptional region (NTS) of the rDNA, in which the *RFB* site is located, is inserted into a non-rDNA region of chromosomes, recombination of a region close to the insertion site is enhanced. This enhancement is called *HOT1* activity and the DNA fragment required for activation is named *HOT1* DNA. We isolated a number of mutants, which are defective in *HOT1* activity, and found that some of them had a concomitant defect in fork blocking activity at the *RFB* site. Both of these defective phenotypes were restored to wild type by introducing a single gene, named *FOB1*, thereby suggesting that fork blockage acts as a trigger for recombination in yeast, as well as in bacteria. In addition to this finding, the detection of *RFB* activity within the rDNA cluster in various higher eucaryotic cells suggests that the fork renewal cycle occurring after the blockage is more general than expected.

The copy number of rDNA repeats is unstable. Although variation in the number of rDNA copies has been observed in various eucaryotes, the underlying mechanism remains unknown. Because fork arrest at the *RFB* site was a trigger for recombination, as mentioned above, we suspected that a fork-blocking event was involved in changing the copy number of rDNA repeats. Neither an increase nor a decrease in rDNA repeats occurred in *fob1* mutants, suggesting that the fork blocking event is required for changing the copy number, probably through a recombination process. As the fork blocking system has only been understood to prevent replication from "colliding" with rDNA transcription so far, this study indicates that the fork block system may have an entirely new function. If this is the case, *RFB* deleted rDNAs should not amplify.

This year, we tested this prediction and also attempted to identify *cis*-sequences, required for amplification of the copy number of rDNA. However, this kind of analysis was not feasible for a repeated gene with high copy number. Therefore, we constructed a strain with a rDNA copy number reduced to two copies of the original genomic locus by using a multi-copy plasmid, carrying a hygromycin B resistant copy of rDNA. Then, each sub-region in a single NTS1 region among the two rDNAs on the genome were replaced with a *URA3* marker gene in order to determine which sequences would be required for rDNA amplification, especially around the *RFB* site. Mutational analysis using this system showed that not only the *RFB* site but also the adjacent ~440 bp region in NTS1 (together called the EXP region) are required for the *FOB1*-dependent repeat expansion. This ~400 bp DNA element is not required for the fork arrest activity or the *HOT1* activity and therefore defines a function unique to rDNA repeat expansion (and presumably contraction) separate from *HOT1* and RFB activity. Our model, which indicates a mechanism by which the copy number of rDNA increases from two to 150, is shown in the accompanying Figure.

II. Enhanced homologous recombination caused by a non-transcribed spacer of the ribosomal RNA gene in *Arabidopsis*.

In yeast, a single rDNA unit structure consists of two rRNA genes, 35S pre-rRNA and 5S rRNA genes, and two non-transcribed spacer (NTS) regions, NTS1 and NTS2. NTS1 is flanked by the 3'-ends of the two rRNA genes and NTS2 is flanked by the 5'-ends of the two rRNA genes. A region containing NTS1-5SrRNA gene-NTS2, named *HOT1* DNA, has activity, that

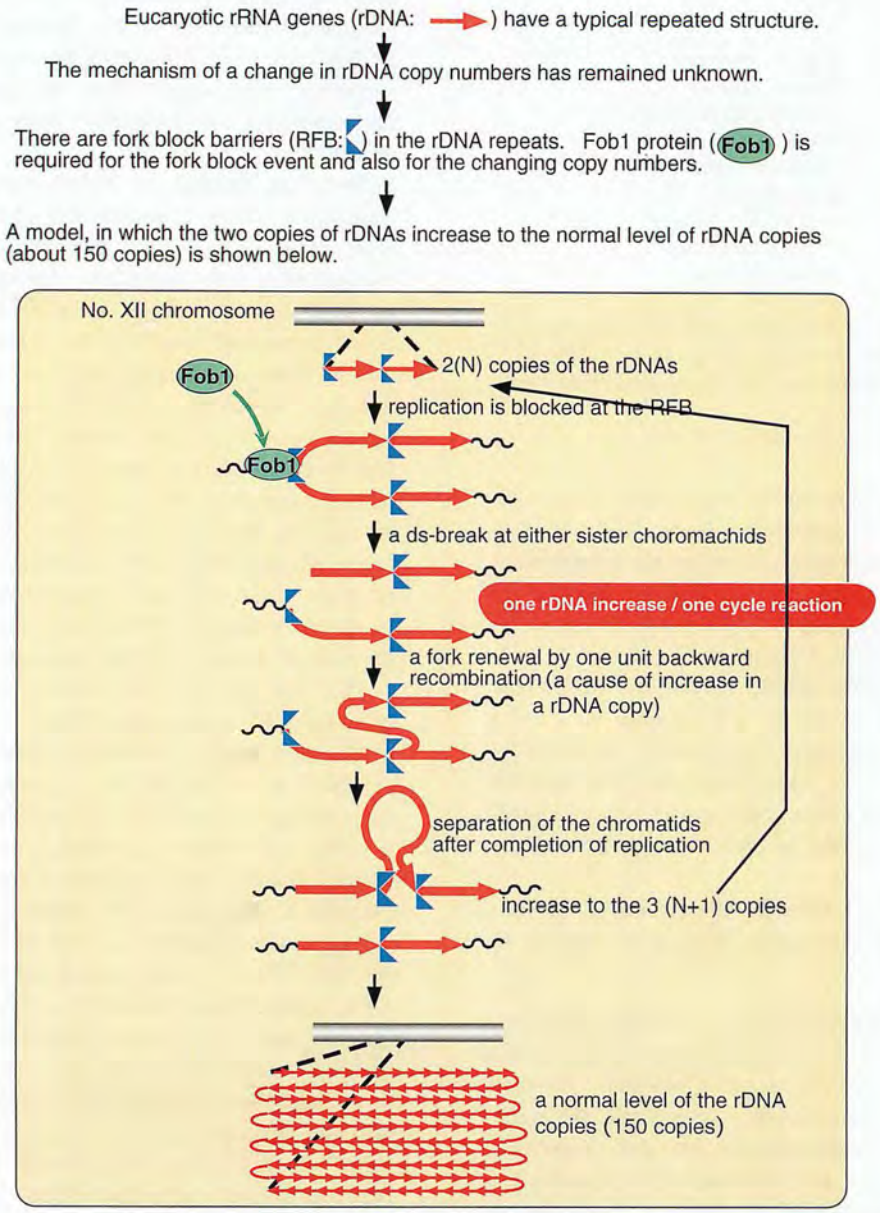

Figure. An increase mechanism for rDNA copy number. The figure shows that one reaction cycle increases the rDNA by one copy. Therefore, 150 reaction cycles can increase the copy number of the rDNA from two to 150.

stimulates recombination at a region close to a non-rDNA locus where the *HOT1* DNA is inserted. The stimulated recombination activity is called *HOT1* activity. Further analysis revealed that only two small non-contiguous regions, called E and I, are required for *HOT1* activity. E and I are located in NTS1 and NTS2, respectively and correspond to enhancer and initiator of RNA polymerase I dependent 35S rRNA transcription. The rDNA repeated structure is essentially the same in various eucaryotes as in yeast, except in other eucaryotes 5S rRNA genes are located at a different locus from the 35S rRNA gene cluster. In addition, fork-blocking activity has been detected in the NTS regions of the rDNA array in all eucaryotes tested so far. Here, we investigated whether the non-transcribed spacer region in the rDNA cluster of higher eucaryotes has hotspot activity similar to that observed in yeast. We chose *Arabidopsis* as an experimental model, because a reliable recombination assay system had been developed and recombination event, if it occurs anywhere-whole body of the plant, is easily visible. To analyze the effect of the NTS on mitotic homologous recombination in the plant, we constructed transgenic lines of *Arabidopsis* containing the NTS region and a recombination substrate, in which two 3'- and 5'- deleted *uidA* genes, with partially overlapping sequences are separated by a Hyg^r gene. Histochemical GUS-staining monitored reconstitution of functional *uidA* genes by homologous recombination. We found that recombination occurred more frequently in all organs tested in F

(Fork Block) lines transgenic for NTS than in C (Control) lines without NTS. The average number of GUS^+ spots on leaves in F lines was more than nine-fold higher than in C lines. Furthermore, by genomic Southern blotting analysis, post-recombinational molecules were detected in a transgenic line, F43, which had an extremely high number of GUS^+ blue spots. These results strongly suggests that NTS-dependent enhancement of homologous recombination could be a common feature in higher plant as well as yeast.

III. *E. coli* genomic structure and function

Analysis of whole genomic sequence of *E. coli* had been completed in 1997. While a strain Japan team sequenced is W3110, the other US team did is MG1655. These strains were separated from a common ancestor during or right after World War II. In order to understand the micro-evolution of these strains, we are sequencing a previously undetermined region of the W3110 genome and will finished this spring. Our goal is to compare this genome with the MG1655 genomes at the nucleotide level.

