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

1999

The cover photograph shows the expression of a T-box gene brachyury in early Xenopus laevis embryos. The radial expression is restricted to the equitorial region of embryo which represents presumptive mesoderm.

Post Doctral Fellow

1 NIBB Research Fellow

2 JSPS Postdoctral Fellow

3 JSPS Research Associate

4 JST Fellow

CONTENTS

INTRODUCTION

The National Institute for Basic Biology (NIBB), a government-supported, basic research institute, was established in 1977. As a center of excellence, NIBB aims to promote the biological sciences by conducting first-rate research on site as well as by cooperating 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 1999, the Center for Bio-environmental Science was established to investigate pollution and other adverse effects of the activities of human beings on the earth, including the so-called "environmental hormones" (endocrine disruptors). The Center, managed by NIBB, is operated jointly with the Institute for Molecular Science (IMS) and the National Institute for Physiological Sciences (NIPS). The Center will be located in the newly constructed center for biosciences in 2000. The Director-General of the NIBB currently acts as the head of the Center. In August, Professors Teizo Kitagawa (IMS) and Minoru Kanehisa (Kyoto University) were appointed Professor and Adjunct Professor of the Center, respectively. Drs. A. Hazama (NIPS) and H. Tsukaya (University of Tokyo) were promoted to Associate Professor of the Center in August and October, respectively. Associate Professor S. Saito (Nagoya University) was appointed Adjunct Associate Professor in November. Professor Taisei Iguchi (Yokohama Municipal University) is scheduled to join this Center soon.

In addition to establishing the Center for Bio-environmental Science, we have replaced 1 research associate, 5 institute research fellows and 1 technician with 7 research associates, 6 institute research fellows and 1 technician. In the past several years, the total number of personnel working at the NIBB, especially graduate students and post doctoral fellows, has increased from about 200 to 300. We would also like to congratulate Associate Professor Ikuko Nishimura of the Division of Cell Mechanism, Department of Cell Biology, on her promotion to Professor at Kyoto University in November.

As a national and 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 held the 42nd NIBB International Conference entitled "Tubulin; 30 Years Later" (Professors Y. Nagahama, R. Kamiya, I. Mabuchi and H. Hotani, organizers) in March to celebrate the thirtieth anniversary of the naming of tubulin

H. Mohri

by H. Mohri. In accordance with the "Memorandum of Understanding between the NIBB and the Biological Research Center of the Hungarian Academy of Sciences", a Japanese-Hungarian Bi-national Symposium entitled "Developmental and Environmental Control of Cell Differentiation" (Professors Naoto Ueno and Denes Dudits, organizers) was held in Szeged, Hungary in October with the support of the Japan Society for Promotion of Science.

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 continue our improvement, the activities and future plan of NIBB, especially in the field of animal science, were subjected to an external peer review. We welcome any suggestions concerning the research activities of NIBB.

Finally, I would like to congratulate Professor Norio Murata for being selected as a Foreign Member of the Hungarian Academy of Sciences.

Hideo Mohri, D.Sc.
Director-General

ORGANIZATION OF THE INSTITUTE

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

Policy and Decision Making

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

Administration

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

Research

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

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

In 1999 the Center for Bio-environmental Science has attached to NIBB.

Research Support Facility

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

Campus

The Okazaki National Research Institutes covers an area of 150,000m^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 facility were also completed in June, 1983. A building for Laboratory of Gene Expression and Regulation (2,577m^2) was newly built in December, 1996.

Department/Laboratry	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
Interim Center for Bio-environmental Science	

GRADUATE PROGRAMS

The NIBB carries out two graduate programs.

1. Graduate University tor Advanced Studies

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

The Department consists of the following Divisions and Fields:

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

2. Graduate Student Training Program

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

OFFICE OF DIRECTOR

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

Phylogeny of outer arm dynein in flagella and cilia

Hideo Mohri

Dynein is a motor protein essential for microtubule-dependent cell motility. In axonemes of flagella and cilia, outer arm and inner arm dyneins attach to outer doublet microtubules. The isolated outer arm dynein molecule is a three-headed bouquet in protists (e.g. *Chlamydomonas* and *Tetrahymena)*, whereas the molecule is two-headed in multicellular animals (e.g. Deuterostomia and Protostomia). The number of heads corresponds to the number of heavy chain subunits. Alpha, β and γ heavy chains compose the outer arm in protists and α and β heavy chains are present in the outer arm of multicellular animals. We examined the outer arm dynein isolated from sperm flagella of a sea anemone, a species located just prior to the divergence of the two main branches in the animal kingdom phylogenic tree. The outer arm was the two-headed molecule typical of multicellular animals. Fig.1 shows schematic drawings of both animal (metazoan) and protist (and some lower plant) flagella and cilia with special reference to the outer arms. Since molecular phylogenetic analyses of dynein heavy chains indicate that animal α and β heavy chains are related to protist γ and β heavy chains, respectively, topographic relationship among these heavy chains in the cross-sections are also indicated in the figure. Based on the phylogenetic tree of dynein genes, *Chlamydomonas* α and β appear to be produced by gene duplication. Combined with other phylogenetic analyses, it is plausible that there was (or is) the organism more ancient than *Chlamydomonas* and *Tetrahymena*, which has two-headed outer arm dynein and consequently, the third heavy chain acquired in protists may have been lost during the evolution of animals from their ancestral unicellular organisms.

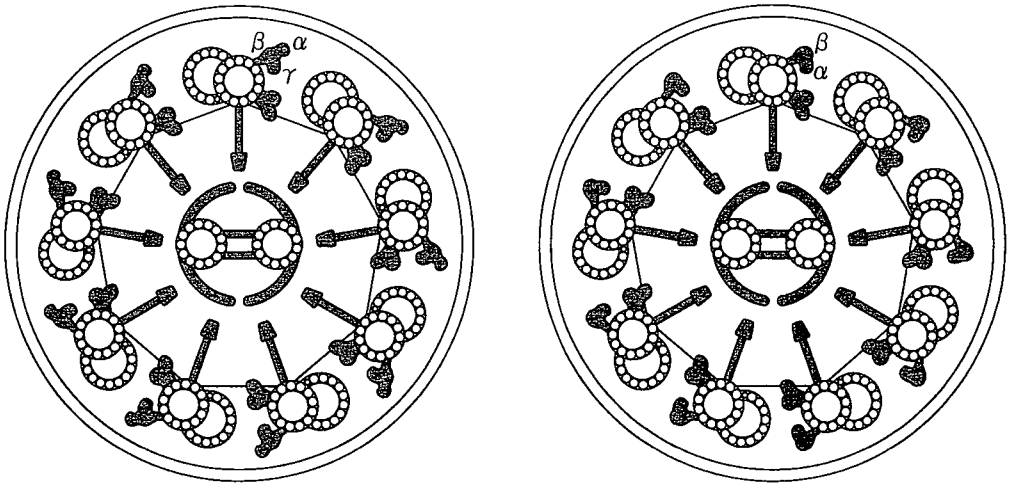

Fig.1. Schematic cross-sections of flagella or cilia in protists and lower plants (left) and in animals (right). Positions of each heavy chain subunit (head) are also shown.

Molecular evolution of photosynthesis.

Shigeru Itoh

We study the molecular evolution of photosynthesis through physicochemical research of various types of photosynthetic organisms. Oxygen-evolving photosynthesis of cyanobacteria seems to be evolved from the anoxygenic bacterial photosynthesis at 2.7-3.5 billion years ago just after the evolution of life. Symbiosis of cyanobateria inside the eukariotic cells, then, produced the first plant 2 billion years ago. The process of evolution from anoxygenic to oxygenic photosynthesis has been our main target of research.

(1)Discovery of anomalous photosynthesis.

Acaryochloris marina, a newly discovered oxygen-evolving cyanobacteria-like unicellular organism, was found to use a far-red absorbing new pigment chlorophyll *d* in its photosystem I reaction center pigment-protein complex in collaboration with Marine Biotechnology Institute Kamaishi (Fig.2). Although chlorophyll *a* that absorbs visible red light has been known to be indispensable for the oxygenic photosynthesis of all the plants and cyanobacteria, *A. marina* efficiently undergoes oxygenic photosynthesis even with far-red light of lower quantum energy compared to the red light absorbed by chlorophyll *a*. We named the special pair chlorophyll *d* of the newly identified photosystem I reaction center *P740*. The organism can be a missing link between the anoxygenic photosynthesis that uses 800-860 nm far-red light and the oxygenic photosynthesis that uses 650-700 nm red light.

Photosynthesis in a newly discovered bacterium *Acidiphilium rubrum* isolated from acidic mine drainage, was also shown to be quite different from the photosynthesis in all the ever-known oxygenic and anoxygenic photosynthesis. *A. rubrum* uses Zn-containing bacteriochlorophyll in its anoxygenic photosynthesis. This was the first case of photosynthesis based on pigments other than chlorophylls that are Mg-containing pigments. These new organisms show the wide variability of photosynthesis.

(2) Evolution of optimization mechanism of photosynthesis.

Structure-function relationships of the photosynthetic light reaction in the newly found organisms as well as plants and in anaerobic green sulfur bacteria *Chlorobium* and *Heliobacteria,* are studied by modern physicochemical techniques. We modified the reaction center complexes of these organisms by replacing quinone (vitamin K1) or chlorophyll cofactors by the artificial compounds or by site-directed mutagenesis, and studied the ultra-fast reaction kinetics by picosecond laser fluorometry, spectroscopy and spin-echo pulse ESR spectroscopy at 4 -280 K. Molecular architectures of plant and bacterial photosynthetic reaction center proteins are shown to be highly optimized in utilization of solar energy, however, in somewhat different direction in each case. The design of ancestral photosynthetic apparatus that is now lost, is to be traced based on new findings.

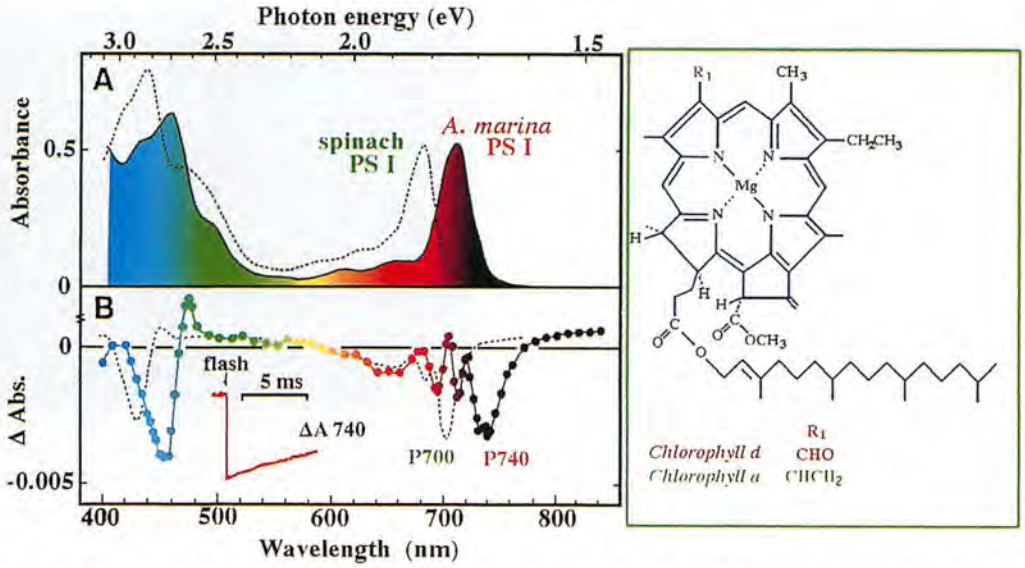

Fig.2. Absorption spectrum (A) and light-induced difference absorption spectrum of reaction center chlorophyll P740(B) of newly identified photosystem I (PS I) reaction center complex of *Acaryochloris marina* that has far-red absorbing chlorophyll *d*. Broken lines in (A) and (B) represent absorption and difference spectra (P700) in plant (spinach) PS I reaction center containing chlorophyll *a*, respectively. Molecular structures of chlorophylls *a* and *d* are also shown.

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 lepidopteren 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.

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 Fig.3 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.3. The tracheoles (fine threads) and the primary trachea (thick tube in the center) at the late stage of the pre-pupa.

Protein palmitoylation and developmental mechanism at embryogenesis in invertebrate and vertebrate

Kohji Ueno

We have studied the molecular mechanisms of the development of cells and organs in the silkworm *Bombyx mori* and have elucidated that the abdominal leg development was regulated by a homeotic gene which specifies the identities of abdominal segments. We 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 thought to be important in regulation of signal transduction. Therefore we speculated that p260/270 may be involved in regulation of signal transduction and may function in abdominal leg development.

To better 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. This result suggested that a homolog of p260/270 was expressed in mouse embryos. *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. The transcripts were detected in the regions of forebrain, midbrain, hindbrain and spinal cord in the central nervous system and also detected in the cranial ganglia and dorsal root ganglia in the peripheral nervous systems. Immunocytochemical analyses of cultured mouse primary embryonic brain cells were performed to identify which cells express mouse p260/270 homolog. This analysis revealed that mouse homolog was expressed specifically in neural cells, but not in neuroepithelial and glial cells. Fig.4 showed a result of immunocytochemical analysis of primary cultured cells. From these results, we speculate that a protein palmitoylase may function in the development of neural cells in the central and peripheral nervous system during mouse embryogenesis.

We found that protein palmitoylase were expressed specifically in a few types of cells during early embryogenesis in invertebrate and vertebrate. Further study is necessary to elucidate the common mechanism how the modification of protein palmitoylation regulates the development of the cells in invertebrate and vertebrate.

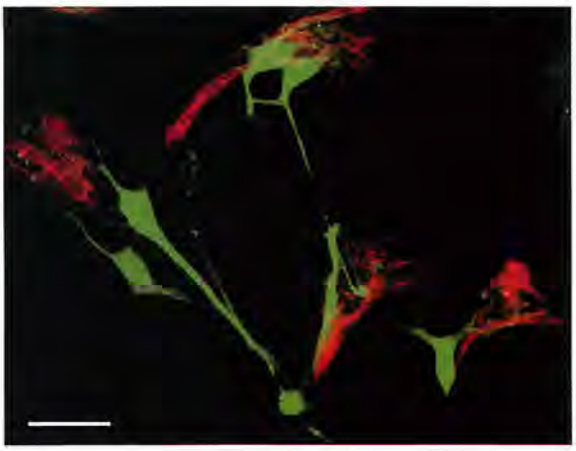

Fig.4. Immunocytochemical analysis of cultured primary cells. Cells were double labeled with an antibody against mouse p260/270 homolog and anti-nestin antibody. Mouse homolog (green) was detected in neurons whereas nestin (red) was detected in neuroepithelial cells. Bar represents 25μm.

Publication Lists:

Akiyama, M., Kobayashi, M., Kise, H., Takaichi, S., Watanabe, T., Shimada, K., Iwaki, M., Itoh, S., Ishida, N., Koizumi, M., Kano, H., Wakao, N. and Hiraishi, A. (1999) *Acidiphilium rubrum* and Zinc-bacteriochlorophyll part I: Molecular structure of the zinc-containing bacteriochlorophyll. In *"Photosynthesis:Mechanism and Effects"* (ed. Garab, G.), pp. 731-734. Kluwer.

Hu.Q., Ishikawa, T., Inoue, Y., Iwasaki, I., Miyashita, H., Kurano, N., Miyachi, S., Iwaki, M. and Itoh, S. (1999) Heterogeneity of chlorophyll *d*-binding photosystem I reaction centers from the photosynthetic prokaryote *Acaryochloris marina*. In *"Photosynthesis: Mechanism and Effects"* (ed. Garab,G.), pp. 437-440. Kluwer.

Itoh, S., Iwaki, M., Hu, Q., Miyashita, H. and Miyachi, S. (1999) A novel photosystem type reaction center from oxygenic prokaryote *Acaryochloris marina* that contains chlorophyll *d*. In *"The Photosynthetic prokaryotes"* (eds. Peschek et al.), pp. 183-186. Kluwer.

Itoh, S., Iwaki, M., Hu, Q., Miyashita, H., Iwasaki, I., Kurano, N., Miyachi, S., Kobayashi, M., and Shimada, K. (1999) New types of reaction centers using chlorophyll-*d* and Zn-bacteriochlorophyll *a*. In *"Photosynthesis: Mechanism and Effects"* (ed. Garab, G.), pp. 521-526. Kluwer

Iwaki, M., Itoh, S., Kamei, S., Matsubara, H. and Oh-oka, H. (1999) Time-resolved spectroscopy of chlorophyll-a like electron acceptor in the reaction center complex of the green sulfur bacterium *Chlorobium tepidum*. *Plant and Cell Physiol.*, **40**, 1021-1028.

Kubo-Irie, M., Ohta, K., Meguro, T., Nakazawa, T. and Mohri, H. (1999) Morphological evidence that spermatogenic cells undergo apoptosis within testis of diapausing swallowtail pupa (*Atropheneura alcinous*). *J. Exp. Zool.***283**, 194-201.

Matsunami, K., Kokubo, H., Ohno, K., Xu, P.-x., Ueno K. and Suzuki, Y. (1999) Embryonic silk gland development in *Bombyx*: molecular cloning and expression of the *Bombyx trachealess* gene. Dev. Genes Evol. **209**, 507 – 514.

Mohri, H. (1999) Tubulin 30 Years Later. *Cell Struct. Funct.* **24**, 265-267.

Mohri, H., Inaba, K., Kubo-Irie, M., Takai, H. and Yano-Toyoshima, Y. (1999) Characterization of outer arm dynein in sea anemone, *Anthopleura midori*. *Cell Motil. Cytoskel.* **44**, 202-208.

Noguchi, T., Kamimura, Y., Inoue, Y. and Itoh, S. (1999) Photoconversion of a water-soluble chlorophyll protein from *Chenopodium Album*: Resonance Raman and FTIR study of protein and pigment structures. *Plant and Cell Physiol.*, **40**, 305-310.

Oh-oka, H. Iwaki, M., and Itoh, S. (1999) The tight coupling of quinol oxidoreductase to the P840 reaction center complex in *Chlorobium*. In *"Photosynthesis:Mechanism and Effects"* (ed. Garab, G.), pp. 1525-1528. Kluwer.

Shimada, K., Itoh, S., Iwaki, M., Nagashima, K. V. P., Matsuura, K. and Kobayashi, M., Wakao, N. (1999) Reaction center complex based on Zn-bacteriochlorophyll from *Acidiphilium rubrum*. In *"Photosynthesis: Mechanism and Effects"* (ed. Garab G.), pp. 909-912. Kluwer.

Wakao, N., Hiraishi, A., Shimada, K., Kobayashi, M., Takaichi, S., Iwaki, M. & Itoh, S. (1999) Discovery, characteristics, and distribution of Zn-BChl in aerobic acidophilic bacteria including *Acidiphilium* species and other related acidophilic bacteria. In *" The Photosynthetic prokaryotes"* (eds. Peschek et al.), pp. 745-750. Kluwer.

Yoshii, T., Hara, H., Kawamori, A., Akabori K., Iwaki, M. & Itoh, S. (1999) ESEEM study of the location of spin-polarized chlorophyll-quinone radical pair in membrane-oriented spinach photosystems I and II complexes. *Appl. Magn. Reson.* **16**, 565-580.

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:	*Ikuko Hara-Nishimura (- Sept. 30))*
Research Associates:	*Makoto Hayashi (- March 31)*
	Tomoo Shimada
	Shoji Mano (July 1-)
Technical Staffs:	*Maki Kondo*
	Katsushi Yamaguchi
Post doctoral fellows:	*Tetsu Kinoshita (- March 31)*
	Yasuko Hayashi-Ishimaru
	Kanae Shirahama
	Shoji Mano (-June 30)
	Hiroshi Hayashi (April 1-)
	Kenji Yamada (April 1-)
Graduate Students:	*Naoto Mitsuhashi*
	Kazumasa Nito
	Etsuko Watanabe (April 1-)
	Yuki Tachibe
	(from Hiroshima University)
	Youichiro Fukao
	(from Fukui Pref. Univ.)
JSPS Technical Staffs:	*Miwa Kuroyanagi*
	Miki Kinoshita (-Oct. 30)
	Chizuru Ueda
	Chihiro Nakamori
	Michiyo Kato (April 1-)
Visiting Scientists:	*Claudio Olivari*
	(from Univ. of Milano, Italy)
	Yasuko Koumoto

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, protein translocation and protein degradation. In 1999, we showed that light dependent alternative splicing is involved in the microbody transition.

Hydroxypyruvate reductase (HPR) is a leaf peroxisomal enzyme that function in the glycolate pathway of photorespiration. We have obtained two highly similar cDNAs (HPR1 and HPR2) for pumpkin HPR. It has been revealed that two HPR mRNAs are produced by alternative splicing from a single type of pre-mRNA. The HPR1 protein, but not the HPR2 protein, was found to have a targeting sequence into leaf peroxisomes at the C-terminus, suggesting that alternative splicing control the subcellular localization of the two HPR proteins. Analysis of transgenic Arabidopsis expressing fusion proteins with green fluorescent protein (GFP) revealed the different subcellular localizations of the two HPR proteins (Fig. 1). RT-PCR analysis showed that the alternative splicing is regulated by light, indicating that the microbody transition is partly controlled in the level of splicing of the mRNA by the light dependent alternative splicing.

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 b-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 b-oxidation during germination and subsequent seedling growth, whereas the ped2 mutant has a defect in the intracellular transport of thiolase from the cytosol to glyoxysomes. Further analysis on identification the mutated genes of these ped mutants are now in progress.

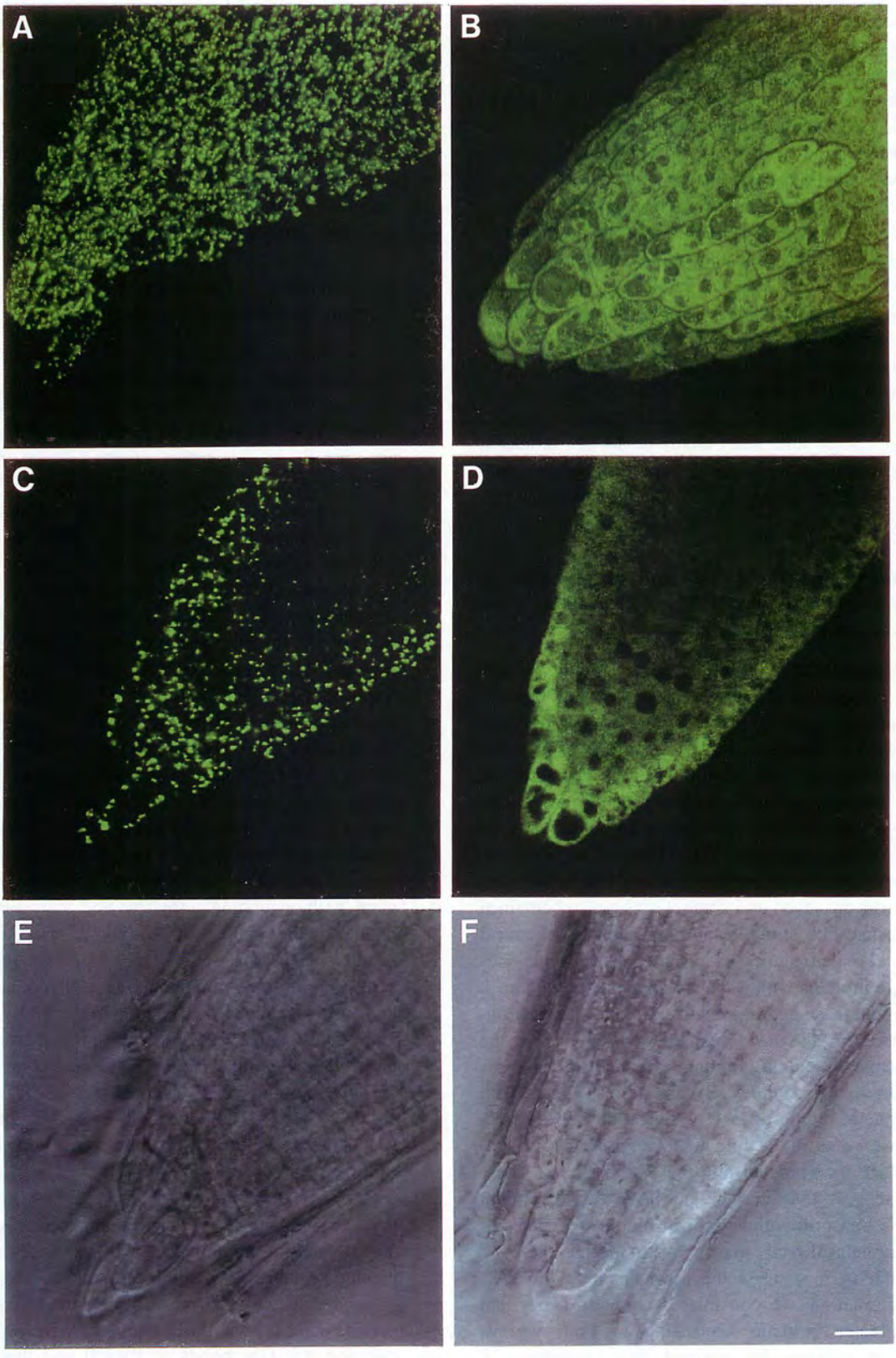

Figure 1. Subcellular localizations of green fluorescent protein (GFP) fusions in root cells of transgenic *Arabidopsis thaliana*.
Root cells were observed in light field (Nomarski optics; E and F) and with blue light excitation (470-490 nm; A, B, C and D). (A, B) Three-dimensional images of SGFP-H1 which has the peroxisomal targeting signal (PTS1) at the C-terminal end of GFP and SGFP-H2 which does not have PTS1, respectively. (C, D) The same samples viewed with a confocal microscope. (E, F) The same samples viewed with a light microscope. Peroxisomes were observed to be distributed diffusely throughout the cytosol of SGFP-H1 (A, C), whereas the fluorescence of GFP accumulated in the cytosol and the nucleus of SGFP-H2 (B, D). Bar indicates 10 μm.

In addition of these genetic approaches, we started to characterize the enzymes of b-oxidation cycle. In glyoxysomes, fatty acids are first activated to fatty acyl-CoA by fatty acyl-CoA synthetase. Fatty acyl-CoA is the substrate for fatty acid b-oxidation, which consists of four enzymic reactions. The first reaction is catalyzed by acyl-CoA oxidase. The second and the third enzymatic reactions are catalyzed by a single enzyme that possesses enoyl-CoA hydratase and β-hydroxyacyl-CoA dehydrogenase activities. The fourth reaction is catalyzed by 3-ketoacyl-CoA thiolase. We have characterized acyl-CoA oxidase that is active on long-chain acyl-CoA and 3-ketoacyl-CoA thiolase in glyoxysomes. In 1999, we reported an evidence that glyoxysomes contain another acyl-CoA oxidase that can metabolize short-chain acyl-CoA. These findings revealed that the short-chain acyl-CoA oxidases function in fatty acid b-oxidation in glyoxysomes, and that by the cooperative action of long- and short-chain acyl-CoA oxidases, plant peroxisomes are capable to performing the complete b-oxidation of acyl-CoA, whereas mammal peroxisomes are not.

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.

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.

To investigate a VPE system in protein-storage vacuoles, we isolated the PAC vesicles and characterized a 100-kDa component (PV100) of the vesicles. Isolated cDNA for PV100 encoded a 97,310-Da protein that was composed of a hydrophobic signal peptide and the following three domains: an 11-kDa Cys-rich domain with four CxxxC motifs (C, Cys), a 34-kDa Arg/Glu-rich domain composed of six homologous repeats, and a 50-kDa vicilin-like domain. Mass spectrometry and peptide sequencing of soluble proteins of the vacuoles showed that two Cys-rich peptides, three Arg/Glu-rich peptides and the vicilin-like protein were produced by cleaving Asn-Gln bonds of PV100 and that all these proteins had a pyroglutamate at their NH_2 terminus. VPE was responsible for cleaving Asn-Gln bonds of a single precursor, PV100, to produce multiple seed proteins. It is likely that the Asn-Gln stretches not only provide cleavage sites for VPE but also produce aminopeptidase-resistant proteins. Cys-rich peptide

function as a trypsin inhibitor and Arg/Glu-rich peptides function as cytotoxic peptides. Our findings suggested that PV100 is converted into different functional proteins in the vacuoles of seed cells.

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. Further analyses on the roles of these chaperonin homologues in differentiation of plastids are under experiments by using transgenic Arabidopsis which was overexpressed or was reduced these chaperonin homologues

Publication List:

Ali, K., Nii, N., Yamaguchi, K. and Nishimura, M. (1999) Levels of nonstructural carbohydrate in leaves and roots and some characteristics of chloroplasts after application of different amounts of nitrogen fertilizer to peach seedlings. *J. Jap. Soc. Hort. Sci.* **68**, 739-745.

De Bellis L., P. Giuntini, Hayashi, H., Hayashi, M. and Nishimura, M. (1999) Purification and characterization of pumpkin long-chain acyl-CoA oxidase. *Physiol. Plant.* **106**, 170-176.

De Bellis, L., S. Gonzali, A. Alpi, Hayashi, H., Hayashi, M. and Nishimura, M. (2000) Purification and characterization of a novel pumpkin short-chain acyl-CoA oxidase which structure resembles acyl-CoA dehydrogenase. *Plant Physiol.* in press.

Hayashi, H., L. De Bellis, Ciurli, A., Kondo, M., Hayashi, M. and Nishimura, M.(1999) A novel oxidase that catalyzes oxidation of short-chain acyl CoA in plant peroxisomes. *J. Biol. Chem.* **274**, 12715-12721.

Hayashi, M., Toriyama, K., Kondo, M., Hara-Nishimura, I. and Nishimura, M. (1999) Induction of precursor-accumulating vesicles by expression of chimeric genes consisting of pumpkin 2S albumin and phosphinothricin acetyltransferase. *Plant Cell Physiol.* **40**, 263-272.

Hayashi, M., Toriyama, K., Kondo, M., Kato, K., Mano, S., L. De Bellis, Hayashi-Ishimaru, Y., Yamaguchi, K., Hayashi, H. and Nishimura, M. (2000) Functional transformation of plant peroxisomes. *Cell Biochem. Biophys.* in press.

Hiraiwa, N., Nishimura, M. and Hara-Nishimura, I. (1999) Vacuolar processing enzyme is activated self-catalytically by sequential removal of the C-terminal and N-terminal propeptides. *FEBS Lett.* **447**, 213-216.

Kato, A., Hayashi, M., Kondo, M. and Nishimura, M. (2000) Transport of peroxisomal proteins that are synthesized as large precursors in plants. *Cell Biochem. Biophys.* in press.

Kato, A., Hayashi, M. and Nishimura, M. (1999) Oligomeric proteins containing N-terminal targeting signals are imported into peroxisomes in transgenic Arabidopsis. *Plant Cell Physiol.* **40**, 586-591.

Kinoshita, T., Yamada, K., Hiraiwa, N., Kondo, M., Nishimura, M. and Hara-Nishimura, I. (1999) Vacuolar processing enzyme is up-regulated in the lytic vacuoles of vegetative tissues during senescence and under various stressed conditions. *Plant J.* **19**, 43-53.

Koumoto, Y., Shimada, T., Kondo, M., Takao, T., Shimonishi, Y., Hara-Nishimura, I. and Nishimura, M. (1999) Chloroplast Cpn20 forms a tetrameric structure in Arabidopsis thaliana *Plant J.* **17**, 467-477.

Mano, S., Hayashi, M. and Nishimura, M. (1999) Light regulates alternative splicing of hydroxypyruvate reductase in pumpkin. *Plant J.* **17**, 309-320.

Mano, S., Hayashi, M. and Nishimura, M.(2000) A leaf-peroxisomal protein, hydroxypyruvate reductase, is produced by light-regulated alternative splicing. *Cell Biochem. Biophys.* in press.

Minami, Y., Nishimura, O., Hara-Nishimura, I., Nishimura, M. and Matsubara, H. (2000) Tissue and intracellular localization of indican and the purification and characterization of indican synthase from Indigo plants. *Plant Cell Physiol.* in press.

Miyagishima, S., Itoh, R., Toda, K., Kuroiwa, H., Nishimura, M. and Kuroiwa, T. (1999) Microbody proliferation and segregation cycle in the single microbody-alga Cyanidioschyzon merolae. *Planta.* **208**, 326-336.

Takemoto, D., Hayashi, M., Doke, N., Nishimura, M. and Kawakita, K. (1999) Molecular cloning of a defense-response-related cytochrome P450 gene from tobacco using a fungal elicitor. *Plant Cell Physiol.* **40**, 1232-1242.

Takemoto, D., Hayashi, M., Doke, N., Nishimura, M. and Kawakita, K. (2000) Isolation of gene for EIR, an elicitor inducible receptor like protein, from tobacco by differential display. *Plant Cell Physiol.* in press.

Yamada, K., Shimada, T., Kondo, M., Nishimura, M. and Hara-Nishimura, I. (1999) Multiple functional proteins are produced by cleaving Asn-Gln bonds of a single precursor protein by vacuolar processing enzyme. *J. Biol. Chem.* **274**, 2563-2570.

DIVISION OF BIOENERGETICS

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This division aims to understand the autophagy in respects to its molecular mechanism and its physiological role in multicellular organisms. 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 biosynthetic process. Autophagy is well conserved in eukaryotes and is a major route for bulk degradation of cytoplasmic constituents and organelles.

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 already isolated a set of autophagy-deficient mutants (*apg*), and have cloned most of *APG* genes essential for autophagy. We are now characterizing these gene products.

II. Apg10 is a unique protein conjugating enzyme

Last year, we reported on novel protein conjugation system required for autophagy. Apg12, one of Apg proteins, is terminated with glycine at its C-terminus. The C-terminal glycine is bound to a lysine residue of Apg5, another Apg protein, via an isopeptide bond. This conjugation reaction is distinct from but quite similar to ubiquitination and the other related reaction such as SUMO-1 and Rub1 system. All of these ubiquitination-like (Ubl) reactions are catalyzed by a series of enzyme system.

First, C-terminal glycine of Ubl protein is activated by binding to active center of E1-like enzyme via a thioester bond. E1-like enzymes are ATPase which share sequence similarity each other, and the ATPase activity is prerequisite for the reaction. Collaborating with Dr. Kominami's laboratory(Juntendo Med. Univ.), we have reported that Apg7 is an E1-like enzyme involved in Apg12 conjugation reaction.

Next, C-terminal glycine of Ubl protein is conjugated to the active center of E2-like enzyme via thioester bond. We characterized Apg10, which is required for conjugation reaction of Apg12. Apg10 binds to Apg12 depending on Apg7 function. We identified active center, Cys residue, of Apg10. C-terminal Gly of Apg12 bind to this Cys via a thioester bond. The active Apg10 is required for Apg12-Apg5 conjugation, and necessary for autophagy. Most notably, all previously known E2-like enzymes show sequence similarity each other, however Apg10 does not show the similarity to any known E2 enzymes. Apg10 is a quite unique E2-like enzyme. These findings prompted us the idea that Ubl conjugation system is more prevailing than ever expected from sequence similarity.

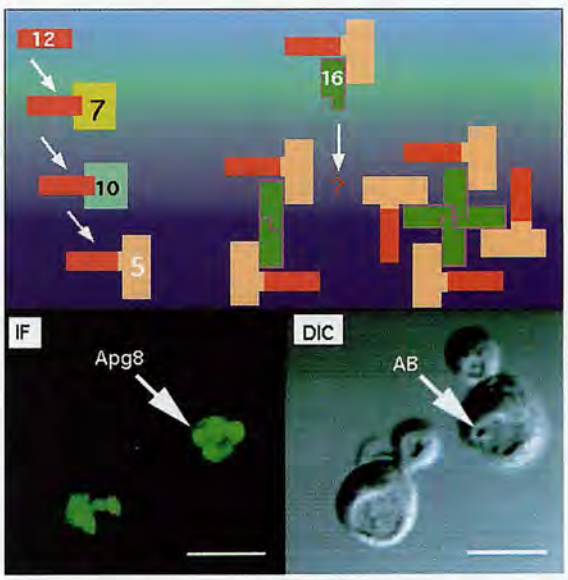

Figure 1 Molecular apparatuses for autophagy in yeast. **Upper Panel**: The diagram of Apg12 system. The numerals in the boxes denotes the corresponding Apg proteins. The number of each subunits in the final complex is still not determined. Arrow related to Apg16 does not necessarily represent the order of the events, rather do show the concept of the organization. **Lower Panel:** Immunofluorescence (Left) and Nomarsky Images (Right) of Apg8 localization. The vacuolar protease deficient mutants were starved and the tagged Apg8 is observed as the FITC signal (the arrow in left panel). Autophagic body is indicated by the arrow in the right panel

III. Apg16 crosslinks multiple Apg12-Apg5 conjugates

Despite these findings of novel Ubl system, the mechanism how the system is involved in autophagic mechanism is still to be determined. However, an accessory part related to this system, which will aid us understanding the point, was discovered.

In a two-hybrid screen with Apg12 as a bait, we identified a novel protein, Apg16, which is required for autophagy. Thereafter, we noticed that the Apg16 is not bound to Apg12 but Apg12-Apg5 conjugate. Moreover, Apg16 is bound to Apg5 alone, but more preferentially to Apg12-Apg5 conjugate. The binding occurs at the N-terminal region of Apg16. On the other hand, Apg16 have a coiled coil domain at its C-terminus, which generally functions for protein interaction. Actually Apg16 forms an homooligomer via the coiled coil domain. In accordance with this scheme, two or more independent Apg12-Apg5 conjugates were shown to be cross-linked via Apg16. Hence, this hetero-oligomeric large protein complex may play a role in autophagic process.

IV. Apg8 as a tracer of membrane flow in autophagosome formation

Autophagosome is a double membrane structure. The outer membrane fuses with the vacuolar membrane, and the inner membrane structure is released into the vacuolar lumen. The released membrane structure, autophagic body, is disintegrated depending on the vacuolar protease activity, and the cytosolic materials inside are degraded. Therefore, when vacuolar protease activity is blocked, autophagic bodies are accumulated inside the vacuole.

Apg8, one of Apg proteins, is transcriptionally upregulated upon starvation via Tor-kinase dependent signaling pathway, which controls induction of autophagy. Indirect immunofluorescence study showed that during a nutrient-rich growing phase, Apg8 is mostly dispersed throughout the cytoplasm as tiny dot structures. Upon starvation, Apg8-positive structure appears adjacent to the vacuole, which represents autophagosome or its intermediate. When vacuolar protease activity is blocked, Apg8 was observed to be accumulated inside autophagic bodies. According to immuno EM analysis in collaboration with Dr. M. Baba (Japan Women's Univ.), Apg8 is mostly enriched on the membrane of forming intermediate of autophagosome. Upon completion of the formation, Apg8 is detached from the membrane and some are released to the cytosol and the others are entrapped within the autophagosome. Thus, Apg8 could be a useful tracer for membrane flow in autophagosome formation. More detailed EM observation clarified that Apg8-positive electron lucent small structures are gathering around forming autophagosome. We postulate that these structures are one of the precursors of autophagosomal membrane. If this is the case, formation process of autophagosome is accompanied with multiple fusion of small precursor structures to the

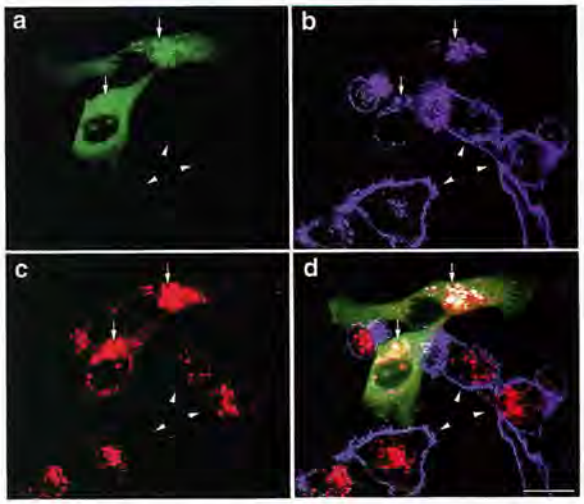

Figure 2. TfR and the endocytosed dextran are accumulated in the compartments where the mutant SKD1 is localized. HeLa cells transfected with the mutant SKD1 fused with GFP were incubated in the presence of 1 mg/ml TRITC-dextran at 37 °C for 8 hours. The cells were then fixed, permeabilized, and subjected to immunofluorescence confocal microscopy for TfR. GFP-mutant SKD1 labeling (a), TfR staining (b), TRITC-dextran labeling (c), and a merged image (d) of a same field are shown. Arrows heads and arrows indicate the untransfected cells and the mutant SKD1-positive compartments in the transfected cells, respectively. Bar, 20 μm.

intermediate structure. This contradicts to the generally accepted scheme that autophagosome is formed by engulfing of the preexisting large membrane structure, such as ER. This hypothesis will be tested in our ongoing project.

V. SKD1 regulates morphology of endosomes and membrane traffic through them in mammalian cells

We also engage in the study of mammalian to pursue cell biological and physiological issues of autophagy. In the course of the study, we encountered an interesting molecule, which regulates membrane dynamics in endosomal system.

The mouse SKD1 is an AAA-type ATPase homologous to the yeast Vps4p/ Csc1p implicated in both autophagy and transport from endosomes to the vacuole. To elucidate a possible role of SKD1 in mammalian endocytosis, we generated a mutant SKD1, harbouring a mutation (E235Q) that is equivalent to the dominant negative mutation (E233Q) in Vps4p/Csc1p. Overexpression of the mutant SKD1 in cultured mammalian cells caused defect in uptake of transferrin and low-density lipoprotein. This was due to loss of their receptors from the cell surface. The decrease of the surface transferrin receptor (TfR) was correlated with expression levels of the mutant protein. The mutant protein displayed a perinuclear punctate distribution in contrast to a diffuse pattern of the wild type SKD1. TfR, the lysosomal protein lamp-1, endocytosed dextran and epidermal growth factor (EGF), but not markers for the secretory pathway were accumulated in the mutant

SKD1-localized compartments. Degradation of EGF was inhibited. Electron microscopy revealed that the compartments were exaggerated multivesicular vacuoles with numerous tubulo-vesicular extensions containing TfR and an endocytosed horseradish peroxidase. The early endosome antigen EEA1 was also redistributed to these aberrant membranes. Taken together, our findings suggest that SKD1 regulates morphology of endosomes and membrane traffic through them.

Publication List:

Original Articles

Tanida, I., Mizushima, N., Kiyooka, M., Ohsumi, M., Ueno, T., Ohsumi, Y. and Kominami, E. (1999). Apg7p/Cvt2p: A novel protein-activating enzyme essential for autophagy. *Mol Biol Cell.* **10**, 1367-1379.

Shintani, T., Mizushima, N., Ogawa, Y., Matsuura, A., Noda, T. and Ohsumi, Y. (1999). Apg10p, a novel protein-conjugating enzyme essential for autophagy in yeast. *EMBO J.* **18**, 5234-5241.

Mizushima, N., Noda, T. and Ohsumi, Y. (1999). Apg16p is required for the function of the Apg12p-Apg5p conjugate in the yeast autophagy pathway. *EMBO J.* **18**, 3888-3896.

Kirisako, T., Baba, M., Ishihara, N., Miyazawa, K., Ohsumi, M., Yoshimori, T., Noda, T. and Ohsumi, Y. (1999). Formation process of autophagosome is traced with Apg8/Aut7p in yeast. *J Cell Biol.* **147**, 435-446.

Tagawa, Y., Yamamoto, A., Yoshimori, T., Masaki, R., Omori, K., Himeno, M., Inoue, K. and Tashiro, Y. (1999). A 60 kDa plasma membrane protein changes its localization to autophagosome and autolysosome membranes during induction of autophagy in rat hepatoma cell line, H-4-II-E cells. *Cell Struct Funct.* **24**, 59-70.

Yabe, I., Horiuchi, K., Nakahara, K., Hiyama, T., Yamanaka, T., Wang, P. C., Toda, K., Hirata, A., Ohsumi, Y., Hirata, R., Anraku, Y. and Kusaka, I. (1999). Patch clamp studies on V-type ATPase of vacuolar membrane of haploid *Saccharomyces cerevisiae*. Preparation and utilization of a giant cell containing a giant vacuole. *J Biol Chem.* **274**, 34903-34910.

Noda T., Kim J., Huang WP., Baba M., Tokunaga C., Ohsumi Y., and Klionsky D.J., (2000) Apg9p/Cvt7p is an integral membrane protein required for transport vesicle formation in the cvt and autophagy pathways. *J Cell Biol.l* **148**, 465-480

Yoshimori T., Yamagata F., Yamamoto A., Mizushima N., Kabeya Y., Nara A., Miwako I., Ohashi M., Ohsumi M., Ohsumi Y., (2000) The mouse SKD1, a homologue of yeast Vps4p, is required for normal endosomal trafficking and morphology in mammalian cells. *Mol Biol Cell.* **11**, 747-763

George, M.D., Baba M., Scott, S.V., Mizushima N., Garrison B., Ohsumi Y., Klionsky D.J., (2000) Apg5p functions in the sequestration step in the cytoplasm to vacuole targeting and macroautophagy pathways. *Mol Biol Cell.* **in press**

Furukawa, K. Mizushima, N., Noda, T., Ohsumi, Y., (2000) A protein conjugation system in yeast with homology to biosynthtic enzyme reaction of prokaryotes. *J Biol Chem.* **275**, 7462-7465

Review Articles

Klionsky, D. J. and Ohsumi, Y. (1999). Vacuolar import of proteins and organelles from the cytoplasm. *Annu Rev Cell Dev Biol.* **15**, 1-32.

Ohsumi, Y. (1999). Molecular mechanism of autophagy in yeast, *Saccharomyces cerevisiae. Philos Trans R. Soc. Lond, B* **354**, 1577-1580; discussion 1580-1581.

Ohsumi, Y. (1999). Pictures in cell biology. Autophagosomes in yeast. *Trends Cell Biol.* **9**, 162.

DIVISION OF CELL PROLIFERATION

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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. Second year of the five-year term, and with new research staff and graduate students, we started a large-scale screening to find strains useful for this purpose.

I. Comprehensive identification and developmental tracing of brain cells

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 the 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 require detailed basic knowledge about when and how the brain structure is formed. Thus, "developmental neuroanatomy" 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. After initial pilot experiments, we employed a two-stage screening process. 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. As of December 1999, ca. 50000 photographs depicting 1612 of the total 4500 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.

Among the 1612 lines screened, 98 % showed GAL4 expression in the brain, suggesting that most of the genes in the genome are expressed in at least some portion of the brain cells. Among them, less than 20 % showed expression in a small enough number of cells for which identification is feasible. In many such strains, the expression pattern changes drastically during neurogenesis. Only a few percent of the whole strains label identical neurons throughout larval, pupal and adult stages. These lines are found valuable to trace the development, path finding and synapse formation processes of the labelled cells.

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. As for visual pathways, for example, five novel types of projection neurones have already been identified by our screen.

The other target is to reveal the formation process of one of the highest-order associative centres called the "central complex". In this area, fibres from various brain regions converge to form the sophisticated array circuit structure, providing a good model system for studying the molecular mechanisms of path finding and circuit formation. We have identified several strains that label central complex neurons from rather early stages and are tracing the developmental processes in more detail (Fig. 1).

II. Tools for comprehensive cell lineage analysis of 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. Though this system proved useful for revealing the clonal architecture of several brain regions, the performance of the transformant lines used for activating the *flippase* gene was not optimal, showing a high level of spontaneous recombi-

nation. To solve this problem, we generated and screened ca. hundred new lines and obtained several that show no background *flippase* expression and sharp heat shock-induced activation. Using these lines, an attempt to make a comprehensive map of the lineage-related circuit structures of the fly brain is underway.

III. Analysis of genes for path finding processes in the adult brain development

Although many genes related to the neuronal path finding processes have been identified and analysed in *Drosophila*, their functions have been studied primarily in the simple motor neurons and the ventral nerve cord of embryos. Little has been investigated about their functions when neurones compose highly complicated circuits of the adult brain.

The latest genetic mosaic system, the "MARCM (mosaic analysis with a repressible cell marker) system", can generate labelled clones with mutant genotype whereas surrounding wild-type cells remain unlabelled. This is a powerful technique for studying the function of a gene in the path finding processes of identified fiber projection. Using this system, we analysed the function of the gene *trio*, which encodes a Dbl family protein. As a model system for analysing its possible role in path finding controle of developing axons, we focused on the brain region called mushroom bodies (MB), whose clonal structure has been revealed in great detail by us and other investigators.

An MB is made by four specific stem cells (neuroblasts) and thus consists of four clones. When one of them were labelled without introducing *trio* genotype, the neurones of the clone extended their axons through the peduncle and arborised into the vertical and medial lobes (Fig. 2A). In contrast, neurons of the *trio* mutant clones appeared to navigate normally through the peduncle till the approximate region of the two lobes, but there they either stalled or are misrouted around the end of the peduncle, forming only sparse arborisation into the lobes (Fig. 2B). On the other hand, branching in the calyx appeared unaffected. These suggest that *trio* plays an essential role in projection and arborisation of axons from the peduncle into lobes.

IV. 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 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 and experimental protocols, as well as meetings and job announcements, are provided.

Publication List:

Awasaki, T., Saito, M., Sone, M., Suzuki, E., Sakai, R., Ito, K., Hama, C. The Drosophilla Trio plays an essential role in patterning of axons by regulating their directional extention . *Neuron*. **in press**.