In order to identify a minimal set of genes required for the duplication of a single cell and to elucidate the function of those genes, we proposed a new project and a established a new collaboration team, headed by Mori (NAIST). Since last year our project has been supported by CREST from JST and is being carried out by the following four groups: (1) resources, (2) informatics, (3) database, and (4) functional analysis. At present, the resources group (1) has cloned all ORF candidates, constructed *E. coli* micro-arrays using the cloned ORFs and started to analyze gene expression under various conditions, obtaining results that will be published. Other plans, such as the construction of a set of mutant strains, in which each gene is disrupted, are in progress. These strains will be distributed to workers world wide, stimulating functional analysis of the *E. coli* genome, and eventually contributing to defining an integrated functional network of genes in *E. coli*.

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DIVISION OF SPECIATION MECHANISMS I

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Our research goal is to understand mechanisms underlying evolution of the nervous system. In order to approach this question, we are studying the genes that are expressed in the specific areas of the primate neocortex. Using differential display method, we have obtained genes that showed marked differences within the primate neocortex.

Our second approach is to understand informational processing in the brain underlying learning behaviors with gene expression techniques. Here, we report our findings in the year of 2000.

I. Genes expressed in specific areas of the neocortex

The neocortex is most evolved in mammals, particularly in primates, and thought to play the major role in higher functions of the brain. It is known to be divided into distinct functional and anatomical areas and has been a matter of debate what extent the areas of the neocortex are genetically and environmentally determined. It is also puzzling why, during the evolution of mammals, the neocortex was most markedly expanded while the number of the genes in the mammal was little changed. To access these questions, we studied gene expression within different areas of the neocortex.

1) In collaboration with Professor Hiroyuki Nawa (Nigata university), we used DNA macroarray technique to examine gene expression in the areas of human prefrontal, motor and visual cortexes. We found almost all the genes among 1088 genes examined showed only less than a factor of two in the difference of their expressions. Only one gene showed more than three fold difference and another one was between two and three fold difference within the three areas. These results suggest that the genes that are expressed among the different areas of the human neocortex are very similar. However, the question remained whether there are any genes that show marked difference within areas of neocortex.

2) In order to answer this question, we employed differential display methods and found at least two genes that indicated the area specific expression.

i) One, designated *occ1* is specifically expressed in the occipital cortex, particularly in V1 area, in the primate brain. Furthermore, the expression of occ1 turned out to be activity dependent, because, in the monocularly deprive–monkeys with being injected TTX into one of eyes, the expression of occ1 is markedly decreased in the ocular dominance columns of the primary visual cortex (V1).

ii) The other gene that showed marked difference within the neocortex, is gdf7, a member of BMP/TGF-β family, which is specifically expressed in the motor cortex of the African green monkey. We are currently examining the detailed expression pattern of the both genes.

3) We have also further isolated several area specific genes with RLCS (Restriction Landmark cDNA Scanning).

II. Gene expression under audio-visual discrimination task

We are studying gene expression of c-Fos under audio-visual discrimination tasks in collaboration with professor Yoshio Sakurai (Kyoto University). We found that the visual and audio tasks enhanced the specific expression of c-Fos in the visual and audio cortex, respectively. Among the early visual and auditory pathways examined, c-Fos was specifically induced in the cortexes but not in the earlier pathways, suggesting the neural modulation of the neocortex depending on the types of the tasks. We are currently identifying the neuronal cell types in the cortical area that induced c-Fos depending on the relevant task.

Publication list:

Original Articles

Watakabe, A., Sugai, T., Nakaya, N., Wakabayashi, K., Takahashi, H., Yamamori, T. and Nawa, H. Similarity and variation in gene expression among human

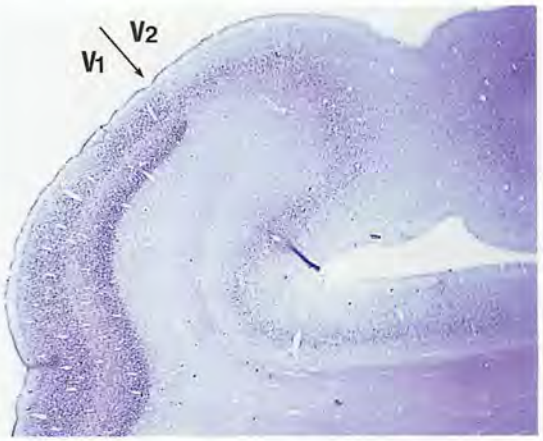

Fig. 1 Expression pattern of occ1 in the visual cortex.
In situ hybridization pattern of *occ1* in the primate visual cortex. Occ1 is markedly expressed in the layer IVcβ and moderately in the layers of II, III and IVa in area V1. The boundary between V1 and V2 is shown by an arrow.

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DIVISION OF SPECIATION MECHANISMS II

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Assistant Professor:	*Tomomichi Fujita*
Post Doctoral Fellow:	*Rumiko Kofuji[1](-Mar. 31)*
	Tomoaki Nishiyama[2] (Apr. 1-)
	Misako Mishima[1] (Dec. 1-)
Technical Staff:	*Naomi Sumikawa (Oct. 1-)*
	Yukiko Tanikawa
	Masae Umeda
	Yoshimi Bitoh
	Makiko Kondo (-Sept.30)
	Mayumi Naruse (Sept. 30-)
	Kazuko Kabeya (July 1-)
Graduate Students:	*Satomi Shindo(-Mar. 31)*
	Yuji Hiwatashi
	Satoko Kobayashi-Arakawa
	Keiko Sakakibara
	Ryosuke Sano (Chiba Univ.)
	Tomoaki Nishiyama
	(Univ. of Tokyo)(-Mar. 31)
	Takako Tanahashi
	(Univ. of Tokyo) (-Mar. 31)
	Hisako Sakaguchi
	(Shinshu Univ.) (Apr. 1-)

All living organisms evolved from a common ancestor more than 35 billion years ago, and accumulated mutations on their genomes caused the present biodiversity. The traces of evolutionary processes are found in the genomes of extant organisms. By comparing the genomes of different organisms, we can infer (1) the phylogenetic relationships of extant organisms and (2) the genetic changes having caused the evolution of morphology and development. The inferred phylogenetic relationships give important insights on problems in various fields of evolutionary biology, and our group focuses on biogeography, evolution of morphological traits, and systematics in wide range of taxa. On the evolution of morphology and development, we aim to explore genetic changes led the evolution of plant body plan. We selected *Arabidopsis* (angiosperm), *Gnetum* (gymnosperm), *Ginkgo* (gymnosperm), *Ceratopteris* (pteridophyte), *Physcomitrella* (bryophyte), and some green algae as models to compare the gene functions involved in development of the reproductive organs and shoot apical meristem of land plants.

I. Evolution of reproductive organs in land plants

A flower is the most complex reproductive organ in land plants and composed of sepals, petals, stamens, and gynoecium. Female haploid reproductive cells are covered with a sporangium (nucellus) and two integuments, and further enclosed in a gynoecium. Male haploid reproductive cells (pollens) are covered with a sporangium (pollen sack). On the other hand, gymnosperms and ferns have simpler reproductive organs than angiosperms and lack sepals and petals. Female sporangia (nucellus) of gymnosperms are covered with only one integument. Sporangia of ferns have no integuments and are naked on the abaxial side of a leaf.

The development of floral organs is mainly regulated by A-, B-, C-function genes, which are members of the MADS-box gene family. These genes are transcription factors containing the conserved MADS and K domains. MADS-box genes of angiosperms are divided into more than 10 groups based on the gene tree. The *LEAFY* gene is the positive regulator of the MADS-box genes in flower primordia.

What kind of changes of the MADS-box genes caused the evolution of the complex reproductive organs in the flowering plant lineage ? Comparisons of MADS-box and *LFY* genes in vascular plants suggest that the following sequential changes occurred in the evolution of reproductive organs. (1) Plant-type MADS-box genes with both MADS and K domains were established. (2) The number of MADS-box genes increased, and the three ancestral MADS-box genes that later generate A-, B-, C-functions genes were likely originated before the divergence of ferns and seed plants. (3) Specifically expressed MADS-box genes in reproductive organs evolved from generally expressed ones in the seed plant lineage. (4) The ancestral gene of the AG group of MADS-box genes acquired the C-function before the divergence of extant gymnosperms and angiosperms. (5) The gene duplication that formed the AP3 and PI groups in MADS-box genes occurred before the diversification of extant gymnosperms and angiosperms. (6) The ancestral gene of angiosperm A-function gene was lost in extant gymnosperm lineage. (7) *LFY* gene becomes positively regulate MADS-box genes before extant gymnosperms and angiosperm diverged. (8) Spatial and temporal patterns of A-, B-, C-function gene expression were established in the angiosperm lineage.