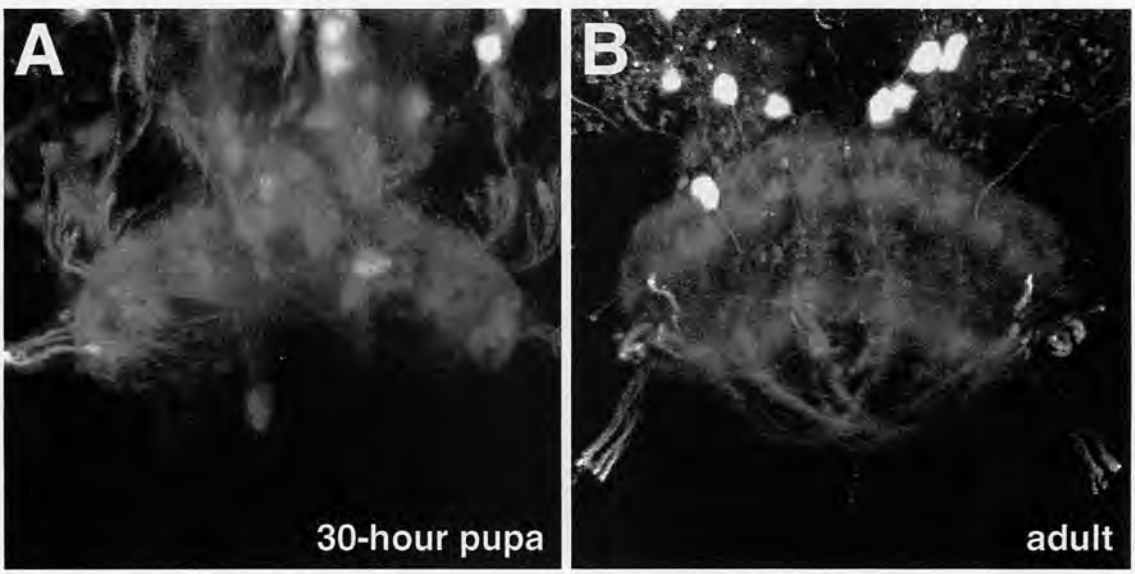

Fig. 1 Development of fibres innervating the central complex
A: In early pupae, only upper part of the fan-shaped body region of the central complex is formed. B: The adult fan-shaped body shows two layers of tangential connections.

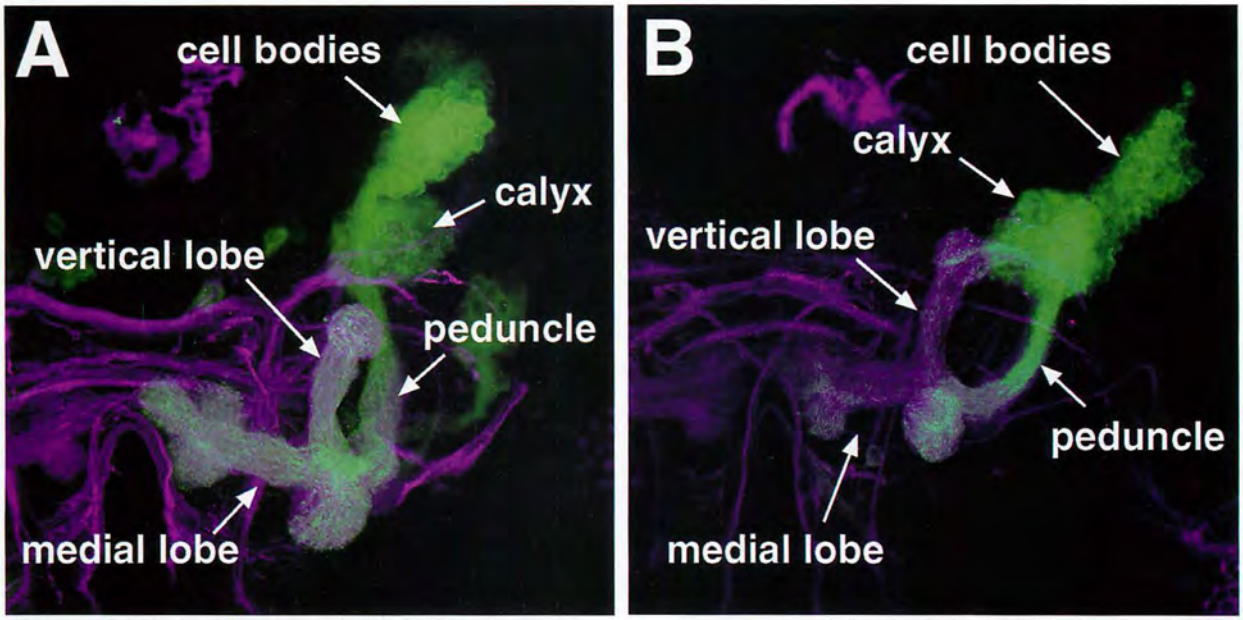

Fig. 2 ***trio*** **clones in the mushroom bodies (MB)**
A: The MB consists of neurons produced by four stem cells. One of the four wild-type clones is made to express *CD8* reporter gene and labelled with anti-CD8 antibody (green). Fibres from all the four clones, together with some other tracts, are labelled with anti-FasII (purple). B: In the *trio* mutant clone (green), neurones extend axons in the peduncle but exhibit poor projection into the vertical and medial lobes. Anti-FasII labelling (purple) shows that the other three clones form essentially normal MB structure.

DIVISION OF CELLULAR COMMUNICATION

(ADJUNCT)

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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 fine glass needles. 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*.

Publication List:

Minoura, I., Yagi, T., and Kamiya, R. (1999). Direct measurement of the inter-doublet elasticity in flagellar axo-

nemes. Cell Struct. Funct. 24:27-33.

Omoto, C. K., Gibbons, I. R., Kamiya, R., Shingyoji, C., Takahashi, K., and Witman, G. B. (1999). Rotation of the central pair microtubules in eukaryotic flagella. Molec. Biol. Cell 10:1-4.

Leivo, T., J. Lohi, A-L. Kariniemi, G. Molander, C. L. Kiraly, M-L. Kotovirta, K. Owaribe, R. E. Burgeson, and Leivo, I. (1999). Hemidesmosomal defect in dermatitis herpetiformis; Decreased expression of BP230 and plectin/HD1 in uninvolved skin. Histochem. J. 31:109-116.

Okumura, M., Uematsu, J., Hirako. Y., Nishizawa, Y., Shimizu, H., Kido, N., and Owaribe, K. (1999). Identification of the hemidesmosomal 500 kDa protein (HD1) as plectin. J. Biochem. 126:1144-1150.

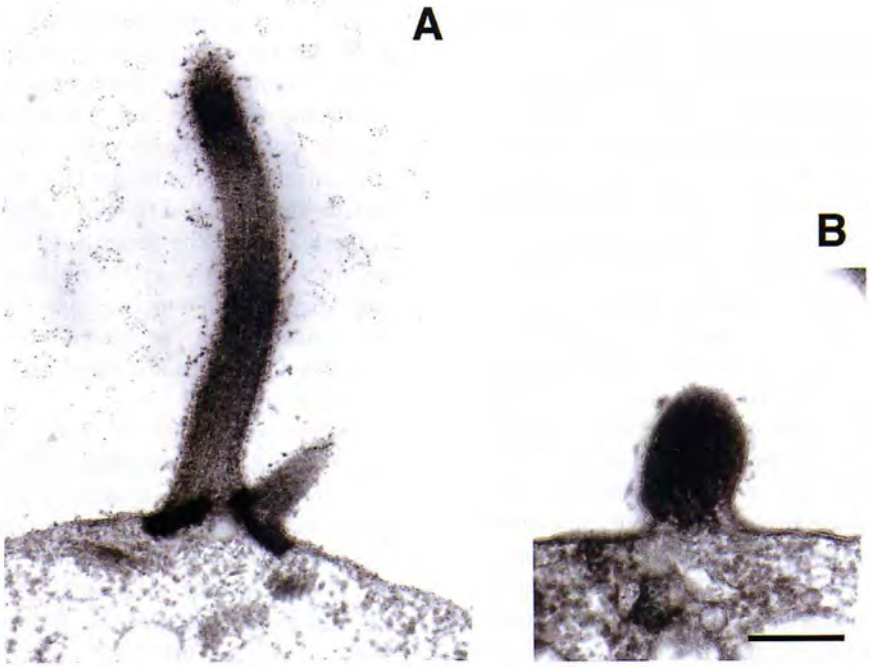

Fig. 1. Fertilization tubules in wild-type (A) and *ida5* (B) mt+ gametes produced in response to a 1 hour exposure to 10 mM dibutyryl-cAMP and 1 mM IBMX. Bar, 0.3 μm. Wild-type fertilization tubules have been shown to contain F-actin bundles.

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 (Fig. 1). 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 preexisting 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.

Publication List:

Suda, M., Fukui, M., Sogabe, Y., Sato, K., Morimatsu, A., Arai, R., Motegi, F., Miyakawa, T., Mabuchi, I., and Hirata, D. (1999). Overproduction of elongation factor 1a, an essential translational component, causes aberrant cell morphology by affecting the control of growth polarity in fission yeast. *Genes to Cells* **4**, 517-527.

Sayers, Lee G., Katayama, S., Nakano, K., Mellor, H., Mabuchi, I., Toda, T., and Parker, P. J. Rho-dependence of Schizosaccharomyces pombe Pck2. *Genes to Cells* in press.

Kusano, K., Abe, H. and Obinata, T. (1999). Detection of a sequence involved in actin-binding and phosphoinositide-binding in the N-terminal side of cofilin. *Mol. Cell. Biochem.* **190**, 133-141.

Tsubakimoto, K., Matsumoto, K., Abe, H., Ishii, J., Amano, M., Kaibuchi, K. and Endo, T. (1999). Small GTPase RhoD suppresses cell migration and cytokinesis. *Oncogene* **18**, 2431-2440.

Okada, K., Obinata, T. and Abe, H. (1999). XAIP1: a *Xenopus* homologue of yeast actin interacting protein 1 (AIP1), which induces disassembly of actin filaments cooperatively with ADF/cofilin family proteins. *J. Cell Sci.* **112**, 1553-1565.

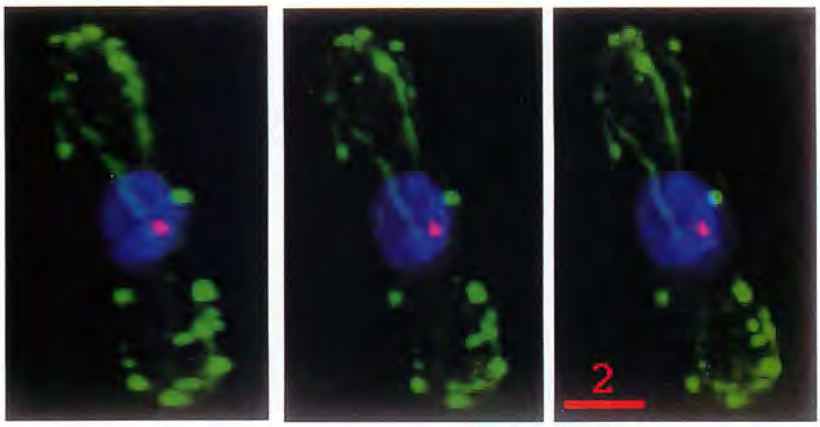

Fig. 1 3-D images of an interphase *S.pombe* cell. Each image is rotated by 12 degree from the neighbor. Green, F-actin. Blue, DNA. Red, spindle pole body. Bar, 2 μm.

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.

DIVISION OF REPRODUCTIVE BIOLOGY

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	Tohru Kobayashi
Institute Research Fellow:	*Yoshinao Katsu*
JSPS Postdoctoral Fellow:	*Mika Tokumoto*
	Daisuke Kobayashi
	Masaru Matsuda
	Craig E. Morrey
	Catherine Dreanno
	Balasubramarian Senthilkumaran
JSPS Research Associate:	*Akihiko Yamaguchi*
	Yuichi Oba
	Toshitaka Ikeuchi
	Fumie Sakai
	Masatada Watanabe
	Gui-Jin Guan
Graduate Student:	*Ryo Horiuchi (Graduate University for Advanced Studies)*
Monbusho Foreign Scientist:	*Srinivas K. Saidapur (Karnatak University)*
Visiting Scientist:	*Shanbhag Bhagyashri (Karnatak University)*
	Ching-Fong Chang (National Taiwan Ocean University)

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 and sex change

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. 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°C. 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. Mitosis of germ cells begins 15-20 days post-hatching in genetic females, but can not be 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 of often sex-specific. In these cases, the sex of the gonad is easily distinguished. In tilapia, gonadal sex is morphologically distinct at 20-25 days post hatching. Ovarian differentiation is initially marked by stromal elongations of the gonad for the formation of the ovarian cavity. Testicular differentiation is characterized by the appearance of a narrow space in the stromal 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 (Fig. 1). In contrast, the ability of steroid-producing cells to synthesize steroid hormones could not be confirmed in the testis during sex differntiation of testicular differentiation.

Sequential, protogynous hermaphroditism, i.e. female to male sex change, provides an ideal comparative model for studies on the endocrine regulation of sexual differentiation. Prior to sex change, the gonad of *Thalassoma duperrey* contains primordial germ cells and oogonia but not spermatogonia. To begin investigating the importance of hormonal signals on germ cell differentiation during sex change, we examined the expression of P450arom and 11β-hydroxylase mRNAs in *T. duperrey*. The ovarian form of P450arom is predominantly expressed in the ovary prior to the initiation of sex change. Shortly thereafter, expression diminished to undetectable levels suggesting ovarian P450arom/estrogen is critical for the maintenance of ovarian function and/or detrimental to testicular differentiation. In contrast, P450 11β-hydroxylase is likely to be a major factor regulating the gonadal changes. P450 11β-hydroxylase mRNA was relatively abundant in the ovary; however, it was up-regulated concomitant with the onset of testicular differentiation indicating the importance of P450 11β-hydroxylase/11-ketotestosterone (11-KT) to spermatogenesis and spermiation. Consequently, the switch from an ovary to a testis corresponds exceptionally well to a switch in the steroidogenic pathway of the gonad thus providing further evidence of the critical nature of steroid hormones in teleost sexual differentiation.

II. Endocrine regulation of spermatogenesis

Spermatogenesis is an extended process of differen-

tiation 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-KT, a potent androgen in fish. In turn, 11-KT activates Sertoli cells to stimulate 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 (eARα and eARβ) were cloned eel testes. The amino acid sequences of these two ARs share low homology. In transient transfection assays of mammalian cells, both eAR proteins showed androgen-dependent activation of transcription with 11-KT being the most effective activator. These results indicate that the cloned eAR cDNAs encode functional eel ARs, whose native ligands are 11-KT. *In situ* hybridization revealed that both eAR mRNAs are present in testes prior to HCG injection, only eARα transcripts increased during HCG-induced spermatogenesis suggesting that eARα and eARβ play different roles in spermatogenesis.

In situ hybridization shows that activin type I and II receptors are localized in spermatogonia. Activin B acts via these receptors on spermatogonia to induce *de novo* synthesis of G1/S cyclins (cyclins A, D, and E) and Cdks (cdc2, cdk2, and cdk4), leading to the initiation of mitosis (spermatogonial proliferation). Despite the above-mentioned progress in the study of hormonal regulation of spermatogenesis (mitosis), our attempts to induce meiosis *in vitro* with recombinant eel activin B have been unsuccessful. One possible explanation is that another factor(s), perhaps a meiosis-initiating substance produced in the testis in response to gonadotropins or 11-KT, may be responsible for the mitosis-meiosis transition. Interestingly, cyclin A1 transcripts were first detected in primary spermatocytes during HCG-induced spermatogenesis in eel testes suggesting an important role of cyclin A1 in the progression to meiosis of male germ cells. Further studies on the transcriptional regulation of cyclin A1 expression may therefore answer some of the basic questions related to the initiation and progression towards meiosis during spermatogenesis.

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) was 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. 17α,20β-DP induces oocyte maturation by acting on a pertussis toxin-sensitive G-protein-coupled membrane receptor. The early steps of l7α,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. A 54-kDa Y box protein and polyadenylation of cyclin B mRNA are thought to be involved in 17α,20β-DP-induced initiation of cyclin B mRNA translation. Upon egg activation, MPF is inactivated by degradation of cyclin B. It was demonstrated that the 26S proteasome initiates cyclin B

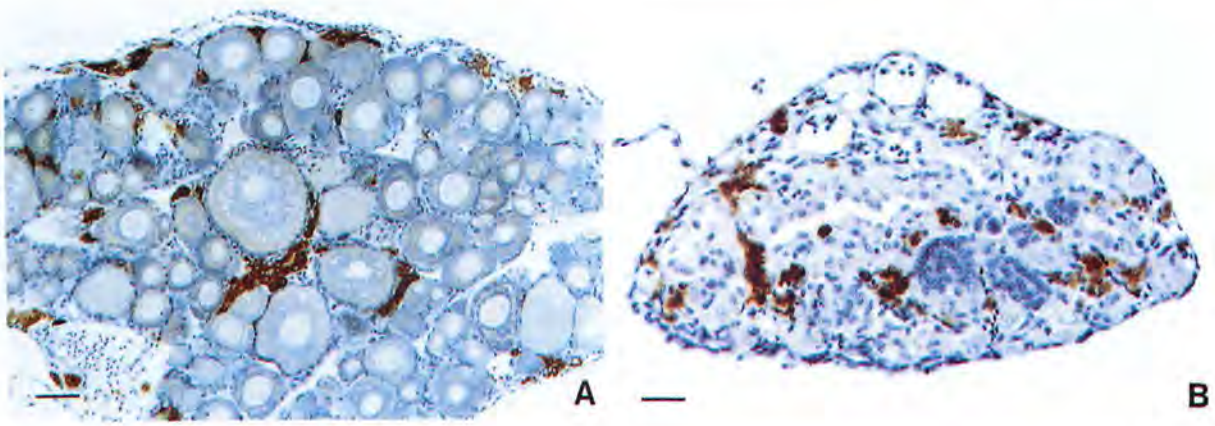

Fig. 1. Masculinization of genetic female (XX) tilapia by inhibition of estrogen synthesis using an inhibitor of cytochrome P450 aromatase (P450arom), fadrozole (Fig. 1). Fadrozole was given to fry at 8 days after hatching for 22 days. A, Control (Female, ovary); B, Fadrozole (Sex reversed male, testis). Gonads were stained with an antibody against P450arom (A) or an antibody against 3β-hydroxysteroid dehydrogenase (3β-HSD) (B).

degradation through the first cut of its NH_2 terminus at lysine 57.

Publication List:

Chang, X.T., Kobayashi, T., Todo, T., Ikeuchi, T., Yoshiura, Y., Kajiura-Kobayashi, H., Morrey, C. and Nagahama, Y. (1999). Molecular cloning of estrogen receptors α and β in the ovary of a teleost fish, the tilapia (*Oreochromis niloticus*). *Zool. Sci.* **16**, 653-658.

Guan, G., Tanaka, M., Todo, T., Young, G., Yoshikuni, M. and Nagahama, Y. (1999). Cloning and expression of two carbonyl reductase-like 20β-hydroxysteroid dehydrogenase cDNAs in ovarian follicles of rainbow trout (*Oncorhynchus mykiss*). *Biochem. Biophys. Res. Comm.* **255**, 123-128.

Hondo, E., Kobayashi, T., Ishiguro, N., Kurohmaru, M., Kitamura, N., Yamada, J. and Nagahama, Y. (1999). Prolactin induces protamine 2 mRNA expression in rat testis. *J. Reprod. Dev.* **45**, 205-212.

Ikeuchi, T., Todo, T., Kobayashi, T. and Nagahama, Y. (1999). cDNA cloning of a novel androgen receptor subtype. *J. Biol. Chem.* **274**, 25205-25209.

Katsu, Y., Carnall, N., Nagahama, Y. and Standart, N. (1999). Phosphorylation of p82 clam CPEB by MAP kinase and cdc2 kinase. *Dev. Biol.* **209**, 186-199.

Katsu, Y., Yamashita, M. and Nagahama, Y. (1999). Translational regulation of cyclin B mRNA during 17α, 20β-dihydroxy-4-pregnen-3-one (maturation-inducing hormone)-induced oocyte maturation in goldfish, *Carassius auratus*. *Mol. Cell. Endocrinol.* **158**, 79-85.

Kitano, T., Takamura, K., Kobayashi, T., Nagahama, Y. and Abe, S.-I. (1999). Suppression of P450 aromatase (P450arom) gene expression in sex-reversed males produced by rearing genetically female larvae at high temperature during a period of sex determination in Japanese flounder (*Paralichthys olivaceus*). *J. Mol. Endocrinol.* **23**, 167-176.

Mita, M., Saneyoshi, M., Yoshikuni, M. and Nagahama, Y. (1999). A methyl donor for 1-methyladenine biosynthesis in starfish ovarian follicle cells. *Mol. Reprod. Develop.* **54**, 63-68.

Mita, M., Yasumasu, I., Yoshikuni, M. and Nagahama, Y. (1999). 1-Methyladenine production from ATP by starfish ovarian follicles. *Biochem. Biophys. Acta* **1428**, 13-20.

Oba, Y., Hirai, T., Yoshiura, Y., Yoshikuni, M., Kawauchi, H. and Nagahama, Y. (1999). Cloning, functional characterization and expression of a gonadotropin receptor cDNA in the ovary and testis of amago salmon (*Oncorhynchus rhodurus*). *Biochem. Biophys. Res. Comm.* **263**, 584-590.

Oba, Y., Hirai, T., Yoshiura, Y., Yoshikuni, M., Kawauchi, H. and Nagahama, Y. (1999). The duality of fish gonadotropin receptors: cloning and functional characterization of a second gonadotropin receptor cDNA expressed in the ovary and testis of amago salmon (*Oncorhynchus rhodurus*). *Biochem. Biophys. Res. Comm.* **265**, 366-371.

Osaki, A., Okida, N., Ishikawa, K., Tokumoto, M., Nagahama, Y., Lippert, T.H., Voelter, W. and Tokumoto, T. (1999). Identification of ubiquitin in seminal plasma from tilapia, *Oreochromis niloticus*. *Biomed. Res.* **20**, 249-252.

Todo, T., Ikeuchi, T., Kobayashi, T. and Nagahama, Y. (1999). Fish androgen receptor: cDNA cloning, steroid activation of transcription in transfected mammalian cells, and tissue mRNA levels. *Biochem. Biophys. Res. Comm.* **254**, 378-383.

Tokumoto, M., Horiuchi, R., Nagahama, Y., Ishikawa, K. and Tokumoto, T. (1999). Two proteins, a goldfish 20S proteasome subunit and the protein interacting with 26S proteasome, change in the meiotic cell cycle. *Eur. J. Biochem.* **266**, 1-8.

Tokumoto, M., Horiuchi, R., Nagahama, Y. and Tokumoto, T. (1999). Identification of the *Xenopus* 20S proteasome *α4* subunit which is modified in the meiotic cell cycle. *Gene* **239**, 301-308.

Tokumoto, M., Nagahama, Y. and Tokumoto, T. (1999). Molecular cloning of cDNA encoding a cyclin-sensitive ubiquitin carrier protein (E2-C) from goldfish (*Carassius auratus*) and expression analysis of the cloned gene. *FEBS Letters* **458**, 375-377.

Tokumoto, T., Tokumoto, M., Seto, K., Horiguchi, R., Nagahama, Y., Yamada, S., Ishikawa, K. and Lohka, M.J. (1999). Disapperance of a novel protein component of the 26S proteasome during *Xenopus* oocyte maturation. *Exp. Cell Res.* **247**, 313-319.

Watanabe, M., Tanaka, M., Kobayashi, D., Yoshiura, Y., Oba, Y. and Nagahama, Y. (1999). Medaka (*Oryzias latipes*) FTZ-F1 potentially binds to promoter regions of P-450 aromatase: cDNA cloning and functional characterization. *Mol. Cell. Endocrinol.* **149**, 221-228.

DIVISION OF CELL DIFFERENTIATION

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From an aspect that differentiation of cells is regulated at least in part by the functions of cell type- or tissue-specific transcription factors, mechanisms underlying differentiation of the steroidogenic tissues such as the gonads (testis and ovary) and adrenal cortex have been under investigation in our division. Based on observations obtained in the past decade, we started in Okazaki new projects to understand mechanisms underlying sex-differentiation of the gonads and mechanisms of sex-differentiation of brain functions necessary for reproductive behaviour. To address these issues, we have focussed our attention on the mechanisms how steroidogenic-tissue specific transcription factors regulate their target genes which are essential for the tissue differentiation and functions, and how the genes encoding the transcription factors are regulated during the tissue differentiation. In addition to the studies above, Dr. Shimono joined to our division as a research associate from April 1999 and has started to investigate mechanisms underlying head formation.

I. Gene regulatory cascade in the steroidogenic tissue differentiation

When a differentiation process of a tissue is considered, it is reasonable to assume a gene regulatory cascade specific for each tissue in which certain genes encoding transcription factors are involved as the components. In the cascades required for adrenal and gonadal differentiation, Ad4BP/SF-1 is locates upstream of some 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 during the tissue differentiation and Ad4BP/SF-1 is an essential transcription factor for the tissue differentiation, identification of the components consisting the cascade as well as their genetical relationship in the cascade are essential for fully understanding the mechanisms of the tissue differentiation.

From these points of view, the regulatory region of the Ad4BP/SF-1 gene was analysed by making transgenic mice or with cultured cells. However, our in vivo investigation 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 the Ad4BP/SF-1 gene, which will be expected to give us a new insight into metabolism of biologically active lipophylic compounds.

Dax-1 is another transcription factor of our interest, which is also implicated in the steroidogenic tissue differentiation. Although our previous study revealed that the factor acts as a suppressor of Ad4BP/SF-1, regulation of the suppressive effect has not yet clarified at the molecular level. We recently uncovered the function of the amino terminal half of Dax-1 containing a unique repeated sequence instead of Zn-finger DNA binding domain, which enables us to understand how Dax-1 suppresses the function of Ad4BP/SF-1 and how the activity of Dax-1 is regulated.

In addition to these transcription factors, factors such as Sox-9, Wt-1, Emx-2, and GATA-4 are known to be implicated in the gonad development. In order to isolate other factors interacting with the transcription factors above, yeast two-hybrid screening was performed with a cDNA library constructed with an mRNA prepared from mouse foetal gonads. Extensive screening resulted in isolation of interacting molecules including coactivators and other type of transcription factors, some of which were novel factors. 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 discrepancy between the two methods, close examination was carried out with a series of adrenal cortex of both sexes at several developing stages from foetal 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 an-

drogen 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.

Investigations of head formation and brain sex differentiation are included as the targets of our study. These studies, same as the studies described above, have been performed based on examination of the functions of transcription factors. Uncovering the molecular mechanisms will be elucidated by these efforts in the near feature.

Publication List:

Morohashi, K., Tsuboi-Asai, H., Matsushita, S., Suda, M., Nakashima, M., Sasano, H., Hataba, Y., Li, C-L., Fukuta, J., Irie, J., Watanabe, T., Nagura, H., & Li, E. (1999) Structural and Functional Abnormalities in the Spleen of *mFtz-F1* Gene Disrupted Mouse. *Blood* **93**, 1586-1594.

Kawabe, K., Shikayama, T., Tsuboi, H., Oka, S., Oba, K., Yanase, T., Nawata, H., & Morohashi, K. (1999) *Dax-1* as One of the Target Genes of Ad4BP/SF-1. *Mol. Endocrinol.* **13**, 1267-1284.

Kabe, Y., Goto, M., Shima, D., Imai, T., Wada, T., Morohashi, K., Shirakawa, M., Hirose, H., & Handa, H. (1999) The Role of Human MBF1 as a Transcriptional Coactivator. *J. Biol. Chem.* **274**, 34196-34202.

Nagamine, C.M., Morohashi, K., Carlisle, C., & Chang, D.K. (1999) Sex Reversal Caused by *Mus musculus domesticus* Y Chromosomes Linked to Variant Expression of the Testis-Determining Gene *Sry*. *Dev. Biol.* **216**, 182-194.

Morohashi, K. (1999) Gonadal and Extragonadal Functions of Ad4BP/SF-1 -Developmental Aspects-. *Trends in Endocrinol. Metab.* **10**, 169-173.

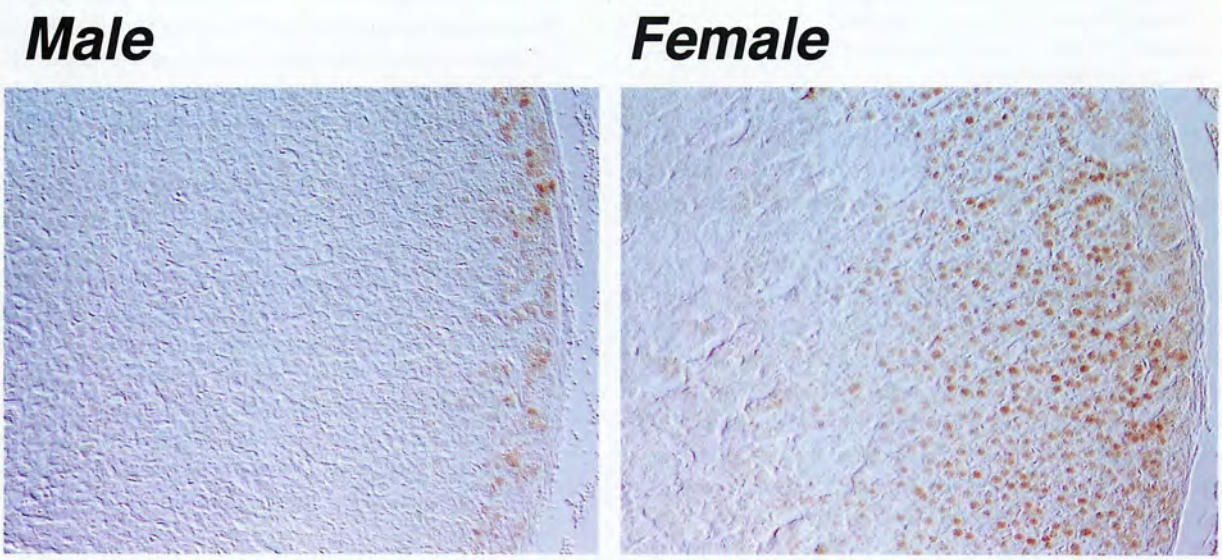

Fig. Sex-dependent distribution of Dax-1 in mouse adrenal cortex.
Adult mouse adrenal glands from both sexes were immunostained with an antiserum for Dax-1.

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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 several groups of molecules called 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 of PGFs. Our main interest is to know how pattern formation in morphogenesis is regulated by PGFs during development. We address this problem using several model animals, including frog, fly and worm with the view of extracellular and intracllular signaling of PGFs. We employ embryology, genetics, molecular and cellular biology and biochemistry. In addition, we have recently introduced a systematic and comprehensive way to screen target genes of PGFs using arrayed cDNAs.

I. Hierarchy of homeobox genes in pattening embryo

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 known to be 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 siginificance 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), which is a fusion protein with the virus-derived VP16 activation domain. Interestingly, VP-Xmsx-1 induced a secondary body axis, complete with muscle and neural tissues, when the fusion protein was overexpressed in ventral blastomeres. The result suggests that Xmsx-1 activity is necessary for both mesoderm and ectoderm to be ventralized. We also examined the epistatic relationship between Xmsx-1 and another ventralizing homeobox protein Xvent-1 and showed that Xmsx-1 is likely to be acting upstream of Xvent-1. We propose that Xmsx-1 is required in the BMP-stimulated ventralization pathway that involves the downstream activation of Xvent-1.

II. Cross-talk of growth factor signaling in early development

Signals generated by the members of the Wnt and TGF-β superfamilies mediate a diverse array of biological responses. These secreted factors, via activation of cell surface receptors, participate in the regulation of cell fate specification and control cell proliferation during development and tissue maintenance. In the early amphibian embryo (*Xenopus laevis*), both Wnt and TGF-β superfamily signaling cascades are required for establishment of a dorsal signaling center, Spemann's organizer. In *Xenopus*, as in other systems, both signaling pathways utilize extranuclear proteins which, upon activation, translocate to the nucleus to participate in transcriptional complexes. Wnt signaling is mediated by translocation of β-catenin into the nucleus together with members of the Lef1/Tcf class of HMG box transcription factors to activate gene expression. Likewise, TGF-β superfamily signaling is mediated by "pathway-specific" Smads which translocate into the nucleus together with the "universal Smad" Smad4 to activate gene expression. Recently, we have demonstrated that Lef1/Tcf, a downstream component of the Wnt signaling cascade can physically interact with Smad4. In *Xenopus*, this interaction directly affects transcriptional activity of the twin (Xtwn) gene during Spemann's organizer formation. This is the first demonstration of a physical interaction between TGF-β and Wnt signaling components, and may represent a direct connection between the TGF-β and Wnt signaling pathways.

III. Genetic dissection of TGF-β signaling in *Drosophila* model system

We have reported that the morphogen gradients of Dpp and Wg provide positional information to cells in the notum region of the wing imaginal disc. dpp is induced at A/P boundary by a Hh dependent mechanism in wing and leg discs, however, wg is induced in various ways. We revealed that initial notal wg induction is regulated by the function of two transcription factors, Pannier and U-shaped. We also found that expression of both *pnr* and *ush* is regulated by Dpp signaling. We propose that the Dpp gradient is utilized not only for anterior/posterior patterning but also contributes for dorsal/ventral patterning through the induction of wg.

We also analyzed *in vivo* function of TAK1 (dTAK1) in *Drosopihla*. We generated transgenic flies which express vertebrate or *Drosophila* homolog of TAK1. Genetic and biochemical analyses indicated that the c-Jun amino-terminal kinase (JNK) signaling pathway is specifically activated by TAK1 signaling. Expression of a dominant negative form of dTAK1 during embryonic development resulted invarious embryonic cuticle defects including dorsal open phenotypes. Our results suggested that TAK1 functions as a MAPKKK in the JNK signaling pathway and participates in such diverse roles as control of cell shape and regulation of apoptosis in *Drosophila*.

IV. TGF-β family in nematode

Nematode *C. elegans* provides powerful genetic approaches to understand the role and mechanism of TGF-β family signaling. We have identified a new member of the TGF-β superfamily, CET-1, from *C. elegans*. *cet-1* null mutants have shortened bodies and male tail abnormal phenotype resembling *sma* mutants. Genetic analysis suggested that CET-1 regulated body length and male ray pattern functioning as ligand in *sma* pathway. *cet-1* is mainly expressed in the ventral nerve cord and other neurons which are thought to secrete CET-1 ligand and induce specific gene expression in target cells. To elucidate molecular mechanism regulating *C. elegans* body length, we employed a differential hybridization of a cDNA array for identification of target genes of the CET-1 signal.

C. elegans cDNAs representing 7,584 independent genes were arrayed on a nylon membrane at high density, and hybridized with ^{33}P-labeled DNA probes synthesized from the mRNAs of wild-type, *cet-1*, *sma-2,* and *lon-2* worms (Fig. 1, top). Signals for all the spots representing hybridized DNA were quantified and compared between strains (Fig. 1, bottom). The screening identified 22 and 2 clones, which were positively and negatively regulated, respectively, by the CET-1 signal. Northern hybridization confirmed the expression profiles of most of the clones indicating good reliability of the differential hybridization using arrayed cDNAs. In situ hybridization analysis revealed the spatial and temporal expression patterns of each clone and showed that at least 4 genes, including the gene for the type I receptor for CET-1, *sma-6*, were transcriptionally regulated by the CET-1 signal.

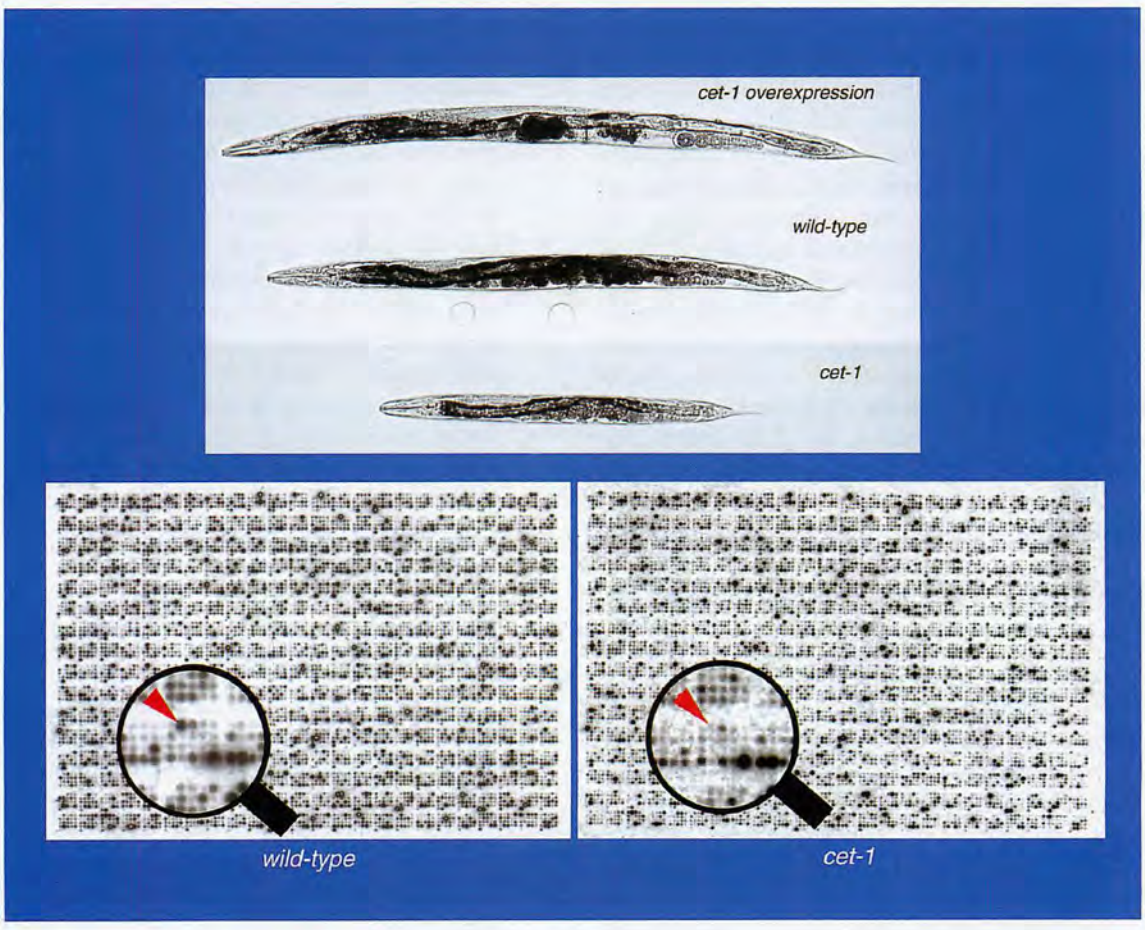

Fig. 1. Screening for TGF-β-regulated genes in *C. elegans*. (Upper panel) Change of body length depending on *cet-1* gene doses. Cet-1 overexpressing, wild-type, and *cet-1* null mutant worms from top. (Bottom panel) Macroarray of *C. elegans* cDNAs. Hybridization signals were compared between wild-type and *cet-1* null mutant worms. The arrowhead points a DNA spot down-regulated in the mutant.

Publication List:

Adachi-Yamada, T., Nakamura, M., Irie, K., Tomoyasu, Y., Sano, Y., Mori, E., Goto, S., Ueno, N., Nishiada, Y., Matsumoto, K. (1999) p38 MAP kinase participates in TGF-β superfamily signal transduction in Drosophila wing morphogenesis. *Mol. Cell. Biol.* **19**, 2322-2329.

Hamada, F., Tomoyasu, Y., Takatsu, Y., Nakamura, M., Nagai, S., Fujita, F., Shibuya, H., Ueno, N. and Akiyama, T. (1999) Negative regulation of the Wingless signaling by a Drosophila homologue of Axin. *Science* **283**, 1739-1742.

Hamada, F., Murata, Y., Nishida, A., Fujita, F., Tomoyasu, Y., Nakamura, M., Toyoshima, K., Tabata, T., Ueno, N., Akiyama, T. (1999) Identification and characterization of E-APC, a novel Drosophila homologue of the tumour suppressor APC. *Genes Cells* **4**, 465-474.

Hanafusa, H., Ninomiya-Tsuji, J., Masuyama, N., Nishita, M., Fujisawa, J., Shibuya, H., Matsumoto, K. and Nishida, E. (1999) Involvement of the p38 MAP kinase pathway in TGF-β-induced gene expression. *J. Biol. Chem.* **274**, 27161-27167.

Hirota, Y., Okabe, M., Imai, T., Kurusu, M. Yamamoto, A., Miyao, S., Nakamura, M., Sawamoto, K., Okano, H. (1999) Musashi and Seven in absentia downregulate Tramtrack through distinct mechanisms in Drosophila eye development. *Mech. Dev.* **87**(1-2) 93-101.

Iemura, S., Yamamoto, T. S., Chiyo, T. Kobayashi, H. and Ueno, N. (1999) Isolation and characterization of bone morphogenetic proteins from the early Xenopus embryo. *J. Biol. Chem.* **274**, 26843-26849.

Ishitani, T., Ninomiya-Tsuji, J., Nagai, S., Nishita, M., Meneghini, M., Baker, N., Waterman, M., Bowerman, B., Clevers, H., Shibuya, H. and Matsumoto, K. (1999) The TAK1-NLK MAP kinase-related pathway antagonizes β-catenin-TCF signalling. *Nature* **399**, 798-802.

Masuyama, N., Hanafusa, H., Kusakabe, M., Shibuya, H. and Nishida, E. (1999) Identification of two Smad4s in Xenopus: Their common and distinct properties. *J. Biol. Chem.* **274**, 12163-12170.

Miyama, K., Yamada, G., Yamamoto, T., Takagi, C., Miyado, K., Sakai, M., Ueno, N. and Shibuya, H. (1999) A BMP-inducible gene, Dlx-5 regulates osteoblast differentiation and mesoderm induction. *Dev. Biol.* **208**,123-133.

Mochii, M., Yoshida, S., Morita, K., Kohara, Y. and Ueno, N. (1999) Identification of transforming growth factor-β-regulated genes in *Caenohabtidis elegans* by differential hybridization of arrayed cDNAs. *Proc. Natl. Acad. Sci. USA* **96**, 15020 –15025.

Morita, K., Chow, K. and Ueno, N. (1999) Body length and male tail ray pattern formation of *Caenohabditis elegans* are regulated by a mamber of TGF-β family. Development **126**, 1337-1347.

Nagaso, H. Tada, M., Nikaido, M. and Ueno, N. (1999) Dual specificity of activin type II receptor ActRIIb in dorso-ventral patterning during zebrafish embryogeneisis. *Dev. Growth Diff.* **41**, 119-133.

Nikaido, M., Masazumi, T. and Ueno, N. (1999) Restricted expression of the receptor serine/threonine kinase BMPR-IB in zebrafish. *Mech. Dev.* **82**, 219-222.

Nikaido, M., Tada, M., Takeda, H., Kuroiwa, A. and Ueno, N. (1999) In vivo analyisis using variants of zebrafish BMPR-IA: range of action and involvement of BMP in ectoderm patterning. *Development* **126**, 181-190.

Nishita, M., Ueno, N. and Shibuya, H. (1999) Smad 8B splice variant lacking the SSXS site that inhibits Smad8 mediated signalling. *Genes Cells* **4**, 583-591.

Nishita, M., Hashimoto, M. K., Ogata, S., Laurent, M. N., Ueno, N. Shibuya, H. and Cho, K.W.Y. Interaction between Wnt and TGF-β signalling pathways during formation of Spemann's organizer. *Nature* (in press)

Shimasaki, S., Zachow, R. J., Li, D., Iemura, S., Ueno, N., Chang, R. J., Sampath, K., and Erickson, G. F. (1999) Expression of a functional bone morphogenetic protein (BMP) system in the ovary. *Proc. Natl. Acad. Sci. USA* **96**, 7282-7287.

Suzawa , M., Takeuchi, Y., Fukumoto, S., Kato, S., Ueno, N., Miyazono, K., Matsumoto, T., Fujita, T. (1999) Extracellular matrix-associated bone morphogenetic proteins are essential for differentiation of murine osteoblastic cells in vitro. *Endocrinology* **140**, 2125-2133.

Takatsu, Y., Nakamura, M., Stapleton, M. Danos, M. C., Matsumoto, K. O'Connor, M. B., Shibuya, H. and Ueno, N. TAK1 participates in JNK signaling during Drosophila development. *Mol. Cell. Biol.* (in press)

Yamaguchi, K., Nagai, S-I., Ninomiya-Tsuji, J. Noshita, M., Tamai, K., Irie, K., Ueno, N., Nishida, E., Shibuya, H. and Matsumoto, K. (1999) XIAP, a cellular member of the inhibitor of apoptosis protein family, links the receptors to TAB1-TAK1 in the BMP signaling pathway. *EMBO J.* **18**, 179-187.

Yamamoto, S.T., Takagi, C. and Ueno, N. Requirement of Xmsx-1 in the BMP-triggered ventralization of Xenopus embryos. *Mech. Dev.* (in press)

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Availability of sugars and the inter-organ transport and distribution of sugars are essential in the growth and development of the plant body. Expression of a variety of plant genes is regulated, either positively or negatively depending on the gene, by the level of sugars at the sugar-importing sink sites as well as at the sugar-exporting source sites of the plant body. Thus sugars are not only important as sources for cellular energy and the synthesis of macromolecules but also as a signal controlling the growth and development of plants by changing the pattern of gene expression. Our research attention is focused to elucidate the mechanisms involved in the regulation of gene expression in response to sugars, especially the activation of gene expression by increased-levels of sugars, and the role of such regulation in the organ development in plants. In addition, mechanisms involved in the accumulation of storage proteins in plant vacuoles are also studied.

I. Molecular and genetic approaches for the analysis of the sugar-signalling during growth and development of *Arabidopsis thaliana*.

During the growth of plants, new organs develop as carbohydrate sink, and many vegetative organs shows sink to source transition after their maturation. Many aspects of the organ development in higher plants are thought to be affected, to some degree, by the levels of sugars. Sugars seem to have influence on the meristematic transition in long day plants from vegetative to reproductive growth. To obtain insights into the role of sugar-regulated gene expression in the growth and organ development in plants, we are screening for mutants of Arabidopsis with defects or anomalies both in the sugar-regulated gene expression and in the developmental processes such as leaf development and the determination of the flowering time. To aid this purpose, we have established more than 7,000 independent lines of Arabidopsis plants transformed with T-DNA containing multiple copies of the enhancer sequence. We have identified more than 20 of mutant lines with defects in the development of leaves or anormalies in the flowering time which also show the altered patterns of the expression of sugar-inducible genes, such as β-amylase gene (*Atβ-Amy*) (Fig. 1). The mutants were named as *uns* (*unusual sugar response*) after their abnormal responses to sugars concerning the gene expression, *in vitro* flowering and chlorophyll content in leaves of *in vitro*-cultured plant.

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

Expression of genes coding for sporamin and β-amylase, two major proteins of the storage roots of sweet potato, is inducible by high levels of sugars in various vegetative tissues. The GUS reporter genes under the control of the promoters of these genes are also inducible by sugars in leaves of transgenic tobacco plants, and these fusion genes are expressed in tubers of transgenic potato plants. Although the induction of expression of these fusion genes requires the activity of hexokinase, phosphorylation of hexose by hexokinase is not sufficient to cause the induction. The induction requires Ca^{2+}-signalling and the activity of protein kinase.

Eight different cDNAs for the isoforms of calcium-dependent protein kinase (CDPK) were isolated from leaves of tobacco, and transcripts of two of them were found to be increased upon treatment of leaves with various metabolizable sugars. Antibodies against a fragment of one of these isoforms cross-reacted strongly with the 57 kDa-protein in the soluble fraction from the young leaves. The level of this 57 kDa-protein decreased significantly as leaf matures, while the level of this 57-kDa protein in mature leaves increased significantly after the treatment of leaves with sugars. The sugar-induction of the 57-kDa protein occured preceding the induction of expression of the β-amylase:GUS reporter gene. In addition, a 54 kDa-protein with autophosphorylation activity in the plasma membrane of mature leaves also increased significantly upon treatment of leaves with sugars. This protein was purified to about 1,000-fold compared to the crude

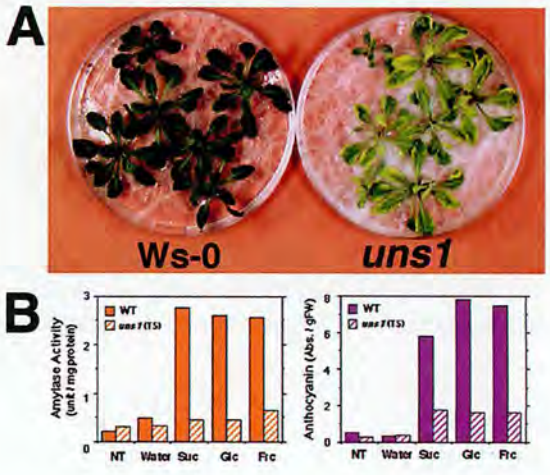

Fig.1 Phenotypes of wild type and homozygous mutant line (*uns1*) grown for 3 weeks. The chlorophyll content of the mutant plants (Fig.1A, right) is much lower than that of wild type plants (Fig.1A, left). Mutant plants also showed the reduced levels of the sugar-inducible increase of β–amylase (Fig.1B, left) and anthocyanin (Fig.1B, right) when leaf explants were treated with high levels (5%) of sugars.

extract. It phosphorylated histone IIIS in a Ca^{2+}-dependent manner and cross-reacted with an antibody against CDPK of *Arabidopsis thaliana*. These results suggest the possible involvement of CDPKs in the sugar-inducible gene expression and the development of leaves.

Selected Publication:

Iwata, Y., Kuriyama, M., Nakakita, M., Kojima, H., Ohto, M. and Nakamura, K., (1998) Characterization of a calcium depedent protein kinase of tobacco leaves that is asociated with the plasma membrane and is inducible by sucrose. *Plant Cell Physiol.* **39**(11): 1176-1183.