Homeobox genes play indispensable roles for development in metazoa, instead of MADS-box genes. This difference is likely caused by the fact that metazoa and land plants established multicellular organs independently after their last common ancestor, which was presumably a unicellular organism or a multicellular organism without multicellular organs. Of note, in both land plants and metazoa, an increase in the number of specific transcription factors (MADS-box genes in land plants and homeobox genes in metazoa) and the subsequent diversification of their expression patterns and regulation of downstream genes are the principal mechanisms for the evolution of body plans.

II. Evolution of vegetative organs in land plants

The ancestor of land plants was primarily haploid. The only diploid cell was the zygote, which immediately underwent meiosis. It is believed that early during land plant evolution, zygotic meiosis was delayed and a multicellular diploid sporophytic generation became interpolated into the life cycle. In the early stages of land plant evolution, sporophytes are epiphytic to gametophytes, as observed in extant bryophytes. During the course of evolution, both generations started to grow independently at the stage of pteridophytes. Finally gametophytes became much reduced and epi-

phytic to sporophytes in seed plant lineage. Molecular mechanisms of development in a diploid generation have been well studied in some model angiosperms, but we have scarce information on those in a gametophyte generation. For example, mosses have leaf- and stem-like organs in their haploid generation, but it is completely unknown whether similar genes involved in angiosperm leaf and stem development are used in the gametophytic generation of mosses or not. To understand the evolution of body plans in diploid and haploid generation at the molecular level, we focus on the comparison of molecular mechanisms governing shoot development between Arabidopsis and the moss *Physcomitrella patens*. *P. patens* is known by its high rate of homologous recombination and suitable for analyze gene functions using the gene targeting. The moss homologs of *SHOOTMERISTEMLESS*, *ZWILLE*, and HD-Zip genes, which are involved in *Arabidopsis* shoot development, have been cloned and their characterization is in progress. We also established enhancer and gene trap lines and tagged mutant libraries of *P. patens* to clone genes involved in the leafy shoot development (Nishiyama et al. 2000).

III. Biogeography of *Coriaria*

Coriaria is, which has been mentioned to be the most conspicuous disjunct distribution in flowering plants distributed in four separate areas in the world. The phylogenetic relationships of 12 *Coriaria* species collected from the representative disjunct areas were inferred by comparing 2416 base pairs of the combined data set of *rbcL* (a large subunit of ribulose 1,5-

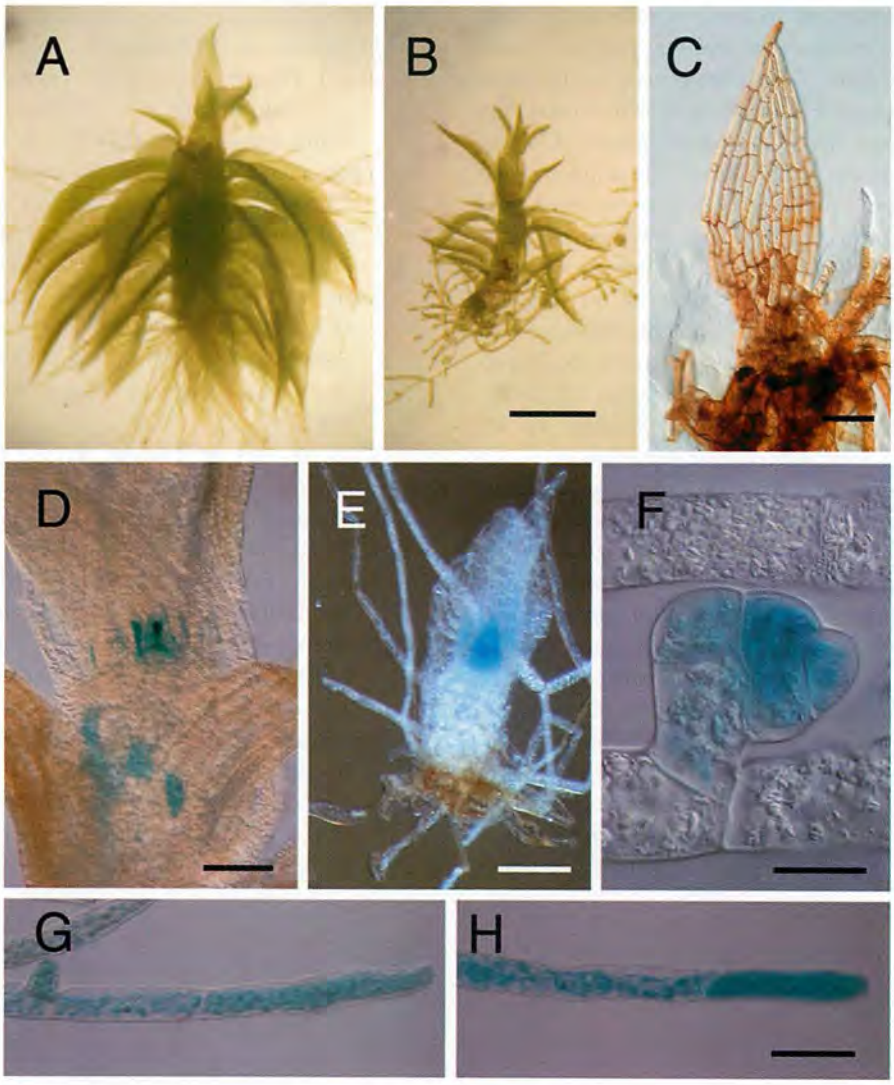

Figure 1. Example of mutant strains and gene-trap lines of *Physcomitrella patens*. A, transformant morphologically indistinguishable from the wild type (19980725084-3). B (TN1) and C (TN2), morphological mutants obtained by shuttle mutagenesis. D-H, histochemical staining of gene-trap lines. D, YH330, mucilage hairs are stained. E, YH78, young leaves are stained. F, YH229, a young bud is stained. G and H, YH206, the chloronema (G) and caulonema (H) are stained. Bar in B = 0.5 mm for A and B. Bars in C and D = 0.1 mm. Bar in E = 0.2 mm. Bar in F = 20 μm. Bar in H = 50 μm for G and H.

bisphosphate carboxylase / oxygenase) and *matK* (maturase K) genes (Yokoyama et al. 2000). The phylogenetic tree shows that the Chile - Papua New Guinea - New Zealand - Pacific islands species and the Central America -northern South America species form a sister group, and the Eurasian clade is more basal to them. The divergence time between the Eurasian group and the other species was estimated as 63 or 59 million years ago using *rbcL* and *matK* molecular clocks, respectively. These results do not support the previously proposed hypotheses to explain the disjunct distribution based on the continental drift, but suggest that the distribution pattern was formed by several geographical migrations and separations in the Cenozoic.

IV. Molecular phylogeny of athyrioid ferns

Nucleotide sequences of the chloroplast gene *rbcL* from 42 species of the fern tribe Physematieae (Dryopteridaceae) were analyzed to gain insights into the inter- and intrageneric relationships and the generic circumscriptions in the tribe. The phylogenetic relationships were inferred using the neighbor joining and maximum parsimony methods, and both methods produced largely congruent trees (Sano et al. 2000). These trees reveal that: 1) *Athyrium*, *Cornopteris*, *Pseudocystopteris*, and *Anisocampium* form a clade and *Athyrium* is polyphyletic; 2) *Deparia sensu lato* is monophyletic and *Dictyodroma formosana* is included in the *Deparia* clade; 3) *Diplaziopsis* forms a clade with *Homalosorus*, which is isolated from the other genera of the Physematieae; 4) *Monomelangium* is included in the monophyletic *Diplazium* clade; 5) *Rhachidosorus* is not closely related to either *Athyrium* or *Diplazium*.

It has been suggested that *Diplazium tomitaroanum* Masam. is a hybrid arising from *Diplazium subsinuatum* (Wall. ex Hook. et Grev.) Tagawa and *Deparia petersenii* (Kunze) M. Kato. *Di. subsinuatum*'s basic chromosome number differs from that of *Diplazium* but is consistent with that of *Deparia*, suggesting that *Di. subsinuatum* is closely related to *Deparia* but not to *Diplazium*. Traditional taxonomy based on morphological characteristics sometimes encounters difficulty in inferring phylogenetic relationships when dealing with taxa having few diagnostic morphological characteristics, such as *Di. subsinuatum* with its simple leaves. We obtained the *rbcL* nucleotide sequences from *Di. subsinuatum* and *Di. tomitaroanum* in order to infer their phylogenetic relationships to other *Deparia* and *Diplazium* species (Sano et al. 2000). We also examined the morphological characteristics of both the scales and leaf axes, which are regarded as diagnostic characteristics for *Deparia* but have not been described in detail for *Di. subsinuatum*. According to the *rbcL* nucleotide sequences, *Di. subsinuatum* and *Di. tomitaroanum* are included in the *Deparia* clade, not in the *Diplazium* clade. Furthermore, the articulate multicellular hairs on leaves, the shape of the rachis groove, the basic chromosome number and the spore morphology are all more similar to *Deparia* rather than to *Diplazium*, indicating that *Di. subsinuatum* should be classified as *Deparia*. We therefore propose a new taxonomic treatment of the two *Diplazium* species.