Koide, Y., Matsuoka, K., Ohto, M. and Nakamura, K., (1999) The N-terminal propeptide and the C terminus of the precursor to 20-kilo-dalton potato tuber protein can function as different types of vaculor sorting signals. *Plant Cell Physiol.* **40**(11): 1152-1159.

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.

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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 (*e.g.* cDNA cloning, site-directed mutagenesis Yeast two-hybrid system), biochemistry (protein, carbohydrate), immunological methods (monoclonal-antibody production), neuroanatomy, cell and organotypic culture (immortalized cell-line production), and embryo manipulation (classical embryology, gene transfer with viral vectors, and gene targeting).

I. Molecular mechanism of retinotectal map formation

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 suggested 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. We examined whether CBF-1 and CBF-2 control the expression of EphA3, a promising candidate for a topographic guidance label in the retina, using *in ovo* electropolation. We obtained results suggesting that these two transcription factors affect the topographic expression of EphA3 indirectly.

In order to further search for topographic molecules in the embryonic retina, we performed a large-scale screening using a new cDNA display system called Restriction Landmark cDNA Scanning (RLCS). A number of molecules displaying various asymmetrical expression patterns along the nasotemporal axis or dorsoventral axis in the retina have been identified. These included already known topographic molecules such as EphA3, CBF-2, etc, expressed along the nasotemporal axis, and ephrin-B2, EphB3, etc, expressed along the dorsoventral axis. We have identified all of the cDNA clones isolated by this screening and examined their expression patterns during development. Furthermore, with respect to the topographic molecules which might have important roles in formation of the retinotectal map and/or the neural network in the retina, we conducted over- and misexpression experiments using viral vectors and *in ovo* electropolation to elucidate the molecular function. In 1999, with the assistance of a computer image-processing software (Fig. 1), we found additional ~30 and ~80 candidate cDNA spots for topographic molecules along the nasotemporal and dorsoventral axes, respectively. Currently, projects to identify them and analyze the expression pattern are underway.

We expect that our studies will lead to elucidation of the molecular mechanism underlying formation of the regional specificity in the retina, and ultimately to uncovering the basic principles for establishing complicated 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

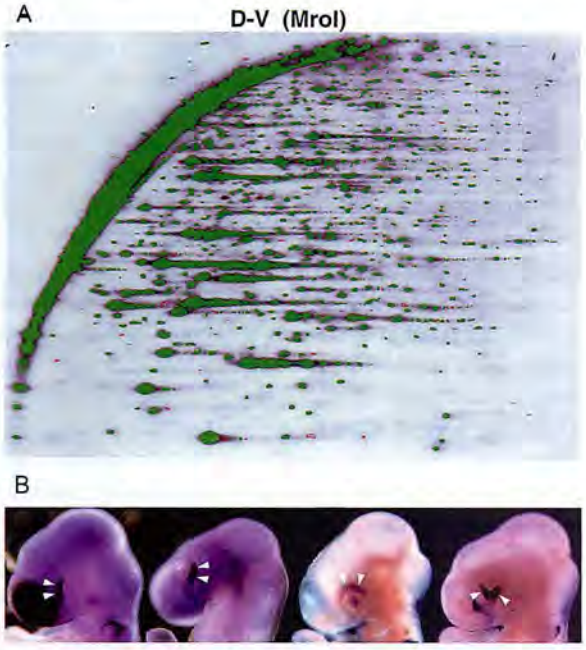

Fig. 1 Genes expressed in a region-specific fashion in the chick retina
(A) RLCS profiles for the dorsal and ventral retina. The dorsal profile (black) was overlaid with the ventral profile (green) using an image-processor. Dorsal specific clones are detectable as rare black spots among abundant green spots.
(B) Region-specific gene expression in the retina. Whole-mount in situ hybridization showed that these genes were specifically expressed in the nasal, temporal, dorsal or ventral region of the retina, respectively.

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-specific RPTP, is expressed as a chondroitin sulfate proteoglycan in the brain. An RNA splice variant corresponding to the extracellular region of PTPζ is secreted as a major proteoglycan in the brain known as 6B4 proteoglycan/phosphacan. The extracellular region of PTPζ consists of a carbonic anhydrase-like domain, a fibronectin-type III-like domain and a serine-glycine-rich region, which is considered to be the chondroitin sulfate attachment region.

In an attempt to reveal the signal transduction mechanism of PTPζ, we tried to identify the ligand molecules of this receptor. To date, we have 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.

In the embryonic rat brain, pleiotrophin and midkine are localized along the radial glial fibers, a scaffold for neuronal migration. On the other hand, PTPζ is expressed in the migrating neurons, suggesting that the ligand-receptor relationship between these molecules plays a role in migration of neurons during brain development. Thus, 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 suggested that PTPζ is involved in migration as a neuronal receptor for pleiotrophin and midkine distributed along radial glial fibers.

Next, in order to reveal the intracellular signaling mechanism of PTPζ, we performed yeast two-hybrid screening using the intracellular region of PTPζ as bait. By screening a rat brain cDNA library, we found that PTPζ interacts with PSD-95/SAP90 family molecules, SAP102, PSD-95/SAP90 and SAP97/hDlg. These proteins are composed of three PDZ domains, a SH3 domain and a guanylate kinase-like domain, and are concentrated in the central synapses mediating protein-protein interactions to form large synaptic macromolecular complexes. Using the yeast two-hybrid binding assay, we found that the C-terminal sequence of PTPζ binds to PSD-95/SAP90 proteins through the second PDZ domain (Fig. 2). Immunohistochemical analysis revealed that PTPζ and PSD-95/SAP90 are similarly distributed in the dendrites of pyramidal neurons of the hippocampus and neocortex (Fig. 2). These results suggest that PTPζ is also involved in the regulation of synaptic function.

To 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 behaviour, circadian rhythm, LTP in the hippocampus, etc.

III. Functional roles of subfamily 2 sodium channels

Voltage-gated sodium channels (NaChs) are responsible for generating action potentials in excitable cells and play many important physiological roles. Cloning

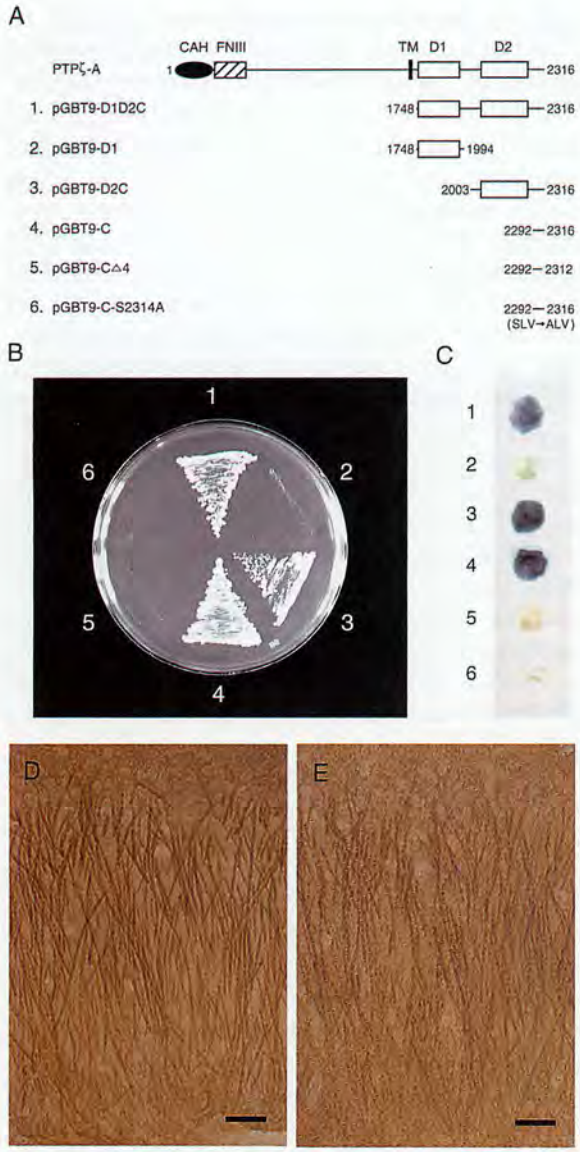

Fig. 2 Interaction of PTPζ with PSD-95/SAP90
Schematic representations of PTPζ and yeast two-hybrid constructs are shown in (A). Various bait constructs of PTPζ were tested for interaction with PSD-95/SAP90 by induction of reporter genes, *HIS3* (B) and *LacZ* (C). Numbers in (B) and (C) correspond to the construct numbers in (A). These results and the other experiments indicated that the C-terminal portion of PTPζ interacts with the second PDZ domain of PSD-95/SAP90. Immunohistochemical staining with the antibodies against PTPζ (D) and PSD-95/SAP90 (E) indicated that both proteins were present in the dendrites of hippocampal pyramidal cells. Scale bars: 40 μm.

of NaChs revealed marked conservation in primary structure that underlies their functional similarity. Thus, all NaChs cloned had been grouped into a single gene family. However, recently, novel NaChs, human Nav2.1, mouse Nav2.3/mNaG and rat SCL11/NaG, were cloned from inexcitable cells such as glial cells. These molecules closely resemble each other but are divergent from the previously cloned sodium channels even in the regions involved in activation, inactivation and ion selectivity. Thus, these molecules have been grouped into a new subfamily of NaChs (subfamily 2). To date, subfamily 2 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 cells expressing subfamily 2 channels and their physiological functions *in vivo*, we planned to generate knock-out mice deficient in the channel gene.

We successfully generated mutant mice in which the mouse subfamily 2 channel gene (*mNaG*) was replaced with the *LacZ* or *neo* gene by gene targeting. From the analysis of *LacZ* knock-in mice, we demonstrated that *mNaG* gene expression is restricted to the dorsal root ganglion and lung during the embryonic stage (see Fig. 1A in the part of Center for Transgenic Animals and Plants). During the postnatal period, in addition to these tissues, Schwann cells in the sensory afferent nerve fibers (*ibid.* Fig. 1B, C) and a subset of neurons in the central nervous system were positive for *mNaG* gene expression. We are currently examining the phenotypes of *mNaG*-null mutant mice to gain insight into the physiological functions of this channel.

Publication List:

Takahashi, M., Yamagata, M. and Noda, M. (1999) Specific expression of ezrin, a cytoskeletal-membrane linker protein, in a subset of chick retinotectal and sensory projections. *Eur. J. Neurosci.* **11**, 545-558.

Yamakawa, T., Kurosawa, N., Kadomatsu, K., Matsui, T., Itoh, K., Maeda, N., Noda, M., and Muramatsu, T. (1999) Levels of expression of pleiotrophin and protein tyrosine phosphatase ζ are decreased in human colorectal cancers. *Cancer Letter*, 135, 91-96.

Maeda, N., Ichihara-Tanaka, K., Kimura, T., Kadomatsu, K., Muramatsu, T. and Noda, M. (1999) A receptor-like protein tyrosine phosphatase PTPζ/RPTPβ binds a heparin-binding growth factor midkine: Involvement of arginine 78 of midkine in the high affinity binding to PTPζ. *J. Biol. Chem.*, **274**, 12474-12479.

Revest, J.-M., Faivre-Sarrailh, C., Maeda, N., Noda, M., Schachner, M. and Rougon, G. (1999) The interaction between F3 immunoglobulin domains and protein tyrosine phosphatase ζ/β triggers bidirectional signalling between neurons and glial cells. *Eur. J. Neurosci.* **11**, 1134-1147.

Kawachi, H., Tamura, H., Watakabe, I., Shintani, T., Maeda, N. and Noda, M. (1999) Protein tyrosine phosphatase ζ/RPTPβ interacts with PSD-95/SAP90 family. *Mol. Brain Res.*, **72**, 47-54.

Yamagata, M., Mai, A., Pollerberg, G.E. and Noda, M. (1999) Regulatory interrelations among topographic molecules CBF1, CBF2 and EphA3 in the developing chck retina. *Dev. Growth Differ.* **41**, 575-587.

Meng, K., Rodorigues-Peña, A., Dimitrov, T., Chen, W., Yamin, M., Noda, M. and Deuel, T.F. (2000) Pleiotrophin signals increased tyrosine phoshorylation of β-catenin through inactivation of the intrinsic catalytic activity of the receptor-type protein tyrosine phosphatase β/ζ. *Proc. Natl. Acad.Sci.* USA **97**, 2603-2608.

Shintani, T., Maeda, N. and Noda, M. (2000) Receptor-like protein tyrosine phosphatase γ (RPTPγ), but not PTPζ/RPTPβ, inhibits NGF-induced neurite outgrowth in PC12D cells. *Dev. Neurosci.*, in press.

Nishiwaki, T., Maeda, N., and Noda, M. (1999) Characterization and developmental regulation of proteoglycan-type protein tyrosine phosphatase ζ/RPTPβ isoforms. *In* Neural Development (Uyemura, K., Kawamura, K., and Yazaki, T. *eds.*) pp.291-297. Springer-Verlag Tokyo.

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The research efforts of this division are aimed at developing a full understanding of the molecular mechanisms by which plants are able to acclimate to and tolerate stresses that arise from changes in environmental conditions, with particular emphasis on temperature stress and salt stress. In 1999, using higher plants and cyanobacteria as our experimental materials, we made significant progress in the following areas.

I. The perception and transduction of low-temperature signals in *Synechocystis*

Low temperature is an important environmental factor that affects the growth of all living organisms. Many organisms are known to acclimate to low temperatures by expressing various low temperature-inducible genes. However, mechanisms for the perception and transduction of low-temperature signals remain to be characterized. In the cyanobacterium *Synechocystis* sp. PCC 6803, expression of the genes for fatty acid desaturases is enhanced by low temperature. Moreover, the decrease in membrane fluidity induced by low 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 putative genes for histidine kinases in *Synechocystis* sp. PCC 6803 and monitored the subsequent response of the promoter of the gene for the ω3 fatty acid desaturase, *desB*, to low temperature. Among 41 mutant lines with disrupted genes for histidine kinases, we identified two mutants, in which, respectively, the *hik19* gene and the *hik33* gene had been inactivated and in which no induction of the reporter gene for luciferase occurred upon exposure of cells to low temperature. This result indicated that mutation of the two genes for histidine kinases abolished the inducibility by low temperature of the activity of the *desB* promoter. Mutation of these two genes also reduced the extent of induction at low temperature of other low temperature-inducible genes, such as the *desD* gene for Δ6 desaturase and the *crh* gene for RNA helicase.

We also introduced an antibiotic-resistance gene cassette randomly into the chromosomes of *Synechocystis* and screened mutants for altered expression of the *desB* gene. In one of the mutants, 2C, in which the *desB* promoter was insensitive to low temperature, we found that the gene for a response regulator, Rer1, had been inactivated. The extent of the low temperature-dependent increase in the level of the *desB* transcript in *Δrer1* cells was reduced to half of that in wild-type cells. By contrast, the inducibility by low temperature of the *desD* and *crh* genes was unaffected by the mutation. Thus, it is possible that Rer1 might regulate the expression of the *desB* gene specifically and might not affect the expression of the other genes examined.

Figure 1 shows a hypothetical scheme for the transduction of low-temperature signals. In this scheme, Hik33 spans the plasma membrane twice and forms a dimer, whose structure is influenced by the physical characteristics of the lipids in the plasma membrane. Such characteristics include fluidity, which is controlled by temperature. When the temperature is decreased, the histidine residue in the histidine kinase domain is phosphorylated. The phosphate group is then transferred to Hik19 and eventually to Rer1 which regulates the expression of the *desB* gene.

II. Genetic modification of plants to enhance stress tolerance: introduction of the capacity for the synthesis of glycinebetaine into *Arabidopsis*

Metabolic acclimation *via* the accumulation of compatible solutes is considered to be a fundamental strategy for the protection and survival of plants when they

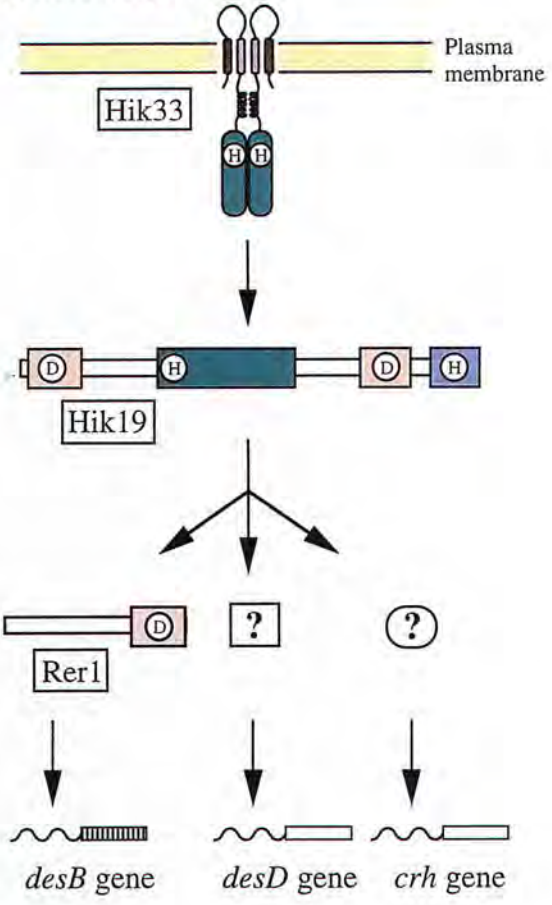

Figure 1. A hypothetical scheme showing the pathway for transduction of low-temperature signals in *Synechocystis*. The histidine kinase domains of Hik33, Hik19, the receiver domains of Hik19 and Rer1, and the histidine phosphate-transfer domain of Hik19 are shown in turquoise, pink and blue, respectively. The plasma membarne is shown in yellow. The histidine and aspartate residues that might be involved in the phospho-relay reaction are indicated by H and D in circles, respectively. Adapted from I. Suzuki, D.A. Los, Y. Kanesaki, K. Mikami and N. Murata, *EMBO J.*, 19, 1327-1334 (2000).

are exposed to extreme environments. In response to stress, such as high levels of salt, cold and drought, certain plants accumulate glycinebetaine (hereafter called betaine), a quaternary amine that protects cellular components, such as complex proteins and membranes, from the harmful effects of high levels of salt or extreme temperatures *in vitro*.

In order to evaluate the role of betaine in stress tolerance *in vivo*, we transformed *Arabidopsis thaliana*, which does not normally accumulate betaine, with the *codA* gene for choline oxidase (which catalyzes the conversion of choline to betaine) from the soil bacterium *Arthrobacter globiformis*. In transformed *Arabidopsis*, the accumulation of betaine, which was the result of the expression of functional choline oxidase in chloroplasts, significantly enhanced the tolerance of the plants to a wide variety of environmental stresses, such as high levels of salt, and low and high temperatures. Furthermore, enhancement of such tolerance was not confined to a specific stage of development but was apparent throughout developmental stages that included the imbibition and germination of seeds and the early and later stages of vegetative growth.

In transformed *Arabidopsis*, enhanced tolerance to stress was also evident at the cellular level. Photosystem II in the chloroplasts of transformed plants was less susceptible than that of wild-type plants to photoinhibition by high-intensity light. We demonstrated that the enhanced tolerance of the photosynthetic machinery to high-intensity light resulted from the accelerated recovery of the photosystem II complex from photoinduced inactivation. The extent of such accelerated recovery was reduced when the synthesis of proteins was blocked by lincomycin, suggesting that betaine, accumulated *in vivo*, might promote protein synthesis *de novo*, which is considered to be crucial for the recovery of cells from stress-induced damage.

III. Mechanisms of inactivation of the photosynthetic machinery by salt stress in cyanobacteria

High-salt stress is an environmental factor of major importance that limits the growth and productivity of plants. We studied the mechanism of the salt-induced inactivation of the photosynthetic machinery in the cyanobacterium *Synechococcus* sp. PCC 7942. Incubation of cells in medium prepared with 0.5 M NaCl resulted first in the rapid and reversible inactivation of photosystems I and II, which was followed by the slow and irreversible inactivation of both photosystems. The rapid inactivation resembled the effects of 1.0 M sorbitol. The slow inactivation was prevented by a blocker of Na^+ channels. The presence of blockers of Na^+ channels and water channels together protected both photosystems I and II against the short-term and the long-term effects of 0.5 M NaCl. Thus, it seems likely that NaCl has both osmotic and ionic effects. Our results suggest the following mechanism for the salt-induced inactivation of photosynthesis. The osmotic effect of NaCl decreases the amount of water in the cytosol, rapidly increasing the intracellular concentrations of salts. The ionic effect of NaCl is caused by an influx of Na^+ ions through K^+/Na^+ channels that also increases concentrations of salts in the cytosol, which results in the irreversible inactivation of photosystems I and II.

We also investigated the role of polyunsaturated lipids in cell membranes in the tolerance of the photosynthetic machinery to high-salt stress by comparing the *desA*$^-$/*desD*$^-$ mutant of *Synechocystis* sp. PCC 6803, which contained monounsaturated fatty acids, with the wild-type strain, which contained the full complement of polyunsaturated fatty acids. The oxygen-evolving activity of *desA*$^-$/*desD*$^-$ cells was more sensitive to high-salt stress than was that of wild-type cells. Moreover, the activity of the Na^+/H^+ antiport in *desA*$^-$/*desD*$^-$ cells was suppressed to a greater extent than that of the antiport in wild-type cells under high-salt stress. These observations suggest that polyunsaturated fatty acids in

membrane lipids might stimulate the activity and/or the synthesis of the Na^+/H^+ antiport system to protect the photosynthetic machinery against salt-induced inactivation.

IV. Acclimation of the photosynthetic machinery to high temperature

High-temperature stress causes the irreversible inactivation of the photosynthetic machinery. However, when photosynthetic organisms have acclimated to moderately high temperatures, their photosynthetic machinery exhibits enhanced thermal stability. We have been studying the molecular mechanisms that underlie the acclimation of the photosynthetic machinery to high temperature in a cyanobacterium and in *Chlamydomonas*.

In the cyanobacterium *Synechococcus* sp. PCC 7002, cytochrome c_{550} and PsbU, the extrinsic proteins of the photosystem II complex, stabilize the oxygen-evolving machinery at high temperatures. To clarify the role of PsbU *in vivo*, we inactivated the *psbU* gene in *Synechococcus* sp. PCC 7002 by targeted mutagenesis. Not only the thermal stability of the oxygen-evolving machinery did not increase in the mutated cells at a moderately high temperature, but these cells were also unable to develop cellular thermotolerance upon acclimation to such temperatures. These results suggest that PsbU might play an important role in enhancing the thermal stability of the oxygen-evolving machinery at high temperatures and, moreover, that the stabilization of the machinery might be crucial for the acquisition of cellular thermotolerance.

In *Chlamydomonas reinhardtii*, enhancement of the thermal stability of the oxygen-evolving machinery was prevented by cycloheximide and lincomycin. The specificity and effects of these drugs suggest that the synthesis of proteins encoded by both the nuclear genome and the chloroplast genome is required for this enhancement. No synthesis of homologs of three heat-shock proteins, namely, Hsp60, Hsp70 and Hsp22, was induced at the moderately high temperatures that induce the enhanced thermal stability of the oxygen-evolving machinery. Thus, it appears likely that heat-shock proteins are not involved in the acclimation of the photosynthetic machinery to high temperature.

Publication List:

(1) Original articles

Alia, Kondo, Y., Sakamoto, A., Nonaka, H., Hayashi, H., Saradhi, P.P., Chen, T.H.H. and Murata, N. (1999) Enhanced tolerance to light stress of transgenic *Arabidopsis* plants that express the *codA* gene for a bacterial choline oxidase. *Plant Mol. Biol.*, **40**, 279-288.

Allakhverdiev, S.I., Nishiyama, Y., Suzuki, I., Tasaka, Y. and Murata, N. (1999) Genetic engineering of the unsaturation of fatty acids in membrane lipids alters the tolerance of *Synechocystis* to salt stress. *Proc. Natl. Acad. Sci. U.S.A.*, **96**, 5862-5867.

Malakhov, M.P., Malakhova, O.A. and Murata, N. (1999) Balanced regulation of expression of the gene for cytochrome c_M and that of genes for plastocyanin and cytochrome c_6 in *Synechocystis*. *FEBS Lett.*, **444**, 281-284.

Nishiyama, Y., Los, D.A. and Murata, N. (1999) PsbU, a protein-associated with photosystem II, is required for the acquisition of cellular thermotolerance in *Synechococcus* species PCC 7002. *Plant Physiol.*, **120**, 301-308.

Papageorgiou, G.C., Govindjee, Govindjee, R., Mimuro, M., Stamatakis, K., Alygizaki-Zorba, A. and Murata, N. (1999) Light-induced and osmotically-induced changes in chlorophyll *a* fluorescence in two *Synechocystis* sp. PCC 6803 strains that differ in membrane lipid unsaturation. *Photosynth. Res.*, **59**, 125-136.

Yamamoto, H., Miyake, C., Dietz, K.-J., Tomizawa, K.-I., Murata, N. and Yokota, A. (1999) Thioredoxin peroxidase in the cyanobacterium *Synechocystis* sp. PCC 6803. *FEBS Lett.*, **447**, 269-273.

(2) Review article

Los, D.A. and Murata, N (1999) Responses to cold shock in cyanobacteria. *J. Mol. Microbiol. Biotechnol.*, **1**, 221-230.

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 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 gametophytes as a model plant 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 for mutant screening to clarify the genes regulating chloroplast photo-relocation. We have begun to use Physcomitrella patens as a model system for which gene targeting is available.

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 various blue-light responses in Adiantum, we are working on the cloning and sequencing of the genes of blue light receptors and on the gene expression and intracellular distributions of the gene products.

1-1 Cryptochromes

We identified two different genes, designated Adiantum cryptochrome4 and 5 (CRY4 and 5), from a genomic DNA library, and found stage specific and light dependent gene expression of the five CRY genes (two newly isolated and three previously isolated genes) in Adiantum. The expression of CRY4 and CRY5 is regulated by light and is under phytochrome regulation. The intracellular distribution of reporter GUS-CRY fusion proteins indicates that GUS-CRY3 and GUS-CRY4 localize in gametophyte nuclei. The nuclear localization of GUS-CRY3 is regulated in a light-dependent manner (Imaizumi et al, Plant Cell 12: 81-96, 2000).

Cryptochrome genes of Physcomitrella patens have also been identified.

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 phototropin has been sequenced. The complete cDNA clone is 3492 bp in length and encodes a protein of 1092 amino acids. Southern blot analysis showed that Adiantum phototropin is likely to be a single copy gene. RT-PCR analysis revealed that it was expressed in various developmental stages (imbibed spores, protonemata, dark grown young leaves, and light grown young leaves). The chromophore attached to LOV (Light, Oxygen, or Voltage) domains was flavin mononucleotide (FMN).

We also cloned and sequenced two cDNAs and respective genomic DNAs of

phototropin from Physcomitrella patens.

1-3 Adiantum Phytochrome 3

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. From the analytical study of fusion protein expressed in E. coli, LOV domains of PHY3 also bind with a FMN, suggesting that phy3 absorbs blue light as well as red/far-red light.

II. Chloroplast relocation

2-1 Arabidopsis

Chloroplasts accumulate at the cell surface under weak light and escape from strong light to optimize photosynthesis. The mechanism of chloroplast relocation, however, is not known. We studied light induced-chloroplast relocation in leaves of Arabidopsis mutants such as cry1, cry2, cry1cry2 and nph1 and found that the chloroplast relocation movement was normal in these mutants, meaning that another (i.e. a new) blue light receptor for chloroplast relocation must exist. To find the photoreceptor for blue light-induced chloroplast relocation, we screened several mutants from T-DNA tagging lines as well as EMS lines of Arabidopsis. The identification of the mutated genes of Arabidopsis mutants is now in progress.

2- 2 Adiantum

A heavy-ion-beam induced deletion mutant of Adiantum which does not show phototropic response and chloroplast photorelocation movement under red light was revealed to lack the PHY3 gene by genomic PCR and Southern blot analysis. It is suggested that phy3 is the photoreceptor mediating red light-induced tropic response and chloroplast photorelocation movement. However, these phenomena induced by blue light are normal, meaning that phy3 might or might not absorb blue light. In the former case, Adiantum phototropin may also work on blue light-induced response as phy3 does.

Publication List:

Christie, J.M., M. Salomon, K. Nozue, M. Wada and W.R. Briggs (1999) LOV (light, oxygen, or voltage) domains of the blue-light photoreceptor phototropin (nph1): Binding sites for the chromophore flavin mononucleotide. Proc. Natl. Acad. Sci. USA 96: 8779-8783.

Imaizumi, T., T. Kiyosue, T. Kanegae and M. Wada (1999) Cloning of the cDNA encoding the blue-light photoreceptor (cryptochrome) from the moss *Physcomitrella patens* (Accession No. AB027528) (PGR99-110) Plant Physiol. 120: 1205.

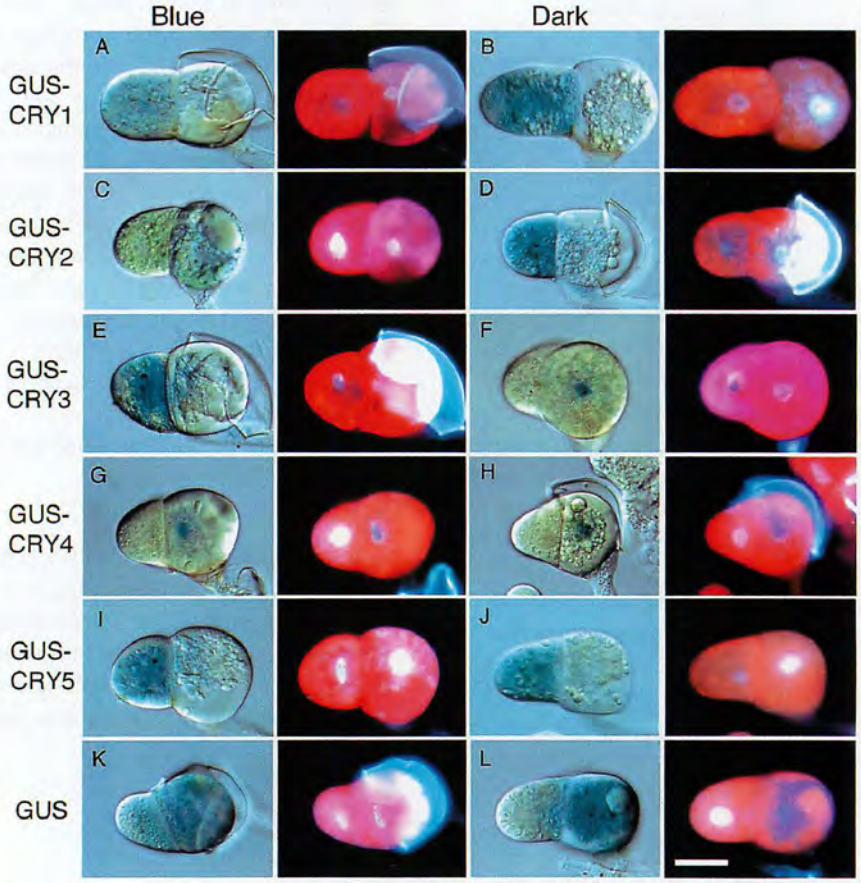

FIG. Representative Images of the Intracellular Distribution of GUS-CRY Fusion Proteins in Germinating Cells of *Adiantum*.
Intracellular distribution of GUS-CRY fusion proteins under different light conditions. GUS-CRY1 (A and B), GUS-CRY2 (C and D), GUS-CRY3 (E and F), GUS-CRY4 (G and H), GUS-CRY5 (I and J), GUS (K and L). Two-celled protonemata expressing various GUS-CRY fusion proteins were incubated under blue light (A, C, E, G, I, and K) or in the dark (B, D, F, H, J, and L) for 16 hr and stained under the same light conditions. The cells showing GUS activity were photographed using Nomarski optics (left panels) and fluorescence micrographs show the position of the nuclei in the same cells stained with 4',6-diamidino-2-phenylindole (right panels). Note that under fluorescence, chlorophyll autofluoresces red and spore coats appear bluish white. The bar shown in L represents 20 μm for all panels.

DIVISION OF BEHAVIOR AND NEUROBIOLOGY (ADJUNCT)

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Associate Professor: *Masato Nakafuku*
Research Associate: *Atsushi Tamada* [1]
Postdoctoral Fellows: *Tatsuro Kumada* [4]
Hiroki Taniguchi [4]
Yumiko Hatanaka
Graduate Student: *Hiroshi Ono*
(from Osaka University)

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.

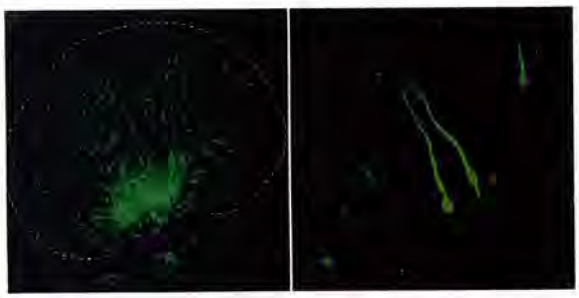

Figure 1. Migrating neocortical neurons visualized with green fluorescent protein (GFP) in vitro.
(Left) Slice culture of rat neocortex that was introduced with GFP cDNA into the ventricular zone by electroporation. A broken line shows the outer margin of the slice. GFP-positive neurons were moving radially 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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	Mary L. Durbin[2)]

[1)] *from Duke University*
[2)] *from University of California, Riverside*

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 genomes, we are currently focused on mobile genetic elements in general and plant transposable controlling elements in particular.

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

The Japanese morning glory is a traditional horticultural plant in Japan, and extensive physiological and genetical studies on the plant have been conducted. A number of mutants related to the color and shape of its flowers and leaves have been isolated since the 17th century, and more than 200 genetic loci including about 20 mutable loci have been documented. We have identified that the mutable *flecked* allele bearing white flowers with colored flecks is the *DFR-B* gene having the *En/Spm*-related transposable element *Tpn1* inserted into its second intron. The non-autonomous *Tpn1* element carries a part of the genomic sequence for an HMG-box. Thus *Tpn1* can be regarded as a specialized transducing transposon.

It is known that the frequency and the timing of the flecking phenotype tend to be heritable by their progeny, although conversion of these phenotypes is sometimes observed. From the variegated flower lines, white variants bearing white flowers free from variegation throughout their plant lives occasionally appeared. Since some of the selfed progeny of the white variants produced a few flecked flowers together with white flowers (others bore only white flowers), the plants were called as variants rather than mutants. We have examined the structure of the *DFR-B* region in the white variants and found that *Tpn1* excision rarely occurs in the variants. DNA methylation in the subterminal repetitive regions of *Tpn1* in the *DFR-B* gene of the white variant appeared to be similar to that of a heavily variegated line, suggesting that the DNA methylation within *Tpn1* is probably not the primary cause of deficiency in excision and that the *Tpn1*-related autonomous element in the white variant is likely to become inactive. The results also suggest that the frequency and the timing of the flecking phenotype of the variegated flowers in the mutable *flecked* line are mainly dependent on the activity of the *Tpn1*-related autonomous element.

The plants with the recessive mutable *speckled* allele, in the presence of the dominant *Speckled-activator*, produce colorless flowers with fine and round colored spots distributed over the corolla, while plants carrying the *speckled* allele without active *Speckled-activator* bear pale yellow flowers. We have found that the *speckled* allele is the *CHI* gene containing a *Tpn1*-related non-autonomous element of 6.5 kb, termed *Tpn2*. Interestingly, *Tpn2* is also a specialized transducing transposon containing a part of the genomic sequence encoding β-galactosidase. The results indicate that the dominant *Speckled-activator* is the *Tpn1*-related autonomous element acting on not only *Tpn2* on the mutable *speckled* allele but also *Tpn1* on the mutable *flecked* allele.

II. Identification of mutable alleles in the common morning glory

The mutable *flaked* line of the common morning glory also bears white flowers with colored flakes and sectors. We showed that the mutable *flaked* allele is caused by insertion of a 3.9 kb transposable element, *Tip100*, into the *CHS-D* gene intron and that *Tip100* belongs to the *Ac/Ds* family. It has been postulated that the timing and the frequency of the variegation are determined by the active state of another genetic element *modulator*. As an initial step to elucidate the genetic system to determine the timing and the frequency of the flower variegation, we examined whether *Tip100* is able to transpose in transgenic tobacco plants and found that *Tip100* can be excised from the introduced vector carrying *Tip100*. The results strongly indicate that *Tip100* is an autonomous element.

III. Characterization of the genes for anthocyanin pigmentation in morning glories.

The *CHS* genes encoding chalcone synthase for flavonoid biosynthesis comprise a multigene family in the common and Japanese morning glories. Among these *Ipomoea CHS* genes, the *CHS-D* gene is the most abundantly expressed in the pigmented young flower buds and is primarily responsible for flower pigmentation. The majority of the remaining *CHS* transcripts in the flower buds are produced from the *CHS-E* gene. Moreover, the *CHS-D* and *CHS-E* genes are expressed predominantly in flower limbs and tubes, respectively. The recombinant CHS-D and CHS-E proteins obtained by expressing the *CHS-D* and *CHS-E* genes in *Escher-*

ichia coli with various expression systems showed CHS activity to produce naringenin chalcone. These results are consistent with the notion that the *CHS-D* and *CHS-E* genes encode the chalcone synthases for anthocyanin biosynthesis in flowers (see also II).

We have characterized the genomic DNA segments of the *CHS-D* and *CHS-E* genes in the Japanese and common morning glories. Both genes have two exons with identical intron positions and carry several copies of two mobile element-like sequences with short terminal inverted repeats, *MELS3* and *MELS6* of around 200 - 300 bp. Small tandem repeats were also found in these *CHS* gene regions. Gene duplication and subsequent divergence are regarded to play important roles in evolution of multiple genes. Comparison of the genomic sequences suggests that gene duplication and major divergence in these *CHS* genes occurred prior to the speciation of the Japanese and common morning glories. Subsequent DNA rearrangements are likely to have taken place after the speciation, and the *MELS* elements appear to play a role in generating such DNA rearrangements.

IV. An efficient transformation system and homologous recombination in rice.

In higher plants, efficient and reliable gene targeting procedures for "reverse genetics" remains to be established. As an initial step to develop such procedures, we are trying to develop an efficient rice transformation system and searching for factors to enhance homologous recombination in rice. We employed an Agrobacterium-mediated transformation system using vigorously dividing calli derived from mature seed scutellum. Under the optimum condition, approximately 1200 independent transformed calli were obtainable from 150 matured seeds.

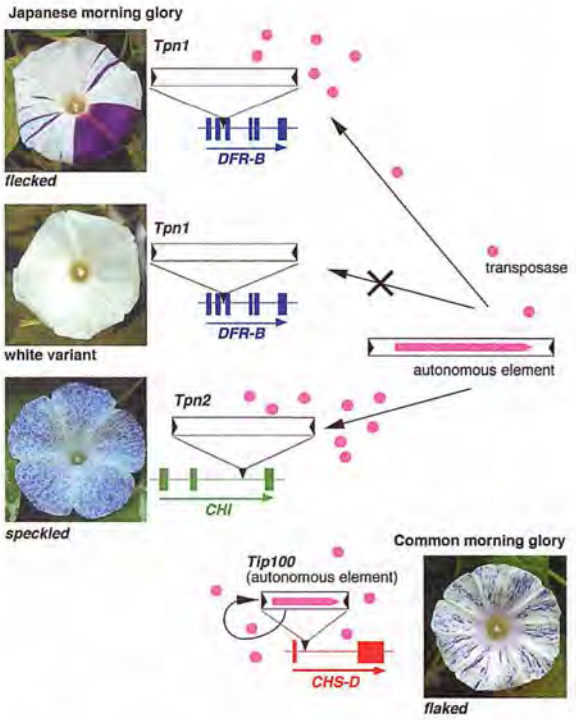

Fig.1. Mutable alleles and transposable elements in the Japanese and common morning glories. In the Japanese morning glory, activities of a putative autonomous element act on the non-autonomous *Tpn1* and *Tpn2* elements and determine the frequency and the timing of flower variegation. In the common morning glory, *Tip100* is an autonomous element.

Publication List:

Hasebe A. and Iida, S. The novel insertion sequences IS*1417*, IS*1418* and IS*1419* from *Burkholderia glumae* and their strain distribution. *Plasmid* in press.

Iida, S., Hoshino, A., Johzuka-Hisatomi, Y., Habu Y. and Inagaki, Y. (1999) Floricultural traits and transposable elements in the Japanese and common morning glories. *Annal. New York Acad. Sci.* 870, 265-274.

Iida S. and Hoshino, A. Spontaneous mutagenesis and transposable elements in the Japanese morning glory. *Gamma Field Symposia* in press.

Inagaki, Y., Johzuka-Hisatomi, Y., Mori, T., Takahashi, S., Hayakawa, Y., Peyachoknagul, S., Ozeki Y. and Iida, S. (1999) Genomic organization of the genes encoding dihydroflavonol 4-reductase for flower pigmentation in the Japanese and common morning glories. *Gene* 226, 181-188.

Johzuka-Hisatomi, Y., Hoshino, A., Mori, T., Habu Y. and Iida, S. (1999) Characterization of the chalcone synthase genes expressed in flowers of the common and Japanese morning glories. *Genes Genet. Syst.* 74, 141-147.

Shiokawa, K., Inagaki, Y., Morita, H., Hsu, T.-J., Iida S. and Noguchi, H. The functional expression of the *CHS-D* and *CHS-E* genes of the common morning glory (*Ipomoea purpurea*) in *Escherichia coli* and characterization of their gene products. Plant Biotechnology in press.

Takahashi, S., Inagaki, Y., Satoh, H., Hoshino A. and Iida, S. (1999) Capturing of a genomic *HMG* domain sequence by an *En/Spm* related transposable element *Tpn1* in the Japanese morning glory. *Mol. Gen. Genet.* 261, 447-451.

DIVISION OF GENE EXPRESSION AND REGULATION II

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Takehiko Kobayashi
Ken-ichi Kodama
**Katsuki Johzuka*
Technical Staff: *Kohji Hayashi*
Yasushi Takeuchi
Graduate Student: *Hiroko Urawa*

**to Department of Biological Chemistry, University of California-Irvine, Irvine, California (Feb.1. 1999-)*

Homologous recombination, which may occur in all organisms, apparently involves genetic exchange between two parent-derived chromatids in addition to the repair of DNA damage induced by physical and chemical reagents. As deduced from 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 1999, work on the two following subjects has advanced our knowledge of both the dynamics and function of genome.

I. Replication fork block protein (Fob1) acts as a rDNA specific recombinator.

We previously found that some recombinational hotspots in *E. coli* are 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 chromosomes. Consequently, this recombination reaction for renewal of the replication fork is a primary cause resulting in 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 rRNA multi-genes (rDNA). It has been reported that in budding yeast (*S. cerevisiae*) when a non-transcriptional region (NTS) of the rDNA, in which *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. In addition to this finding, the detection of *RFB* activity within the rDNA cluster in various higher eucaryotic cells indicates that the fork renewal cycle occurring after the blockage is more general than expected.

A copy number of rDNA repeats is unstable. Until recently, though an increase and a decrease in a number of rDNA copies have been observed in various eucaryotes, the underlying mechanism remains unknown. Because fork arrest at the *RFB* site was a trigger of recombination, as mentioned above, we suspected that a fork-blocking event was involved in changing the copy number. Upon examination it turned out that in a *fob1* mutant neither an increase nor a decrease in the rDNA copy number took place; thereby suggesting that the fork blocking event is required for changing the copy number, probably through recombination process. Since fork blocking system has only been understood to prevent replication from "colliding" with rDNA transcription so far, this study sheds light a novel physiological role for fork blocking system. The newly discovered role may be quite a common in most of eucaryotes.

This year we conducted a molecular examination as to whether the fork blocking step is actually essential for homologous recombination within the rDNA repeated region. In this case, recombination means intra- or inter-sister chromosomal exchange. Because the intra-sister chromosomal recombination between two repeats of the rDNA produces a covalently closed circular (ccc) molecule, its recombination activity can be assayed easily by measuring the quantity of ccc molecules produced. In the case of inter-chromosomal recombination, since the structure of the rDNA cluster remains unchanged whether inter-recombination occurs or not, the recombination product is hard to detect. However, because rDNA has a repeated structure, if any selective maker (*URA3* marker in this experiment) is inserted anywhere in the rDNA region, unequal inter-sister chromosomal recombination can be assayed by detecting duplicated molecules of the inserted marker. Using these two assay methods, we investigated whether *FOB1* or other recombination genes were involved in recombination of the rDNA region and the following results were obtained: (1) the frequency of intra- and inter-recombination in both *fob1* and *rad52* mutants decreased to one tenth of those in the wild type strain (Figure A), (2) while the *RAD52* gene was a general recombinator, the *FOB1* gene was a rDNA region-specific recombinator, (3) a major type of recombination was of the inter-sister chromosomal type, while intra-recombination was minor, (4) at least an inter-recombination occurred uniformly throughout the rDNA region, (5) enhancement of rDNA region-specific recombination by the *sir2* (silencer gene 2) mutation was found to be *FOB1*-dependent.

Guarente's group at MIT has reported that the life span of budding yeast is determined by the number of ccc molecule of rDNA accumulated in mother cells. ccc rDNA molecules, which is excised from the rDNA cluster on the chromosome, are not segregated equally into daughter and mother cells but are accumulated only

in the mother cells. Thus, mother cells "die" after about 20 generations. If the group's speculation is correct, the *fob1* mutant is expected to have a longer life span than the wild type strain. Actually, we found that the *fob1* mutant could live 60 % longer than the wild type strain (Figure B). Independently, Guarente's group also reported a similar result. These findings raised the possibility that the ccc rDNA molecule might also be involved in determining the life span of multicellular organisms. In order to investigate this possibility, isolation and analysis of their *FOB1*-like gene defective mutants should prove to be a successful approach.

II. *E. coli* genomic structure and function

A project named "Structure and function of the *E. coli* genome", supported by a grant termed CREST from JST for a duration of five years, began in January 1999. The team is composed of twelve independent research groups that are divided into four main categories of research. Namely, these divisions are: (1) resource, (2) bio-informatics, (3) database, and (4) systematic functional analysis. At present, we are determining the sequences of a remaining region (about 370 kb) of the genome of *E. coli* W3110 strain and will complete it until this summer. The resource group has constructed a large number of gene disruptants using a random transposon insertion method, in which the insertion sites will be determined by sequencing in part in this laboratory. Our immediate goal is to establish a systematic method for screening the phenotypes of about 4000 different disruptants, each of which has disrupted mutation in each of about 4000 nonessential genes.

Publication List:

Taki, K., Horiuchi, T
The SOS response is induced by replication fork blockage at a *Ter* site located on a pUC-derived plasmid: dependence on the distance between *ori* and *Ter* sites. Mol. Gen. Genet. (1999) 262, 302-309.

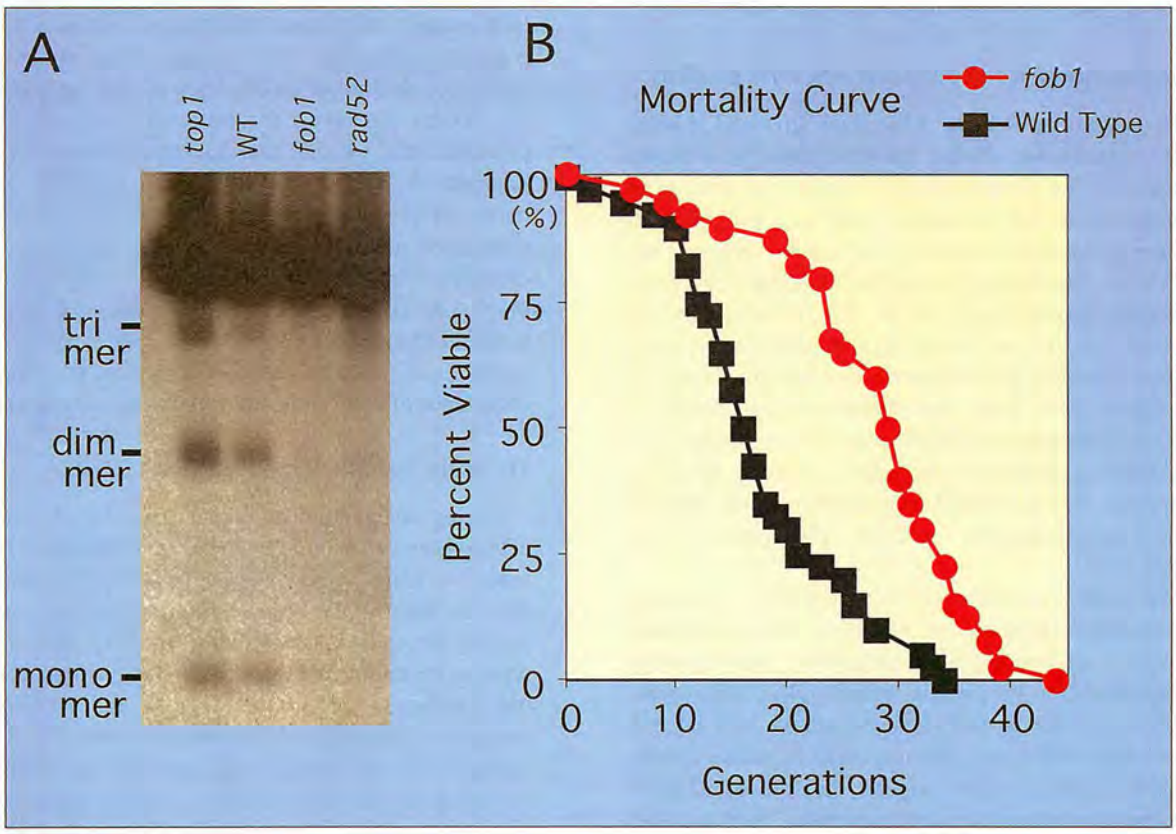

Figure: *fob1* is a long life mutant.
(A) Production of covalently closed circular (ccc) rDNA molecules (mono-, di-, and trimmer) of wild type, *fob1* and other mutants. Guarente's group have been argued that amount of accumulated ccc rDNA molecules in a mother cell determines her life span. (B) Mortality curves of wild type and *fob1* mutants. Life span was determined by scoring the number of daughter cells produced by each mother cell until she lost her ability to bud. Average life spans were as follows: wild type (black square), 17.9 generations and *fob1* (red circle), 28.2 generations.