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CENTER FOR INTEGRATIVE BIOSCIENCE

Interim Head: Hideo Mohri

The center is jointly managed by NIBB and two other institutes in Okazaki, IMS and NIPS. The following projects will be the main focus of the center: 1) Development, Differentiation and Regeneration. 2) Strategic Methodology. 3) Bio-environmental Science.

DEPARTMENT OF BIOENVIRONMENTAL RESEARCH I

Professor:	*Taisen Iguchi*
Associate Professor:	*Hajime Watanabe*
Research Associate:	*Yoshinao Katsu*
Technical Staffs:	*Takeshi Mizutani*
Institute Research Fellow:	*Hiroki Okumura*
Post doctoral fellow:	*David Buchanan*
Graduate Students:	*Hiroshi Urushitani* [1)]
	Satomi Khono[1)]

[1)] *Graduate School of Yokohama City University*

Synthetic chemicals found in the environment have the capacity to disrupt endocrine system development and function in both wildlife and humans. This has drawn public concern since many of these chemicals may bind to estrogen receptors and evoke estrogenic effects. Early evidence that estrogenic chemicals could pose a threat to human health during development came from studies of diethylstilbestrol (DES), which was used to prevent premature birth and spontaneous abortion. Laboratory experiments have demonstrated that exposure of animals to sex hormones during perinatal life can cause permanent and irreversible alterations of the endocrine and reproductive systems as well as the immune system, nervous system, bone, muscle, and liver in both sexes. Although many of these chemicals may bind to estrogen receptors and evoke estrogenic effects in wildlife and humans, the effects of estrogen are not well understood even now. Thus, understanding the effects of sex hormones at the molecular level, especially during development, is very important to resolve these problems.

I Estrogen-induced irreversible changes

Perinatal sex-hormone exposure has been found to induce lesions in reproductive tracts in female mice. The possible relevance of the mouse findings to the development of cancer in humans has been emphasized. In the early seventies, a close correlation between occurrence of vaginal clear cell adenocarcinoma in young women and early intrauterine exposure to diethylstilbestrol (DES) was demonstrated. Many chemicals released into the environment have the potential to disrupt endocrine function in wildlife and humans. Some of these chemicals induce estrogenic activity by binding to the estrogen receptor (ER). The neonatal mouse model has been utilized especially to demonstrate the long-term effects of early sex hormone exposure on the female reproductive tract. Neonatal treatment of female mice with estrogens induces various abnormalities of the reproductive tract: ovary-independent cervicovaginal keratinization, adenosis, uterine hypoplasia, epithelial metaplasia, oviductal tumors, polyovular follicles and polyfollicular ovaries. Female reproductive tracts in mice exposed prenatally to estrogen show altered expression of Hoxa genes and Wnt genes and the analysis of knockout mice lacking Hoxa-10 or Wnt7a show uterine hypoplasia. The growth response of neonatally DES-exposed reproductive organs to estrogen is reduced, as are ER levels and EGF receptor levels, in addition to other hormone receptor levels.

Estrogenic compounds such as bisphenol A (BPA) and nonylphenol as well as dioxins and PCBs were found in the human umbilical cord. BPA can easily cross the placenta and enter the fetus in Japanese monkey and mice. Bisphenol-A (BPA) can be found in fetal brain, testis and uterus when given to pregnant mice. Neonatal exposure to a high BPA dose induced ovary-independent vaginal changes, polyovular follicles and infertility lacking corpora lutera. Thus, the developing mammal is sensitive to exposure to estrogenic agents as evident by the induction of long-term changes in female reproductive organs.

In order to clarify the molecular mechanisms of these effects, we are studying changes in gene expression patterns induced by perinatal exposure to chemicals or estrogen using differential display and DNA microarry techiniques. We have found genes possibly related to the ovary-independent changes by differential display. We also have clustered groups of genes that are respon-

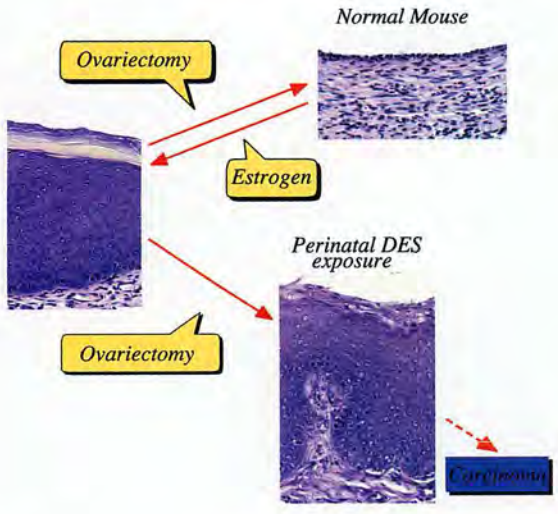

FIG. 1

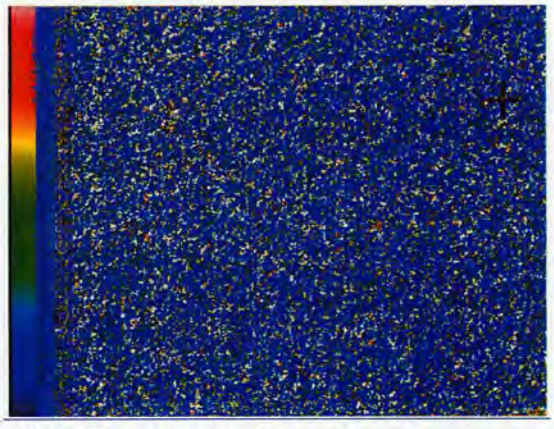

FIG. 2 Fluorescence image of an array

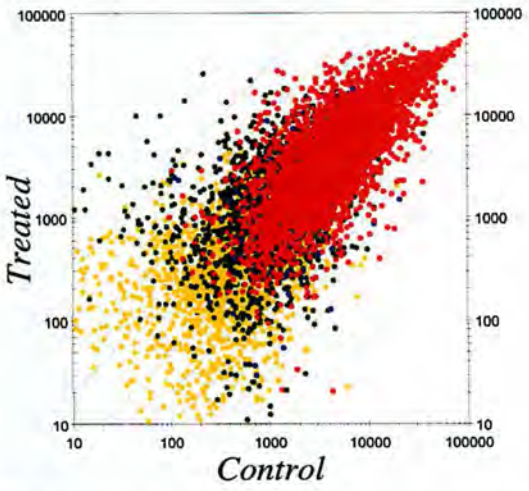

FIG.3 Scatter plot of average expression levels in control and chemical-treated uterus

sive to estrogenic stimuli in uterus by using the DNA microarray system. We need to understand the molecular background of the critical period during development, the low dose effect of estrogenic chemicals and the molecular metabolism of hormones and hormone-like agents in animals including humans.

II Effect of estrogen on amphibian and fishes.

During embryogenesis, exogenous estrogen exposure induces abnormal sex differentiation and the abnormal bone formation in African clawed frog, *Xenopus laevis* and the cyprinodont fish, mummichog (*Fundulus heteroclitus*). In these animals estrogen receptor is present in the embryo. To analyze the function of estrogen, we have isolated cDNA clones of estrogen receptor α and β. The estrogen-responsive genes must play important roles. So, we try to isolate the estrogen-responsive genes to understand the molecular physiology of estrogen action. Vitellogenin has been well characterized in avian, amphibian, and fish as a precursor of egg yolk. As the vitellogenin gene is responsive to estrogen, we can examine the effect of endocrine disruptors in the environment using a vitellogenin-specific and sensitive enzyme-linked immunosorbent assay (ELISA). Japanese tree frog (*hyla japonica*) takes water through ventral skin. We found that sex steroids and endocrine disruptors interfere with water absorption through ventral skin in frogs. Further, using the amphibian and fish as model animals we aim to analyze the effects of numerous chemicals released into the environment on endocrine system function in wildlife.