DIVISION OF SPECIATION MECHANISMS I

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Technical Staffs:	*Hideko Utsumi*
	Kaoru Sawada
NIBB Postdoctoral Fellow:	*Rejan Vigot*
Visiting scientist:	*Yoshinori Shirai*
Graduate student:	*Shiro Tochitani*
	Katsusuke Hata
	Yusuke Komatsu
	Shuzo Sakata
	(kyoto University)
	Ryohei Tomioka
	(Kyoto University)

Our research goal is to understand mechanisms underlying evolution of the nervous system. In order to approach this question, we are studying in two major subjects. 1) One approach is to understand informational processing in the brain underlying learning behaviors. 2) The second approach is to study the genes that are expressed in the specific areas of the monkey neocortex. Here, we report our findings of this year (1999).

I. Gene expression and cerebellar long-term plasticity

In order to know roles of the genes involved in long-term memory, we choose the cerebellum as a model system. In the cerebellum the conjunctive stimuli of parallel fibers and a climbing fiber to a Purkinje cell induce prolonged reduction of a synaptic efficacy between the paralleled fiber to the Purkinje cell (LTD; long-term depression, Ito et al., 1982). Previously, we examined the expression of 10 immediate early genes (IEGs) including all the known Fos and Jun family in cerebellar slices under the pharmacological condition that cause long-term desensitization of the Purkinje cell to AMPA (a glutamate analogue). Among the IEGs examined, Fos and Jun-B were predominantly induced under the conjunctive condition (Nakazawa et al., 1993).

We further examined Jun-B expression in vivo under a conjunctive protocol of AMPA, a pharmacological substitute for parallel fiber stimulation, and climbing fiber stimulation via electric Inferior Olive stimulation. June-B are predominantly induced around the local area where the AMPA and climbing fiber stimulation were conjunct. These results suggest that the coincidence mechanism may exist at gene expression level and lead to a cerebellar long-term plasticity. However, it has not been still clear how these newly synthesized gene products are involved in cerebellar LTD. We are currently investigating the mechanisms.

II. Gene expression under audio-visual discrimination task

We are studying the gene expression of c-Fos under audio-visual discrimination tasks in collaboration with Dr. 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 (manuscript in preparation).

III. Color-defective (dichromat) monkeys

Several percent of humans, most of whom are X-chromosome linked, have been reported to be color deficient. However, despite the color pigment genes of macaque monkeys are very similar to human in the nucleotide sequence, no color-deficient monkeys have been found. We have examined 3153 macaque monkeys using an assay to identify a deletion of exon 5 of the long wave (red) pigment and middle wave (green) pigment genes by PCR amplification with a common primers in both genes and discriminating them by the following *Mbo* I restriction enzyme digestion, and found three monkeys that lacked the exon five of the long wave pigment genes. Further genetic analysis revealed that the three monkeys possessed a hybrid of long (exon 1 to 4) and middle wave (exon 5 and 6) pigment genes (Fig. 1). Analysis of photobleaching difference absorption spectra showed that the absorbency maxima (λmax) of reconstituted L, M and hybrid pigments are 564, 532 and 538 nm, respectively. The λmax for the hybrid was red-shifted by 6 nm from that of the M pigment, indicating that these monkeys are almost protanopic (insensitive to red). The ratio of dichromats in macaque monkeys (approximately 0.1%=3/3153) is significantly lower than that of 1% in humans. These results suggest that there may be some mechanisms which do not exist in humans to reduce the occurrence of color-deficiency in macaque monkeys.

IV. Brain Specific Repetitive (Bsr) RNA

During our attempt to isolate LTD-related genes in cultured cerebellar Purkinje cells, we accidentally found repetitive genes which were specifically expressed in the rat brain (Bsr: brain specific repetitive gene). Among the cells of the rat brain Bsr RNA tends to express in the relatively large cells and in phylogenetically old structures, such as the pareo- and archicortex, amygdala, thalamus and hypothalamus (see Fig. 2., for example). To our surprise, the genes are only found in the rattus but no other species so far examined including murine species. Although we do not know the function of this new type of gene at the moment, it may play some important role in the rat brain and we hope further characterization of the gene reveals it.

kenaka, O., Hanazawa, A., Komatau, H., Mikami, A.,

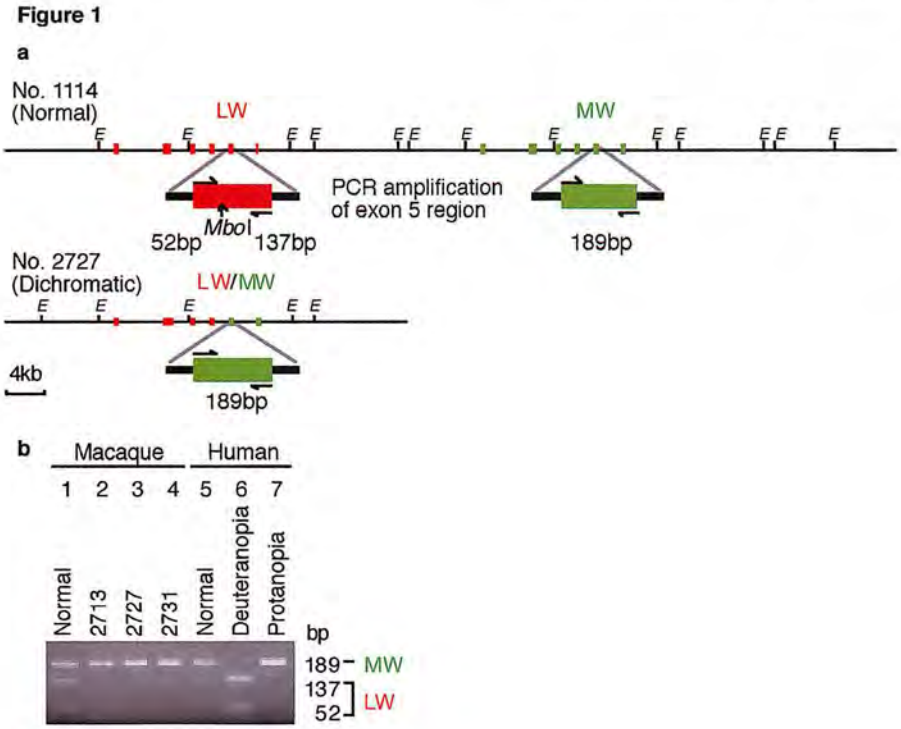

Fig. 1 Physical maps of the L and the M genes. a, Top, the 77-kilobase (kb) region of a wild-type macaque, showing exons of the L (red) and M (green) genes. Enlarged exon-5 regions are shown underneath. The PCR primers and products for exon 5 of the L and M genes are shown in red (137 and 52 base pairs (bp), cleaved by MboI digestion) and green boxes (189 bp, uncleaved), respectively. An Mbo I site specific for exon 5 of the L gene is conserved among Old World monkeys. Bottom, the 36-kb region of a dhichromatic candidate. The enlarged PCR-amplified fragment is shown underneath. B, Gel elctrophoresis analysis. The bands of 189, 137 and 52 bp shown in a are indicated as M (green) or L (red). Lane 1, macaque wild type; lanes 2-4, dichromats, showing only the exon 5 fragment of the M gene; lane 5, human wild type; lanes 6 and 7, deuteranopic (M cone absent) and protanopic (L cone absent) humans, respectively. [Published in Onishi et al., *Nature* 402, 139-140, 1999]

V. 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. The neocortex in the mammal can be divided into distinct functional and anatomical areas. It has been a matter of debate what extent the areas of the neocortex are genetically and environmentally determined. To access this question, we started to isolate the genes that are expressed in specific areas of the brain. Using differential display methods, we found at least two genes that indicated the area specific expression. One, designated OCC-1 is expressed in the occipital cortex in the brain and the other gene is expressed in the motor area. We are currently examining the detailed expression pattern of the genes. We are also further isolating area specific genes with RLCS (Restriction Landmark cDNA Scanning).

Fig. 2 Expression of Bsr in the rat hippocampus. In situ hybridization with an antisense probe of Bsr. Inset: higher magnification. [published in Komine et al., *Mol. Brain Res.*, 66, 1-13, 1999]

Publication List:

Liang, F., Hatanaka, Y., Saito, H., Yamamori, T. and Hashikawa, T. Differential expression of gamma-aminobutyric acid type B receptor-1a and 1b mRNA variants in GABA and non-GABAergic neurons of the rat brain. (2000) *J. Comp. Neurol.* 416, 475-495

Onishi, A., Koike, S., Ida, M., Imai, H., Shichida, Y., Tagoto, S., Suryobroto, B., Kitahara, K. and Yamamori, T. (1999) Dichromatism in macaque monkeys. *Nature* 422, 140-141.

Tochitani, S., Watakabe, A., Liang, F. and Yamamori, T. (1999) A molecular marker for a neuronal population dominantly distributed in the macaque occipital neocortex. *29th Society for Neuroscience (North America) Abstract* 2190.

Komine, Y., Tanaka, N., Yano, R., Takai, R., Yuasa, S.,

Shiroishi, T., Tsuchiya, K. and Yamamori, T. (1999) A novel type of non-coding RNA expressed in the rat brain. *Mol. Brain Res.*, 66, 1-13.

Matsuzawa, M., Muramatsu, T., Yamamori, T., Knoll, W. and Yano, R. (1999) Novel neuronal activities of midkine were examined on embryonic cerebellar neurons using a defined culture system. *Cell. Mol. Neurobiol.*, 19, 209-220.

Kawasaki, T., Kitsukawa, T., Bekku, Y., Matsuda, Y., Sanbo, M., Yagi, T., Fujisawa, H. (1999) A requirement for neuropilin-1 in embryonic vessel formation. *Development* 126, 4895-902

DIVISION OF SPECIATION MECHANISMS II

Associate Professor: *Mitsuyasu Hasebe*
Assistant Professor: *Tomomichi Fujita*
Technical Staff: *Yukiko Tanikawa*
Masae Umeda
Yoshimi Bitoh
Makiko Kondo
Post Doctoral Fellow: *Rumiko Kofuji*[1]
Graduate Students: *Satomi Shindo*
Yuji Hiwatashi
Satoko Kobayashi-Arakawa
Keiko Sakakibara
Ryosuke Sano (Chiba Univ.)
Tomoaki Nishiyama
(Univ. of Tokyo)
Takako Tanahashi (Univ. of Tokyo)

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 remained in the genomes of extant organisms, and we can infer (1) the phylogenetic relationships of organisms and (2) the genetic changes having caused the phenotypic evolution by comparing the genomes of different organisms. The inferred phylogenetic relationships give important insights on problems in various fields of evolutionary biology, and our group is now focusing on biogeography, evolution of morphological traits, and systematics in wide range of taxa. On the phenotypic evolution, we are especially interested in the morphological evolution and aim to explore genetic changes led the evolution of plant body plan. We selected *Arabidopsis* (angiosperm), *Gnetum* (gymnosperm), *Ginkgo* (gymnosperm), *Ceratopteris* (pteridophyte), and *Physcomitrella* (bryophyte) as models to compare the genetic cascades regulating morphogenesis especially in 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 (Hasebe and Ito 1999, Hasebe 1999). (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. New hypothesis on the homology of *Gnetum* reproductive organ

Gnetales, one of the extant gymnosperm orders has traditionally been recognized to be most closely related to flowering plants, because the reproductive organ of Gnetales has some morphological characters similar to flowering plants. Most recent molecular phylogenetic studies do not support the sister relationship of the Gnetales and flowering plants, but instead support a close relationship between Gnetales and other extant gymnosperms. The MADS-box genes are transcription factors, some of which are involved in reproductive organ development in flowering plants. To resolve the discrepancy in phylogenetic inferences, and to provide insights into the evolution of reproductive organs in seed plants, four MADS-box genes (*GpMADS1 - 4*) were cloned from *Gnetum parvifolium* (Fig. 1A, B).

GpMADS2 is likely to be a pseudogene and the other three genes were characterized. A MADS-box gene tree based on partial amino acid sequences showed that *GpMADS3* is included in the AGL6 group, but the other two genes do not cluster with any previously reported MADS-box gene. The three *GpMADS* genes were expressed during the early stage of ovule development in the differentiating nucellus and three envelopes (Fig. 1C-F). A comparison of MADS-box gene expression among conifers, *Gnetum*, and flowering plants suggests that the comparable reproductive organs in *Gnetum* and flowering plants evolved in parallel, and is likely to support the homology between the ovule-ovuliferous scale complex of conifers and the *Gnetum* ovules including the three envelopes (Fig. 1G).

III. Evolution of gametophytic and sporophytic generations

Plants have the haploid gametophytic and diploid sporophytic generations, both of which are usually multicellular and morphologically diversified each other. Origin and evolution of the two generations have been discussed and some hypotheses are proposed, although the molecular basis of the both generations have not been studied. For example, it is not known whether different sets of genes contribute to the morphological differences of the two generations or similar genes are differently used. Ferns are good materials to study the evolution of gametophytic and sporophytic generations, because individuals of the both generations grow independently and it is much easier to analyze than other plats in which one of the two generations is enrolled in the tissue of another generation.

We have cloned 11 homeodomain - leucine zipper (HD-Zip) genes of the fern *Ceratopteris richardii* (*Crhb* genes) encoding a transcription factor and are characterized by the presence of a homeodomain closely linked to a leucine zipper motif. Most of *Crhb* genes belonging to the HD-ZIP I or II subfamilies are almost ubiquitously expressed in both sporophytes and gametophytes, while the amounts of expression are different among the tissues. The similarly ubiquitous expression patterns among different sporophytic tissues have been reported in some angiosperm HD-Zip genes, although the patterns of expression in gametophytic tissues in angiosperms are not known. Our study clearly showed that most of *Ceratopteris* HD-Zip genes are ubiquitously expressed even in gametophytes from which morphologically different tissues and organs of sporophytes develop. It may indicate that *Crhb* genes have unknown functions common in the both generations. On the contrary, *Crhb1* and *Crhb3* genes are expressed only in the gametophytes and it may suggest the existence of gametophyte specific regulatory systems which cause morphological diversity between the two generations. Further studies on the regulatory elements of the *Crhb* genes and on more detailed spatial patterns of expression will give useful information to reveal the evolutionary aspects of differences between sporophytic and gametophytic generations.

IV. Establishment of tagged mutant library of the moss *Physcomitrella patens.*

Mosses have the different body plan from flowering plants. Leafy shoots of mosses are similar to the ones of angiosperms, but develop in the gametophytic generation instead of the sporophytic generation as angiosperms. Organs of mosses are much simpler than flowering plants. For example, the leaves are composed of one layer of cells. Therefore, the body plan of mosses may be regulated by different genes from angiosperms. In addition to analyses of the homologs of angiosperm genes governing morphogenesis, it is necessary to screen specific genes in the moss. We established enhancer and gene trap lines and tagged mutant libraries of *Physcomitrella patens* to clone genes related to leafy shoot development. *P. patens* is known by its high rate of homologous recombination and suitable for molecular biological analyses using the gene targeting. Our libraries should be also useful for other purposes.

Publication List:

Hasebe, M. (1999) Evolution of reproductive organs in land plants. J. Plant Res. **112**: 463-474.

Hasebe, M. and Ito, M. (1999) Evolution of reproductive organs in vascular plants. *In* The Biology of Biodiversity (M. Kato ed.), pp.243-255. Springer-Verlag, Tokyo.

Shindo, S., Ito, M., Ueda, K., Kato, M. and Hasebe, M. (1999) Characterization of MADS genes in the gymnosperm *Gnetum parvifolium* and its implication on the evolution of reproductive organs in seed plants. Evolution and Development **1** : 180-190.

Aso, K., Kato, M., Banks, J.A. and Hasebe, M. (1999) Characterization of homeodomain-leucine zipper genes in the fern, *Ceratopteris richardii* and the evolution of the homeodomain-leucine zipper gene family in vascular plants. Mol. Biol. Evol. **16**: 544-552

Salzman, R., Fujita, T., Zhu-Salzman, K., Hasegawa, M. and Bressan, R.A. (1999) An improved RNA isolation method for plant tissues containing high levels of phenolic compounds or carbohydrates. Plant Mol. Biol. Repter **17**: 11-17.

Nishiyama, T. and Kato, M. (1999) Molecular phylogenetic analysis among bryophytes and tracheophytes based on combined data of plastid coded genes and the 18S rRNA gene. Mol. Biol. Evol. **16**: 1027-1036.

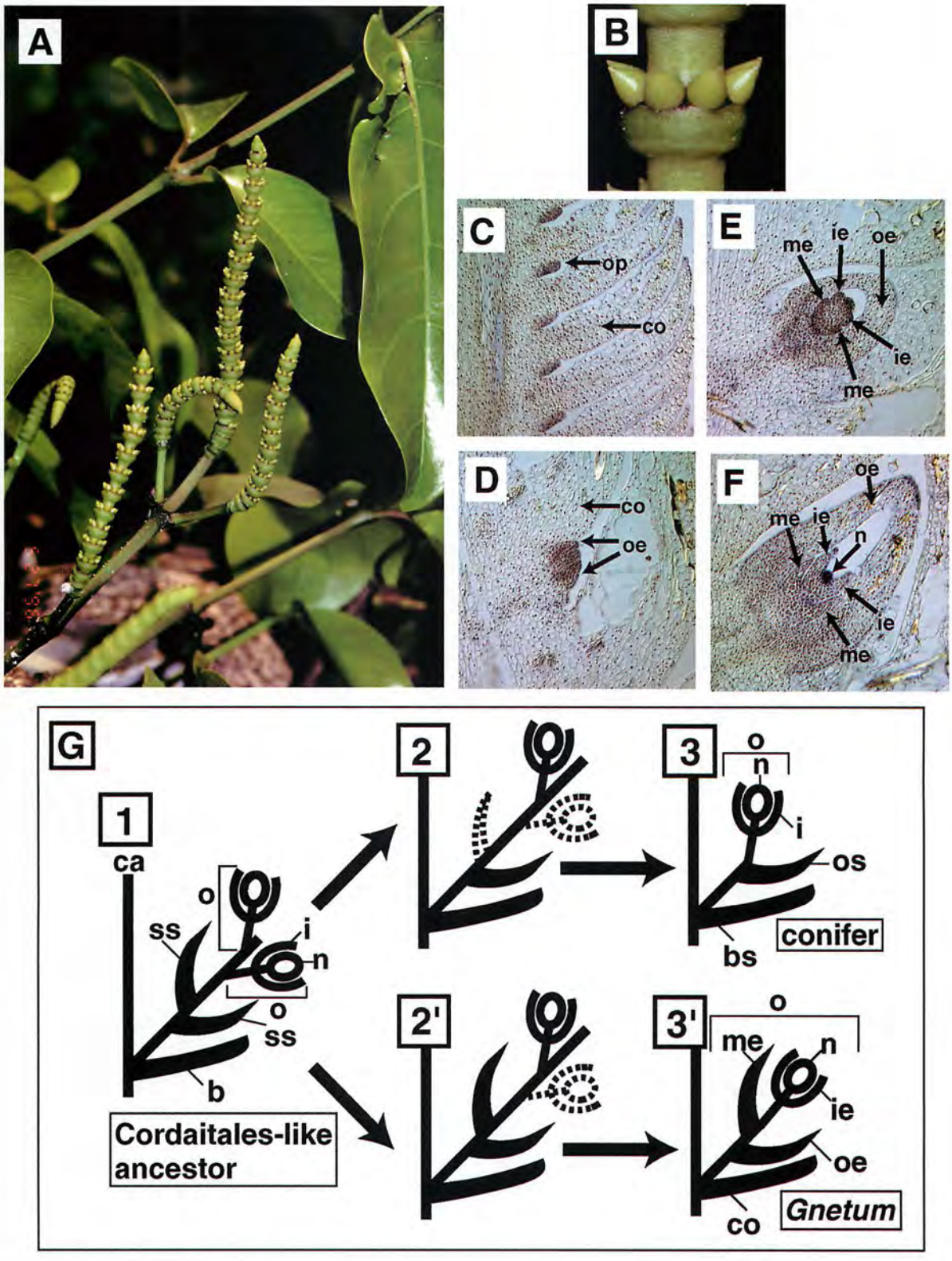

Fig. 1 New hypothesis on the homology between *Gnetum* and conifer reproductive organs. (A) *Gnetum parvifolium.* Female strobili and vegetative branches with leaves. (B) A whorl of nearly mature ovules subtended by annular collars. (C-F) *In situ* localization of *GpMADS3* mRNA in developing ovules of *Gnetum parvifolium.* (G) Scenario for the evolution of ovule-bearing structures in *Gnetum* and conifers from a Cordaitales-like prototype. b, bract; bs, bracteole scale; ca, cone axis; co, collar; i, inner integument; ie, inner envelope; me, middle envelope; n, nucellus; o, ovule; op, ovule primordium; oe, outer envelope; os, ovuliferous scale; ss, sterile scale.

CENTER FOR BIO-ENVIRONMENTAL SCIENCE

Interim Head: Hideo Mohri

The center is currently managed by NIBB and is scheduled to be included in a new center for biosciences which will be 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) Endocrine disrupters. 2) Other chemicals affecting the environment. 3) Biomolecular sensors of environmental factors. 4) Database of bio-environmental science.

Professor : Teizo Kitagawa

I. Biomolecular sciense

Elucidation of a structure-function relationship of metalloproteins is a current subject of this group. The primary technique used for this project is the stationary and time-resolved resonance Raman spectroscopy monitored by near IR to UV lasers. The main themes that we want to explore are (1) mechanism of oxygen activation by enzymes, (2) mechanism of active proton translocation and its coupling with electron transfer, (3) coupling mechanism of proton- and electron transfers by quinones in photosynthetic reaction center, (4) higher order protein structures and their dynamics, and (5) reactions of biological NO.

In category (1), we have examined a variety of terminal oxidases, cytochrome P450s, and peroxidases, and also treated their enzymatic reaction intermediates by using the mixed flow transient Raman apparatus and the Raman/absorption simultaneous measurement device. For (2) the third generation UV resonance Raman (UVRR) spectrometer was constructed and we are going to use it to the peroxy and ferryl intermediates of cytochrme c oxidase. In (3) we succeeded in observing RR spectra of quinones A and B in bacterial photosynthetic reaction centers for the first time last year, but we focused our attention on tyrosine radical this year. For (4) we developed a novel technique for UV resonance Raman measurements based on the combination of the first/second order dispersions of gratings and applied it successfully to 235-nm excited RR spectra of several proteins including mutant hemoglobins and myoglobins. Nowadays we can carry out time-resolved UVRR experiments with nanosecond resolution to discuss protein dynamics. We have succeeded in isolating the spectrum of β145-Tyr, β35-Tyr and α140-Tyr of Hb A separately and their changes upon quaternary structure transition. For (5) we purified soluble guanylate cyclase from bovine lung and observed its RR spectra. To understand the implication, we examined Raman spectra of NO adducts of various mutant Mbs.

II. Fast dynamics of molecules in a solution phase

Picosecond time-resolved resonance Raman (ps-TR^3) spectroscopy is a promising technique to investigate ultrafast structural changes of molecules. However, this technique has not been used as widely as nanosecond TR^3 spectroscopy, mainly due to the lack of light source which has suitable repetition rates of pulses and wavelength tunability. In order to obtain qualified TR^3 spectra, first we need two independently tunable light sources for pump and probe pulses. Second, the repetition rate should be higher than kilohertz to keep a moderate average laser power without allowing the photon density of probe pulse to be too high. We succeeded in developing light sources for ps-TR^3 spectroscopy having wide tunability and kHz repetition, and applied them to study fast dynamics of photo-excited molecules. For carbonmonoxy myoglobin (MbCO), vibrational relaxation with the time constant of 1.9 ps was observed for CO-photodissociated heme. For Ni-octaethylporphyrin in benzene, appreciable differences in the rise times of population at vibrationally excited levels among various modes were observed in the anti-Stokes spectra for the first time. For the same molecule in piperidine, coordination of two solvent molecules was observed in the transient (d,d) excited state. The ps-TR^3 experiments were also applied to Zn-porphyrin dimers, for which some evidence for the π–π interaction in the S_1 state was obtained. The UV ns-TR^3 experiments on MbCO demonstrated the presence of a transient open form of the ligand pathway.