III Molecular Target Search

Abnormalities caused by endocrine disrupting chemicals are reported but the molecular mechanisms of the effects are not well studied. Although estrogen receptor is one of the strongest candidates possibly responsible for the endocrine disrupting function of many chemicals, it alone cannot explain the variety of phenomena induced by endocrine disrupting chemicals. Thus, we are also looking for new target molecules that may be responsible for endocrine disruption. In parallel, we also are studying the ligand-binding mechanisms of nuclear receptors to hormones and other chemicals using Surface Plasmon Resonance technology.

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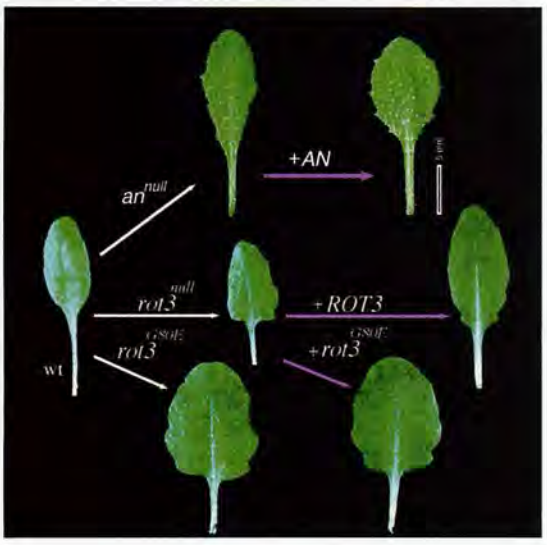
DEPARTMENT OF BIOENVIRONMENTAL RESEARCH II

Associate Professor: *Hirokazu Tsukaya, Ph.D.*
Assistant Professor: *Gyung-Tae Kim, Ph.D.*
Technical Staff: *Makiko Kondo*
Post Doctoral Fellow: *Kiuhyon Cho, Ph.D.*
Graduate Student: *Takeshi Furuhashi (Nagoya Univ.)*
Secretary *Kazuko Kabeya*

Diversity of plant form is mostly attributable to variation of leaf and floral organs, which are modified, leaves. The leaf is the fundamental unit of the shoot system, which is composed with leaf and stem. So the leaf is the key organ for a full understanding of plant morphogenesis. However, the genetic control of development of these shapes had remained unclear. Recently, studies of leaf morphogenesis has been in a turning point, after our successful application of the techniques of developmental and molecular genetics to it, using model plants, *Arabidopsis thaliana* (L.) Heynh. Our purpose is to understand Plants from view point of molecular genetic control of leaf morphogenesis.

Focusing on mechanisms that govern polarized growth of leaves in a model plant, *Arabidopsis thaliana*, we found that the two genes act independently to each other on the processes of polar growth of leaves: the *AN* gene regulates width of leaves and the *ROT3* gene regulates length of leaves. The *AN* gene controls the width of leaf blades and the *ROT3* gene controls length. The *AN* gene seems to control orientation of cortical microtubules in leaf cells. Cloning of the *AN* gene revealed that the gene is a member of gene family found from animal kingdom (Tsukaya et al., in prep). The *ROT3* gene was cloned by us in 1998. Transgenic experiments proved that the *ROT3* gene regulates leaf-length without affect on leaf-width (Kim et al., 1999). We are trying to identify molecular function of the above genes which are essential for leaf morphogenesis.



While *ROT3* regulates the length of both leaf blades and petioles, *ACL2* appears to regulate petiole length exclusively. Genes for perception of environmental stimuli such as light and/or phytohormone perception also affect the petiole length relative to the length of the leaf blade. Genetic studies suggested that petioles and leaf blades share some regulatory pathways but petioles also have their own developmental programs that are independent of those of leaf blades (Tsukaya and Kim, submitted).

Apart from polar elongation, we identified the following genes involved in leaf expansion process. The *AS1* and *AS2* genes are needed for proportional growth of the leaf. Molecular and anatomical analysis of the *as2* mutant is now underway, in collaboration with Prof. Machida, Nagoya University (Endang et al., submitted).

On the other hand, we are trying to identify molecular mechanisms which distinguish developmental pathway of leaves from that of shoots. For such purposes, we introduced tropical plants having queer developmental program for leaf morphogenesis, namely, *Chisocheton, Guarea* and *Monophyllaea*, as materials for molecular studies.

In addition, we are interested in roles of such genes for environmental adaptation, from view point of biodiversity. Leaf index, relative length of leaf to width, is one of the most diverse factor of leaf shape. For instance, rheophytes are characterized by narrow leaves, which represent an adaptation to their habitats. Are *AN* and *ROT3* genes are involved in regulation of adaptive change of leaf index in natural condition? Are these genes the responsible for evolution of rheophytes? So called "Evo/Devo" study of leaf morphogenesis is also one of our research project in NIBB.

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DEPARTMENT OF BIOENVIRONMENTAL RESEARCH III

Professor (Adjunct):Minoru Kanehisa (Kyoto University)

Though intermediary metabolism common to most organisms has been deeply investigated so far, variety of species specific pathways in secondary metabolism, which may work only in specific environmental conditions, are still unclear. The aim of this laboratory is to develop a database system for environmental biology, which integrates knowledge about organic compounds, chemical reactions between these compounds *in vivo*, enzymes (genes) involved in these reactions, and species whose genomes contain these genes. Through this database combining with data from transcriptome or proteome analyses in various environmental conditions, we intend to elucidate the principle of interactions between organisms and environmental chemical compounds to predict or design novel interactions.

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RESEARCH SUPPORT

CENTER FOR TRANSGENIC ANIMALS AND PLANTS

Head: Masaharu Noda

Associate Professor: *Eiji Watanabe*
Supporting Staff: *Mie Yasuda*
Ayako Tozaki (June 1, 2000~)

I. Research supporting activity

NIBB Center for Transgenic Animals and Plants to established in April 1998 to support researches using transgenic and gene targeting techniques in NIBB. We are now planning on the construction of the center building.

The expected activities of the Center are as follows:

1. Provision of information, materials and techniques to researchers.
2. Equipment of various instruments to analyze mutant animals and plants.
3. Development of novel techniques related to transgenic and gene targeting technology.

II. Academic activity

We are studying the functional role of Na_v2 ion channel in collaboration with Division of Molecular Neurobiology. Na_v2 belongs to a group of voltage-gated sodium channels (NaChs) that serve to generate action potentials in electrically excitable cells such as neuronal and muscle cells. Comparing with the other NaChs, Na_v2 has unique amino acid sequences in the regions, which are known to be involved in ion selectivity and voltage-dependent activation and inactivation, suggesting that it must have specific functional properties. To clarify the functional role of Na_v2 *in vivo*, the Na_v2-deficient mice were generated by gene targeting and the physiological phenotypes have been examined. It was suggested that the Na_v2 channel plays an important role in the central sensing of body-fluid sodium level and regulation of salt intake behavior. Details of this study are described in the part of Division of Molecular Neurobiology.

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Watanabe, E., Fujikawa, A., Matsunaga, H., Yasoshima, Y., Sako, N., Yamamoto, T., Saegusa, C., Noda, M. (2000) Na_v2/NaG channel is involved in control of salt intake behavior in the CNS. *J. Neurosci.* 20, 7743-7751.

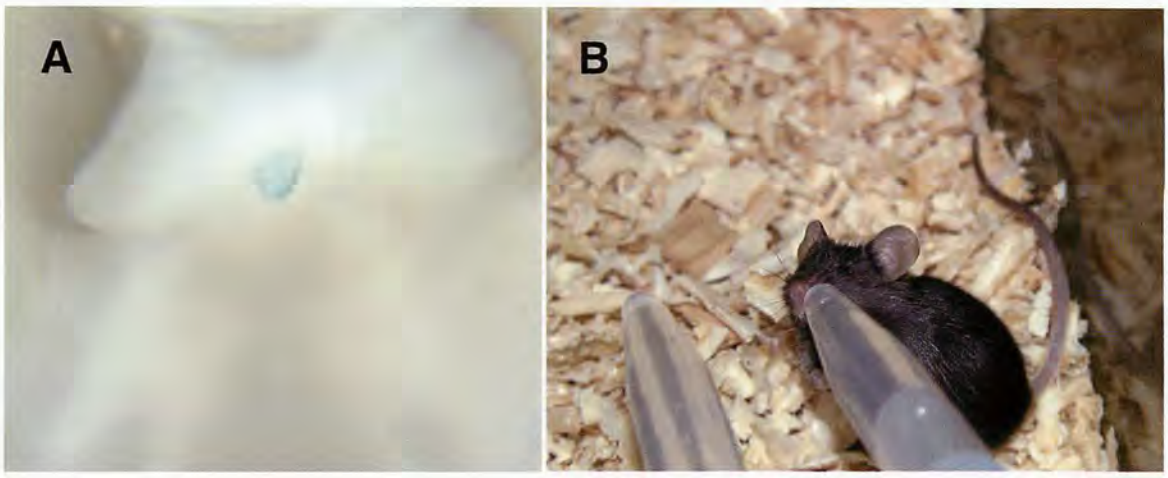


Fig. 1 Na_v2 channel is expressed in specialized neurons and ependymal cells in the adult CNS (A), and the null mutant shows an abnormal ingestion of hypertonic saline (B).
The expression pattern of mouse Na_v2 channel gene was revealed by *lacZ* gene expression in Na_v2+/- mice. The blue signals represent the cells expressing *lacZ* gene. Fig. 1A shows the specific expression of Na_v2 in the subfornical organ. The null mutants showed excessive ingestion of hypertonic saline under both thirst and acute salt appetite conditions. Fig. 1B shows the null mutant ingesting hypertonic saline under the thirst condition.

TECHNOLOGY DEPARTMENT

Head:Hiroyuki Hattori

Common Facility Group

Chief : Kazuhiko Furukawa

Reseach Support Facilities
Shoichi Higashi(Unit Chief)
Chieko Nanba(Subunit Chief)
Hiroyo Nishide
Makiko Itoh(Technical Assistant)
Keiko Suzuki(Technical Assistant)
Yasuyo Kamiya(Technical Assistant)
Yumi Hashimoto (Technical Assistant)
Ayako Tosaki(Technical Assistant)
Nobuko Hattori (Technical Assistant)
Yukiko Tanigawa(Technical Assistant)

Radioisotope Facility
Yoshimi Matsuda(Unit Chief)
Yousuke Kato(Subunit Chief)
Naoki Morooka
Takayo Itoh(Technical Assistant)

Center for Analytical Instruments
Sonoko Ohsawa(Unit Chief)
Tomoko Mori(Subunit Chief)
Yumiko Makino
Hatsumi Moribe(Technical Assistant)

Glassware Washing Facility
(Tomoko Mori)
(Kazuhiko Furukawa)

Research Support Group

Chief : Hiroko Kobayashi

Cell Biology Group
Maki Kondo(Unit Chief)
Yukiko Kabeya

Developmental Biology Group
Chiyo Takagi
Sanae Oka
Chiyo Noda

Regulation Biology Group
Hideko Iinuma
Katsushi Yamaguchi
Yasushi Takeuchi
Shigemi Takami
Kaoru Yamada

Gene Expression and Regulation Group
Tomoki Miwa(Unit Chief)
Sachiko Tanaka(Subunit Chief)
Kaoru Sawada(Subunit Chief)
Koji Hayashi
Hideko Utsumi
Naomi Sumikawa

Integrated Bioscience Group
Takeshi Mizutani
Makiko Kondo(Technical Assistant)

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 institutes throughout Japan. At this meeting, the participants present their own activities and discuss technical problems.The Proceedings are published soon after the meeting.

RESEARCH SUPPORT FACILITY

Head of Facility: *Norio Murata*
Associate Professor: *Masakatsu Watanabe*
Research Associates: *Yoshio Hamada (Tissue and Cell Culture)*
Ikuo Uchiyama (Computer)
Technical Staff: *Sho-ichi Higashi (Large Spectrograph)*
Tomoki Miwa (Computer)
Chieko Nanba (Plant Culture, Farm, Plant Cell)
Hiroyo Nishide (Computer)
Nobuko Hattori (Large Spectrograph)
Makiko Ito (Large Spectrograph)
Yasuyo Kamiya (Tissue and Cell Culture)
Misayo Masuda (Computer)
Keiko Suzuki (Plant Culture, Farm, Plant Cell)

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 200 $\mu m\ s^{-1}$ in tracking speed, down to 2 μm in beam diameter) with an infrared phase-contrast observation system.

2. *Tissue and Cell Culture Laboratory*

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

3. *Computer Laboratory*

Computer laboratory maintains several computers to provide computation resources and means of electronic communication in this Institute. Currently, the main system consists of three servers and two terminal workstations: biological information analysis server (SGI Origin 2000), database server (Sun Enterprise 450), file server (Sun Enterprise 3000), data visualization terminal and molecular simulation terminal (both are SGI Octanes). Some personal computers and color/monochrome printers are also equipped. On this system, we provide various biological databases and data retrieval/analysis programs, and support large-scale data analysis and database construction for the Institute members.

Computer laboratory also provides network communication services in the Institute. Most of PCs in each laboratory as well as all of the above service machines are connected each other with local area network (LAN), which is linked to the high performance multimedia backbone network of Okazaki National Research Institute (ORION). Many local services including sequence analysis service, file sharing service and printer service are provided through this LAN. We also maintain a public World Wide Web server that contains the NIBB home pages (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 50 t) and several smaller tanks. The laboratory also includes a building with office, storage and work space.

6. *Plant Cell Laboratory*

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

7. *Laboratory of Stress-Resistant Plants*

This laboratory was founded to study transgenic plants with respect to tolerance toward various enviromental stresses. It is located in the Agricultural Experimental

Station of Nagoya University (30 km from 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 unicellular 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. Photo-receptive and signal transduction mechanisms of algal gene expression were also studied by action spectroscopy.

(2) Developmental Biology: Replacement of the ankyrin repeats of mouse Notch2 gene with E.coli b-gactosidase gene induces early embryonic lethality around E10.5. The lethality was suggested due to defects in extraembryonic tissues, because the mutant embryo grew and differentiated further in vitro. Histological examination and in situ hybridization analysis with trophoblast subtype-specific probes revealed that the development of giant and spongiotrophoblast cell layers are normal in the mutant placenta, while vasculogenesis in the labyrinth layer apperaed compromised at E9.5. Since the lethality was circumvented by production o

f chimeric mice with tetraploidy wild type embryos, we concluded that the embryonic lethality is due to defect in growth and/or differentiation of labyrinthine trophoblast cells. The mutant embryo, however, could not be rescued

in the tetraploid chimeras beyond E12.5 because of insurfficient development

of umbilical cord, indicating another role of Notch2 signaling in the mouse development. Chimeric analysis with diploid wild type, however, revealed contribution of mutant cells to these affected tissues by E13.5. Thus, Notch2 are not cell autonomously required for the early cell fate determination of labyrinthine trophoblast cells and allantoic mesodermal cells, but plays an indispensable role in the further formation of functional labyrinth layer andumbilical cord.

(3) Computational Biology: Comparative genomics is a useful approach to find clues to understanding complex and diverse biological systems from rapidly growing genome database. We have constructed a database system for comparative analysis of many of microbial genomes ever sequenced and are developing new computational techniques for large-scale genome sequence comparison. Especially, we are developing a method for orthologous grouping among multiple genomes, which is a crucial step for comparative genomics. Since considerable number of genes consist of multiple domains, we have developed a hierarchical clustering algorithm that can automatically split fusion genes into orthologous domains.

In parallel, we are developing a tool to incorporate various sequence features such as G+C contents, codon usage bias and locations of repetitive elements into the genome comparison. By this approach, we make detailed comparison of closely related microbial genomes to investigate the genomic polymorphisms or evolutionary changes in collaboration with Dr. I. Kobayashi's group (Univ. Tokyo). By comparing genomes of two *Helicobacter pyroli* strains and two *Pyrococcus* species, we could find interesting insertion/deletion patterns that frequently include restriction-modification genes.

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*

Chinen A., Uchiyama, I., Kobayashi, I. (2000). Comparison between *Pyrococcus horikoshii* and *Pyrococcus abyssi* genome sequences reveals linkage of restriction-modification genes with large genome polymorphisms. *Gene* **259**, 109-121.

Kumano, K ., Saito, T., Kurokawa, M.m Kanda, Y., Hamada, Y. and Hirai, H. (2000) Binding of Delta1, Jagged1, and Jagged2 to Notch2 rapidly induces cleavage, nuclear translocation, and hyperphosphorylation of Notch2. *Mol. Cell. Biol.* **20**, 6913-6922.

Nobusato, A., Uchiyama, I., Kobayashi, I. (2000). Diversity of restriction-modification gene homologues in *Helicobacter pylori. Gene* **259**, 89-98

Nobusato, A., Uchiyama, I., Ohashi, S., Kobayashi, I. (2000). Insertion with long target duplication: a mechanism for gene mobility suggested from comparison of two related

bacterial genomes. *Gene* **259**, 99-108.

Uchiyama, I. (2000). Hierarchical clusterng procedure for grouping orthologous domains in multiple genomes. *Currents in Computational Molecular Biology*, 146-147.

Uchiyama, I., Miwa, T., Nishide, H., Suzuki, I., Omata, T., Ikeuchi, M., Murata, N., Kanehisa, M. (2000). Data submission system for cyanobacterial DNA chip consortium, *Genome Informatics* **11**, 235-236.

Uchiyama, I., Higuchi, T., Kobayashi, I. (2000). CGAT: Comparative genome analysis tool for closely related microbial genomes. *Genome Informatics* **11**, 341-342.

Takinami, S., Mochizuki, M., Hayatsu, H., Nikaido, O., Kubota, M., Watanabe, M., Hieda, K. and Negishi, T. (2000) Somatic cell mutation and photoproduct formation in *Drosophila* induced by monochromatic UV light in sunlight, *Environmental Toxicology*, **15**, in press

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

Hada, M., Iida,Y.,and Takeuchi,Y.(2000). Action spectra of DNA photolyases for photorepair of cyclobutane pyrimidine dimers in sorghum and cucumber. *Plant Cell Physiol.* **45**: 644-648 .

Kawai, H. and Kreimer, G. (2000). Sensory mechanisms: Phototaxes and light perception in algae. In "The Flagellates: Unity, Diversity and Evolution." (Edited by J. Green and B.S.C. Leadbeater), pp. 124-146. Taylor & Francis Publishing.

Shinomura, T., Uchida, K., and Fruuya, M. (2000). Elementary process of photoperception by phytochorome A for high-irradiance response of hypocotyl elongation in *Arabidopsis*. *Plant Physiol.* **122**, 147-156.

Tada, Y., Wakasugi, T., Nishikawa, A., Furuhashi K. and Yamada K. (2000) Developmental regulation of a gene coding for a low-molecular-weight heat shock protein during haustorium formation in the seedlings of a holoparasitic plant, *Cuscuta japonica. Plant Cell Phisiol.* **41**, 1373-1380.

Takeuchi, Y., Iida, Y., Nakajima, N. and Nikaido, O. (2000). Formation of DNA lesions in cucumber cotyledons exposed to solar UV radiation. *Environ. Sci.* **13**, 351-355

Takinami, S., Mochizuki, M., Hayatsu, H., Nikaido, O., Kubota, M., Watanabe, M., Hieda, K. and Negishi, T. (2000) Somatic cell mutation and photoproduct formation in *Drosophila* induced by monochromatic UV light in sunlight, *Environmental Toxicology*, **15**, in press

Torikai, A., (2000), Wavelength sensitivity of photodegradation of polymers. In "Handbook of Polymer Degradation Second Ed.," (Edited by S. Halim Hamid), pp.573-603, Marcel Dekker, Inc. New York.

THE CENTER FOR ANALYTICAL INSTRUMENTS

(managed by NIBB)

Head of Facility: *Tetsuo Yamamori*
Technical Staffs: *Sonoko Ohsawa*
Tomoko Mori
Yumiko Makino
Technical Assistant: *Hatsumi Moribe*

The Center serves for amino acid sequence analysis, and chemical syntheses of peptides and nucleotids to support researchers in NIBB and NIPS. Instruments of the Center can be used by researchers outside the Institute upon proposal.

Figure 1. Procise 494 Protein Sequencer.

Figure 2. Biomek 2000 Laboratory Automation System.

Representative instruments are listed below.

Protein Sequencers (ABI Procise 494, ABI 473A)
Amino Acid Analyzer (Hitachi L8500A)
Peptide Synthesizers (ABI 433A, ABI 432A)
Plasmid Isolation Systems (Kurabo PI-100 Σ)
Automatic Nucleic Acid Isolation System (Kurabo NA-2000)
DNA Sequencers (ABI 377, 373S, ABI 310)
DNA/RNA Synthesizers (ABI 394, ABI 392)
Thermal Cyclers (Perkin Elmer PJ-9600, Takara TP-300)
Integrated Thermal Cyclers (ABI CATALYST Turbo 800)
Particle Delivery System (Bio-Rad BiolisticPDS-1000/He)
Gas Chromatograph (Shimadzu GC-14APF-SC)
Glycoprotein Analysis System (Takara Glyco-Tag)
High Performance Liquid Chromatographs (Shimadzu LC-10AD, 6AD, Waters 600E)
Integrated Micropurification System (Pharmacia SMART)
Flow Cytometer (Coulter EPICS XL)
Biomolecular Interaction Analysis Systems (Pharmacia BIACORE 2000, Affinity Sensors IAsys)
Laboratory Automation System (Beckman Coulter Biomek 2000)
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)
Bio Imaging Analyzers (Fujifilm BAS2000)
Fluorescence Bio Imaging Analyzer (Takara FMBIO)
Electrophoresis Imaging Systems (BIOIMAGE)
Microscopes (Carl Zeiss Axiophot, Axiovert)
Microscope Photometer (Carl Zeiss MPM 03-FL)

Center for Radioisotope Facilities (CRF)

Head (Professor, concurrent post): *Shigeru Iida*
Associate Professor: *Kazuo Ogawa*
Technical Staffs: *Yoshimi Matsuda (Radiation Protection Supervisor)*
Yosuke Kato (Radiation Protection Supervisor)
Naoki Morooka (Radiation Protection Supervisor)
Supporting Staff: *Takayo Ito*
Yumi Iida

I. Research supporting activity

In this year, the Radioisotope Facility managed by NIBB (National Institute for Basic Biology) was reorganized to the CRF which is included in one of the Common Research Centers belonged to ONRI (Okazaki National Research Institutes).

Technical and supporting staffs of the CRF are serving the purchase of radioisotopes from JRA (Japan Radioisotope Association) and the transfer of radioisotope wastes to JRA. The physical maintenance of the controlled areas where radioisotopes are used is also one of our business.

The CRF consists of four controlled areas: Center, NIBB-sub, LGER (Laboratory of Gene Expression and Regulation)-sub, and NIPS (National Institute for Physiological Science)-sub. Users going in and out the controlled areas counted by the monitoring system are 6,273 in 2000. This count is comparable to that (7,912) in 1999. The items in each controlled area is presented in Figure 1.

II. Academic activity

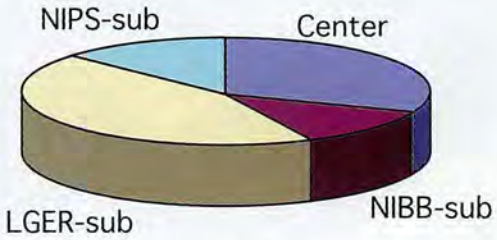
Academic activity by teaching staff is focused on the analysis of the structure and function of a dynein motor protein. Dyneins are a group of microtubule-activated ATPases that serve to convert chemical energy into mechanical energy and divided into axonemal and cytoplasmic dyneins. Figure 2 shows the localization of two isoforms of dynein in the outer arms of sperm axonemes (Ogawa et al., 1977) and the mitotic apparatus of cleaving egg (Mohri et al., 1976) visualized by anti-axonemal dynein (Fragement A) antibodies.

The native dyneins are very large and range in molecular mass up to 1 to 2 mega Da. They are complex proteins containing heavy, intermediate, and light chains defined by the molecular mass. Our prensent project is the molecular cloning of polypeptides contained in outer arm dynein of sea urchin sperm flagella to understand the mechanism how dynein interacts with microtubules, resulting in producing the force.

Outer arm dynein consists of two heavy chains with ATPase activity. The motor activity is closely related to this polypeptide. The first successful molecular cloning of this huge polypeptide (520 kDa) was performed in our laboratory in 1991. Since then cDNA clones for axonemal and cytoplasmic dyneins have been isolated in a variety of organisms. The sequences of heavy chains, without exception, contain four P-loop motives referred to as ATP-binding sites in the midregion of the molecules. Figure 3A and B draw the structure of heavy chain deduced from the amino acid sequence (Ogawa, 1992). Taking the recent works by Koonce et al. (1998) and Vallee et al. (1998) into consideration, this model might be seen as depicted in Figure 3C. In particular, Vallee et al. (1998) have described the importance of a hairpin structure formed between M and C domains which binds to microtubules and presented a novel mechanism for dynein force production different from that of myosin and kinesin.

Outer arm dynein contains three intermediate chains (IC1, IC2, and IC3) that range in molecular mass from 70 to 120 kDa. IC2 and IC3 were cloned by Ogawa et al. (1995) and contain the WD repeats in the carboxy-terminal halves of the molecules. By contrast, IC1 is not a member of the WD family. IC1 has a unique sequence such that the N-terminal part is homologous to the sequence of thioredoxin, the middle part consists of three repetitive sequences homologous to the sequence of NDP kinase, and the C-terminal part contains a high proportion of negatively charged glutamic acid residues (Ogawa et al., 1996). Thus, IC1 is a novel dynein intermediate chain distinct from IC2 and IC3 and may be a multifunctional protein.

Six light chains with molecular masses of 23.2, 20.8, 12.3, 11.5, 10.4, and 9.3 kDa are associating with outer arm dynein. We have already isolated cDNA clone of five LCs. LC1 (23.2 kDa) and LC3 (12.3 kDa) 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 germ cells. Our finding raises the possibility that axonemal dynein proteins are involved in this phenomenon. TRD may be caused by the dysfunction of multiple axonemal dynein proteins.



	Actual count
Center	1,917
NIBB-sub	756
LGER-sub	2,780
NIPS-sub	820
total	6,273

Figure 1. Percentage of users going in and out the controlled areas during April to December, 2000.

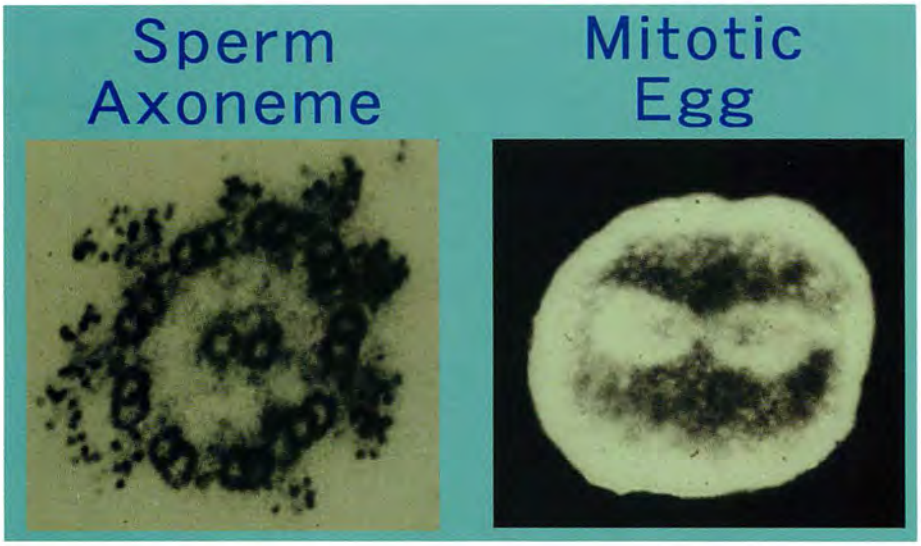


Figure 2. Localization of two dynein isotypes on outer arm of sperm axonemes and mitotic apparatus of cleaving egg.

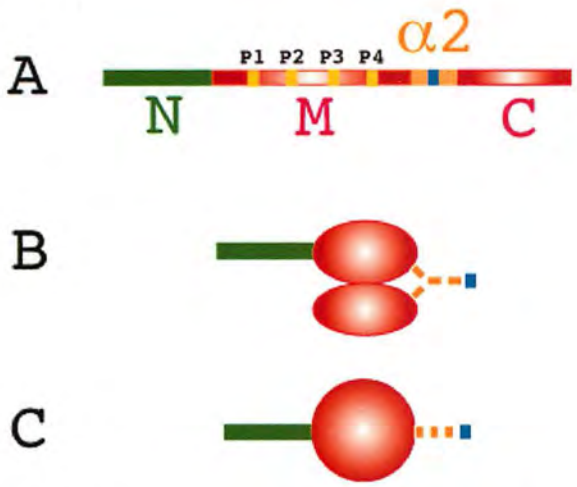
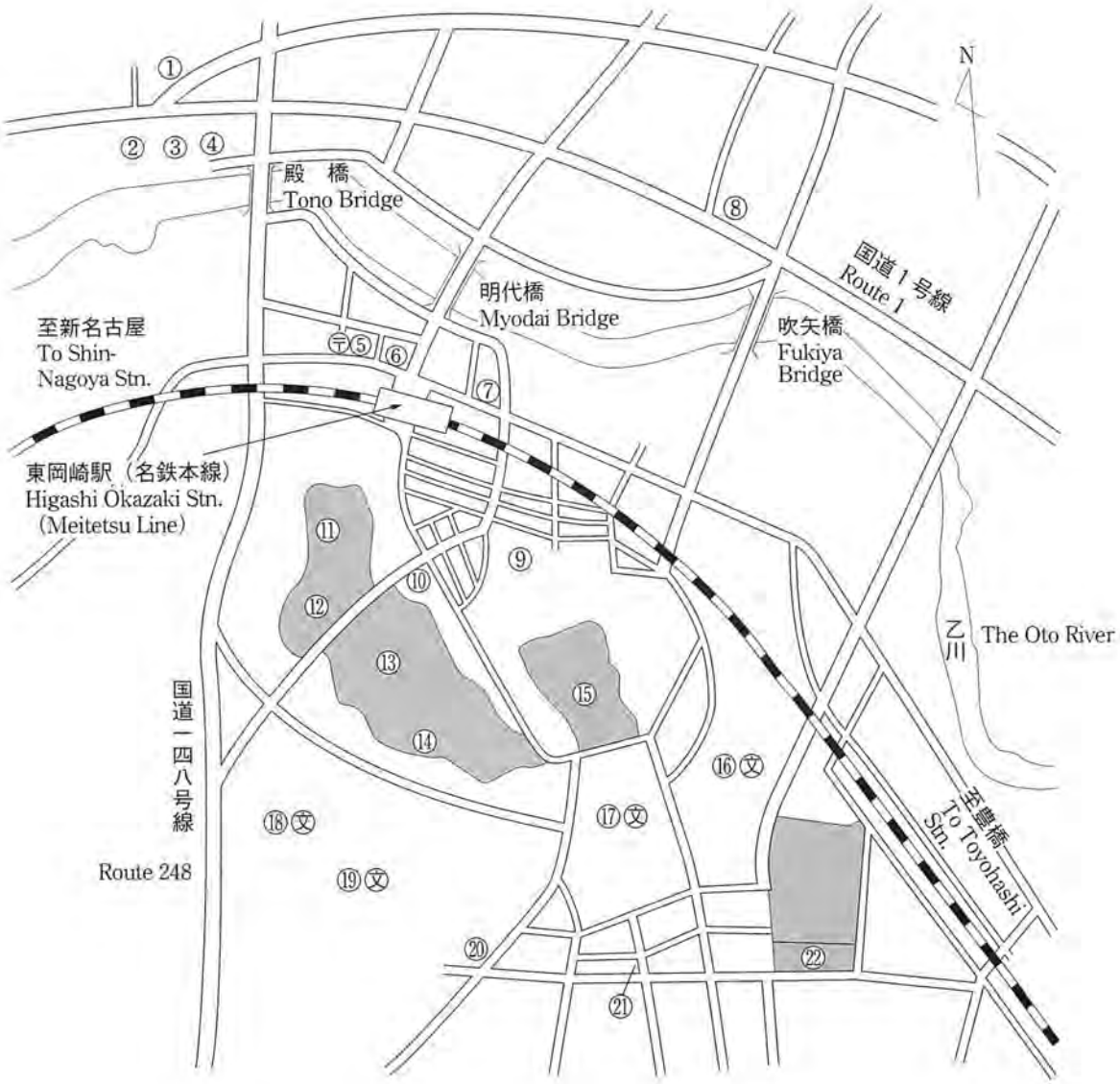


Figure 3. Structure of dynein heavy chain. A; Analysis of amino acid sequence of heavy chain reveals that it consists of three major domains referred to as N, M, and C from the N-terminus. B; M and C domains make larger domain (motor domain) by intramolecular association. C, According to Koonce et al. (1998) showthat recombinant motor domain would be spherical. Vallee et al. (1998) propose that a2 region corresponds to the B-link which is the stalk projected from the globular head structure of dynein, by demonstrating that the recombinant a2 actually binds to microtubules.



① 名鉄岡崎ホテル
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

⑤ 郵便局
Post Office

⑥ 銀行
Bank

⑦ スーパーマーケット“ユニー”
Super Market “Uny”

⑧ 市役所
Okazaki City Office

⑨ 六所神社
Rokusho Shrine

⑩ 岡崎国立共同研究機構（看板）
Signboard of Okazaki National Research Institutes

⑪ 基礎生物学研究所
National Institute of Basic Biology

⑫ 生理学研究所
National Institute for Physiological Sciences

⑬ 管理局
Administration Bureau

⑭ 分子科学研究所
Institute for Molecular Science

⑮ 三島ロッジ・岡崎コンファレンスセンター
Mishima Lodge ・Okazaki Conference Center

⑯ 三島小
Mishima Elementary School

⑰ 岡崎高校
Okazaki High School

⑱ 愛教大附中
Jr. High School affiliated to Aichi Univ. of Education

⑲ 竜海中
Ryukai Jr. High School

⑳ コンビニエンスストア
Convenience Store

㉑ 薬局
Pharmacy

㉒ 山手ロッジ
Yamate Lodge

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