Publication List:

Itoh, S., Bandoh, H., Nagatomo, S., Kitagawa, T. and Fukuzumi, S. (1999) Aliphatic hydroxylation by a bis(μ-oxo)dinickel(III) complex. *J. Am. Chem. Soc.* **121**, 8945-8946.

Itoh, S., Taki, M., Kumei, H., Takayama, S., Nagatomo, S., Kitagawa, T., Sakurada, N., Arakawa, R. and Fukuzumi, S. (1999) Model complexes of the active form of galactose oxidase. Physicochemical properties and reactivity of Cu(II)- and Zn(II)-phenoxyl radical complexes of the novel organic cofactor. *Angew. Chem.Intl. Ed.* **38**, 2775-2776.

Iwase, T., Varotsis, C., Shinzawa-Itoh, K., Yoshikawa, S. and Kitagawa, T. (1999) Infrared evidence for Cu_B ligation of photodissociated CO of cytochrome *c* oxidase at ambient temperatures and accompanied deprotonation of a carboxyl side chain of protein. *J. Am. Chem. Soc.* **121**, 1415-1416.

Kaminaka, S., Imamura, Y., Shingu, M., Kitagawa, T. and Toyoda, T. (1999) Studies of bovine enterovirus structure by ultraviolet resonance Raman spectroscopy. *J. Virol. Met.* **77**, 117-123.

Kohzuma, T., Inoue, T., Yoshizaki, F., Sasakawa, Y., Onodera, K., Nagatomo, S., Kitagawa, T., Uzawa, S., Isobe, Y., Sugimura, Y., Gotowda, M. and Kai, Y. (1999) The structure and unusual pH-dependence of plastocyanin from the fern *Dryopteris crassirhizoma*: The protonation of an active-site histidine is hindered by π-π interactions. *J. Biol. Chem.* **274**, 11817-11823.

Mizutani, Y., Uesugi, Y. and Kitagawa, T. (1999) Intramolecular vibrational energy redistribution and intermolecular energy transfer in the (d,d) excited state of nickel octaethylporphyrin. *J. Chem. Phys.* **111**, 8950-8962.

Nagai, M., Wajcman, H., Lahary, A., Nakatsukasa, T., Nagatomo, S. and Kitagawa, T. (1999) Quaternary structure sensitive tyrosine residues in human hemoglobin: UV resonance Raman studies of mutants at α140, β35, and β145 tyrosine. *Biochemistry* **38**, 1243-1251.

Nagatomo, S., Nagai, M., Tsuneshige, A., Yonetani, T. and Kitagawa, T. (1999) UV resonance Raman studies of α-nitrosyl hemoglobin derivatives: Relation between the α1-β2 subunit interface interactions and the Fe-histidine bonding of α heme. *Biochemistry* in press,

Nakashima, S., Taniguchi, S., Okada, T., Osuka, A., Mizutani, Y. and Kitagawa, T. (1999) Evidence for π-π interac-

tions in the S_1 state of zinc porphyrin dimers revealed by picosecond time-resolved resonance Raman spectroscopy. *J. Phys. Chem. A* **103**, 9184-9189.

Suga, M., Oku, J., Masuda, H., Takaki, M., Mukai, M. and Kitagawa, T. (1999) Mechanism of the anionic cyclopolymerization of bis(dimethylvinylsilyl)methane. *Macromolecules* **32**, 1362-1366.

Suzuki, N., Higuchi, T., Urano, Y., Kikuchi, K., Uekusa, H., Ohashi, Y., Uchida, T., Kitagawa, T. and Nagano, T. (1999) Novel iron porphyrin-alkanethiolate complex with intramolecular NH...S hydrogen bond: Synthesis, spectroscopy, and reactivity. *J. Am. Chem. Soc.* **121**, 11571-11572.

Tomita, T., Hirota, S., Ogura, T., Olson, J. S. and Kitagawa, T. (1999) Resonance Raman investigation of Fe-N-O structure of nitrosylheme in myoglobin and its mutants. *J. Phys. Chem.B* **103**, 7044-7054.

Wada, A., Ogo, S., Watanabe, Y., Mukai, M., Kitagawa, T., Jitsukawa, K., Masuda, H. and Einaga, H. (1999) Synthesis and characterization of novel alkylperoxo mononuclear iron(III) complexes with a tripodal pyridylamine ligand: A model for peroxo intermediates in reactions catalyzed by non-heme iron enzymes. *Inorg. Chem.* **38**, 3592-3593

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.

Publication list:

Goto, S., Nishikoka, T., and Kanehisa, M. (1999) LIGAND database for enzymes, compounds, and reactions. *Nucleic Acids Res.* **27**, 377-379.

Wackett, L.P., Ellis, L.B.M., Speedie, S.M., Hershberger, C.D., Knackmuss, H.-J., Spormann, A.M., Walsh, C.T., Forney, L.J., Punch, W.F., Kazic, T., Kanehisa, M., and Berndt, D.J. (1999) Predicting microbial biodegradation pathways. *ASM News* **65**, 87-93.

Associate Professor : Akihiro Hazama
Research Associate : Kensuke Nakahira
Research Associate : Takuya Takahashi

This division has just started in August, 1999. The cells which constitute our bodies accomadate their environment by sensing the conditions surrounding them. The cells use the special membrane proteins, such as receptors, channels, and transporters, for sensing not only many substances but also heat, osmolarity, and mechanical stimuli. The aim of this division is to understand the sensing mechanims of such membrane proteins sense and how the cells accomodate their environment after sensing the outside conditions.

Publications:

Yasui M, Hazama A, Kwon TH, Nielsen S, Guggino WB, Agre P. Rapid gating and anion permeability of an intracellular aquaporin. Nature. 402:184-187, 1999

Hazama A, Shimizu T, Ando-Akatsuka Y, Hayashi S, Tanaka S, Maeno E, Okada Y. Swelling-induced, CFTR-independent ATP release from a human epithelial cell line: lack of correlation with volume-sensitive Cl- channels. J Gen Physiol. 114:525-533.1999

Baba H, Akita H, Ishibashi T, Inoue Y, Nakahira K, Ikenaka K. Completion of myelin compaction, but not the attachment of oligodendroglial processes triggers K+ channel clustering. J Neurosci Res. 58:752-764. 1999

Shibata R, Wakazono Y, Nakahira K, Trimmer JS, Ikenaka K. Expression of Kv3.1 and Kv4.2 genes in developing cerebellar granule cells. Dev Neurosci. 21:87-93. 1999

Associate Professor: *Hirokazu Tsukaya, Ph.D.*
Assistant Professor: *Gyung-Tae Kim, Ph.D.*

Diversity of plant form is mostly attributable to variation of leaf and morphology of 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 genetics and molecular genetics to it, using model plants such as *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.

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. 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.

Publication List:

Tsukaya, H., Shoda, K., Kim, G.-T. and Uchimiya, H. (2000) Heteroblasty in Arabidopsis thaliana (L.) Heynh. *Planta* **210** : 536-542.

Figure: Single gene, the *ROT3,* controls leaf form (modified from Kim et al., 1999)

RESEARCH SUPPORT

CENTER FOR TRANSGENIC ANIMALS AND PLANTS

Head: Masaharu Noda

Associate Professor: *Eiji Watanabe*
Supporting Staff: *Mie Yasuda (Sept. 1, 1999~)*

I. Research supporting activity

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

The expected activities of the Center at present 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 subfamily 2 channels in collaboration with Division of Molecular Neurobiology. Subfamily 2 channels are a group of voltage-gated sodium channels (NaChs) that generate action potentials in electrically excitable cells such as neurons and muscle cells. Comparing with the other NaChs, this channel species 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 subfamily 2 channels must have specific functional properties. To clarify the functional role *in vivo*, the subfamily 2 channel-deficient mice were generated by gene targeting. We are now examining the physiological phenotypes.

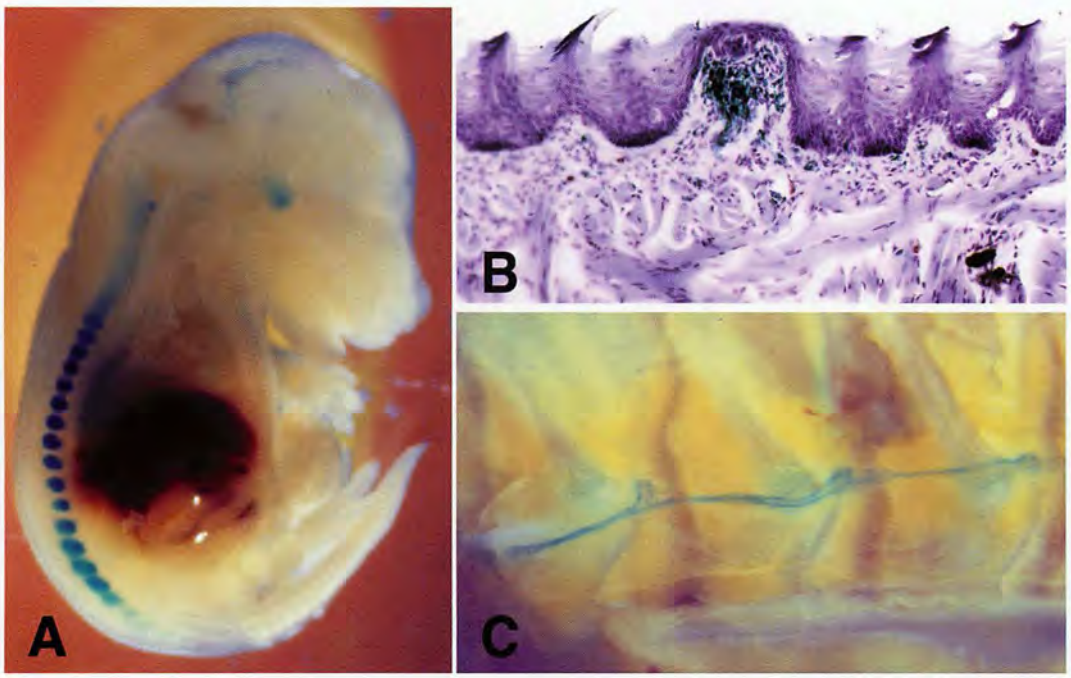

Fig. 1 *LacZ* expression in mNaG+/- mice (A-C)
The expression pattern of mouse subfamily 2 channel gene (*mNaG*) was revealed by *LacZ* gene expression in mNaG+/- mice. The blue signals represent the site expressing *LacZ* gene. (A) E15 whole embryo, (B) tissue section of adult tongue, and (C) adult sympathetic nerve trunk in the thoracic region. In (B), the tissue section was counterstained with cresyl violet.

TECHNOLOGY DEPARTMENT

Head:Hiroyuki Hattori

Common Facility Group

Chief : Kazuhiko Furukawa

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Shoichi Higashi(Unit Chief)
Tomoki Miwa(Subunit Chief)
Chieko Nanba(Subunit Chief)
Hiroyo Nishide
Makiko Itoh(Technical Assistant)
Keiko Suzuki(Technical Assistant)
Misayo Masuda(Technical Assistant)
Hideyuki Gotoh(Thecnical Assistant)
Yasuyo Kamiya(Thecnical Assistant)

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

Center for Analytical Instruments
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Research Support Group

Chief : Hiroko Kobayashi

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

Developmental Biology Group
Chiyo Takagi
Sanae Oka
Naomi Sumikawa

Regulation Biology Group
Hideko Iinuma
Akiko Oda
Shigemi Takami

Gene Expression and Regulation Group
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Koji Hayashi
Yasushi Takeuchi
Kaoru Sawada
Hideko Utsumi
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 insititutes throughout Japan. At this meeting, the participants present their own activities and discuss technical problems.The Proceedings are published soon after the meeting.

RESEARCH SUPPORT FACILITY

Head of Facility: *Yoshinori Ohsumi*
Associate Professor: *Masakatsu Watanabe*
Research Associates: *Yoshio Hamada (Tissue and Cell Culture)*
Ikuo Uchiyama (Computer) (March 1, 1999-)
Technical Staffs: *Sho-ichi Higashi (Large Spectrograph)*
Mamoru Kubota (Large Spectrograph) (-March 31, 1999)
Tomoki Miwa (Computer)
Chieko Nanba (Plant Culture, Farm, Plant Cell)
Hiroyo Nishide (Computer) (June 1, 1999-)
Toshiki Ohkawa (Computer) (-May 31, 1999)
Kaoru Sawada (Tissue and Cell Culture) (-June 30, 1999)
Hideyuki Goto (Large Spectrograph) (May 17, 1999-)
Makiko Ito (Large Spectrograph)
Yasuyo Kamiya (Tissue and Cell Culture) (May 17, 1999-)
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*

Notch is an integral cell surface memberane protein that is known to play a key role in developmental cell-cell interactions in *Drosophila*, particulaly in lateral specification of neural versus epidermal cell fates, a process described thus far only in invertebrates. It is thought to act by a direct signaling pathway rather than through one of the classical signal transduction cascades. The mammalian genome is known to contain four Notch homologues but their developmental significance is not clear. To investigate their role in mammalian development, we have sequenced the murine Notch2 cDNA, determined the primary sequence of its protein, and have produced Notch2 mutant mice by gene trageting procedures. The mutant mice die prior to embryonic day 11.5. Thus, Notch2 palys an essential role in postimplantation development in mice. Chimeric analysis revealed that embryonic lethality of the mutant mice is due to defect in placenta development and Notch2 function is involved in formation of the roof plate of diencephalon and mesencephalon in development later than the lethal stage.

3. *Computer Laboratory*

Computer laboratory maintains several computers to provide computation resources and means of electronic communication in this Institute. This year, new computer systems were introduced to meet requirement of large scale biological data analysis, and now the system mainly 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 (Macintoshes and Windows PCs) and color/monochrome printers are also equipped. Various biological databases and data retrieval/analysis programs are available on this system.

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 for the Institute members 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 cleanbenches, 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 (Horiguchi et al., 1999; Matsunaga et al., 1999; Choi et al., 1999). Photo-receptive and signal transduction mechanisms of algal gene expression were also studied by action spectroscopy (Leblanc et al., 1999).

(2) Computational Biology: Explosively growing biological data produced from various genome projects should contain many clues to understanding complex and diverse biological systems. Comparative genomics is a useful approach to find such clues. We are constructing a database system for comparative analysis of many of microbial genomes ever sequenced and developing new computational techniques for large-scale genome sequence comparison. Currently, our research aim is focused on the identification of orthologous genes between multiple genomes, which is a crucial step for comparative genomics. Since considerable number of genes consist of multiple domains, we are now developing a hierarchical clustering algorithm that can automatically split fusion genes into orthologous domains using all-against-all homology search results. In parallel, we also 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, 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*

Choi, J-S., Chung-Y-H, Moon, Y-J., Kim, C., Watanabe, M., Song, P-s., Joe, C-O, Bogorad, L., and Park, Y. M. (1999) Photomovement of the gliding cyanobacterium *Synechocystis* sp. PCC 6803. *Photochem. Photobiol.* **70**, 95-102.

Hamada,Y., Kadokawa,Y., Okabe, M., Ikawa, M., Coleman, J.R. & Tsujimoto, Y(1999). Mutation in ankyrin repeats of the mouse Notch2 gene induces early embryonic lethality. *Development* **126**, 3415-3424.

Horiguchi, T., Kawai, H., Kubota, M., Takahasi, T. and Watanabe, M.(1999). Phototactic responses of four marine dinoflagellates with different types of eyespot and chloroplast. *Phycol. Res.* **47**, 101-107.

Kobayashi, I., Nobusato, A., Kobayashi-Takahashi, N., Uchiyama, I. (1999) Shaping the genome – restriction-modification systems as mobile genetic elements. *Curr. Opin. Genet. Dev.* **9**, 649-656.

Leblanc, C., Falciatore, A., Watanabe, M. and Bowler, C. (1999). Semi-quantitative RT-PCR analysis of photoregulated gene expression in marine diatoms. *Plant Mol. Biol* **40** (6), 1031-1044.

Matsunaga, S., Takahashi. T., Watanabe, M., Sugai, M. and Hori, T. (1999). Control by ammonium ion of change from step-up to step-down photophobically responding cells in the flagellate alga Euglena gracilis. *Plant Cell Physiol.* **40**(2) , 213-221.

Shimizu, K., Chiba, S., Kumano, K., Hosoya,N., Takahashi,

T., Kanda,Y., Hamada,Y., Yazaki, Y. & Hirai, H.(1999). Mouse Jagged1 physically interacts with Notch2 and other Notch receptors. *J. Biol Chem.* **274**, 32961-32969.

Tanaka,M., Kadokawa, Y., Hamada,Y. & Marunouchi, T.(1999). Notch2 expression negatively correlates with glial differentiation in the postnatal mouse brain. *J. Neurobiol.* **41**, 524-539.

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

Andrady, A. L. and Torikai, A. (1999). Photoyellowing of mechanical pulps III. Intensity effects and dose-response relationships. *Polym. Degradn. Stab.*, **66**, 317-322.

Andrady, A. L., Hamid, S. H., Hu, X. and Torikai, A. (1999). Effects of icreased solar ultraviolet radiation on materials. *J. Photochem. Photobial. B:Biol.* **46**, 96-103.

Choi, J-S., Chung-Y-H, Moon, Y-J., Kim, C., Watanabe, M., Song, P-s., Joe, C-O, Bogorad, L., and Park, Y. M. (1999) Photomovement of the gliding cyanobacterium *Synechocystis* sp. PCC 6803. *Photochem. Photobiol.* **70**, 95-102.

Fujiwara, A., Tazawa, E., Kamata, Y. and Yasumasu, I. (1999). Photo-actvation of respiration in degenerated sperm of echiuroid, oyster and sea urchin. *Zool. Sci.* **16**, 237-246.

Hashimoto, T. and Shichijo, C. (1999). Amplification of phytochrome-induced photomorphogenesis by red light pre-irradiation, and a cryptic red-light signal production. *Trend in Photochem. and photobiol.* vol. **6**, 15-27.

Horiguchi, T., Kawai, H., Kubota, M., Takahashi, T. and Watanabe, M.(1999). Phototactic responses of four marine dinoflagellates with different types of eyespot and chloroplast. *Phycol. Res.* **47**, 101-107.

Ishigaki, Y., Takayama, A.,Yamashita, S. and Nikaido, O. (1999). Development and characterization of a DNA solar dosimeter. *J. Photochem. Photobiol. B: Biol.* **50**, 184-188.

Leblanc, C., Falciatore, A., Watanabe, M. and Bowler, C. (1999). Semi-quantitative RT-PCR analysis of photoregulated gene expression in marine diatoms. *Plant Mol. Biol* **40** (6), 1031-1044.

Matsunaga, S., Takahashi. T., Watanabe, M., Sugai, M. and Hori, T. (1999). Control by ammonium ion of change from step-up to step-down photophobically responding cells in the flagellate alga Euglena gracilis. *Plant Cell Physiol.* **40**(2) , 213-221.

Shichijo, C., Onda, S., Kawano, R., Nishimura, Y. and Hashimoto, T. (1999). Phytochrome elicits the cryptic red-light signal which results in amplification of anthocyanin biosynthesis in sorghum. *Planta* . **208**, 80-87.

Torikai, A. and Hasegawa, H. (1999). Accelerated photodegradation of poly(vinyl chloride). *Polym. Degradn. Stab.* **63**, 441-445.

Torikai, A. and Shibata, H. (1999). Effect of Ultraviolet radiation on photodegradation of collagen. *J. Appl. Polym. Sci.* **73**, 1259-1265.

RADIOISOTOPE FACILITY (managed by NIBB)

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

I. Research supporting activity

Technical and Supporting Staffs of this facility 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 by the registered users of NIBB (National Institute for Basic Biology) and NIPS (National Institute for Physiological Science) for research is also one of our business.

This facility consists of four controlled areas: Center, NIBB-sub, LGER (Laboratory of Gene Expression and Regulation)-sub, and NIPS-sub. Users going in and out the controlled areas counted by the monitoring system were 7,912 in 1999. This count was comparable to that (8,483) in 1998. The items in each controlled area are presented in Figure 1.

II. Academic activity

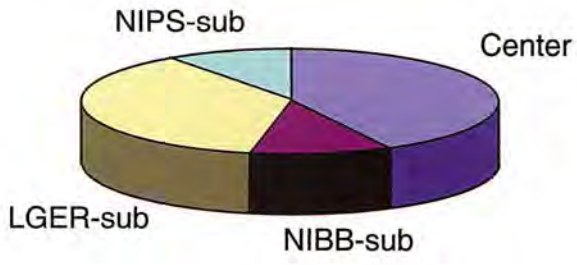
Academic activity by Associate Professor is focused on the analysis of the structure and function of a dynein motor protein. Dyneins are a group of microtubule-activated ATPases that serve to convert chemical energy into mechanical energy and divided into axonemal and cytoplasmic dyneins. Figure 2 shows 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 (Fragment 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 present 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	3,336
NIBB-sub	871
LGER-sub	2,885
NIPS-sub	820
total	7,912

Figure 1. Percentage of users going in and out the controlled areas during April to December, 1999.

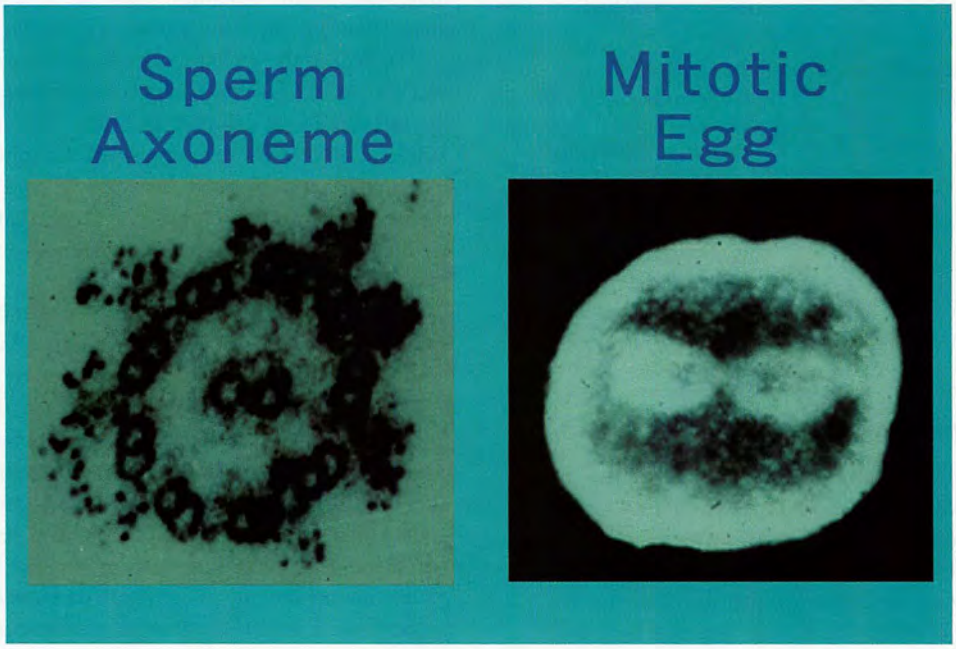


Figure 2. Localization of two dynein isotypes on outer arm of sperm axonemes and mitotic apparatus of cleaving egg.

Publication List:

S. Watabe, Y. Makino, K. Ogawa, T. Hiroi, Y. Yamamoto, and S. -Y. Takahashi. Mitochondrial thioredoxin reductase In bovine adrenal cortex: Its purification, properties, nucleotide/amino acid sequences, and Identification of selenocysteine. *Eur. J. Biochem.* **204**, 74-84 (1999).

K. Inaba, O. Kagami, and K. Ogawa. Tctex2-related outer arm dynein light chain Is phosphorylated at activation of sperm motility. *Biochem. Biophys. Res. Commun.* **256**, 177-183 (1999).

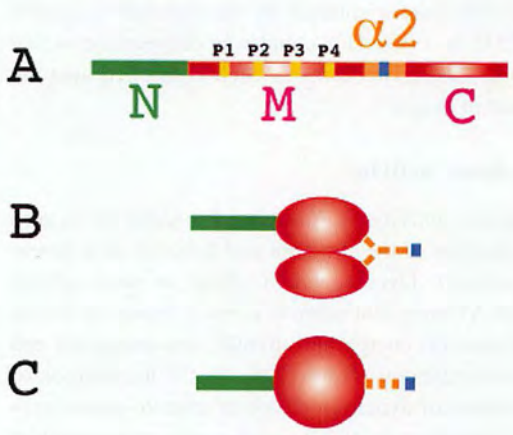


Figure 3. Structure of dynein heavy chain. A; Analysis of amino acid sequence of heavy chain reveals that it consists of three major domains referred to as N, M, and C from the N-terminus. B; M and C domains make larger domain (motor domain) by intramolecular association. C, According to Koonce et al. (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.

THE CENTER FOR ANALYTICAL INSTRUMENTS

(managed by NIBB)

Head of Facility: *Tetsuo Yamamori*
Technical Staffs: *Mamoru Kubota*
Sonoko Ohsawa
Tomoko Mori
Yumiko Makino
Takeshi Mizutani (~Feb. 15, 2000)
Technical Assistant: *Hatsumi Moribe*

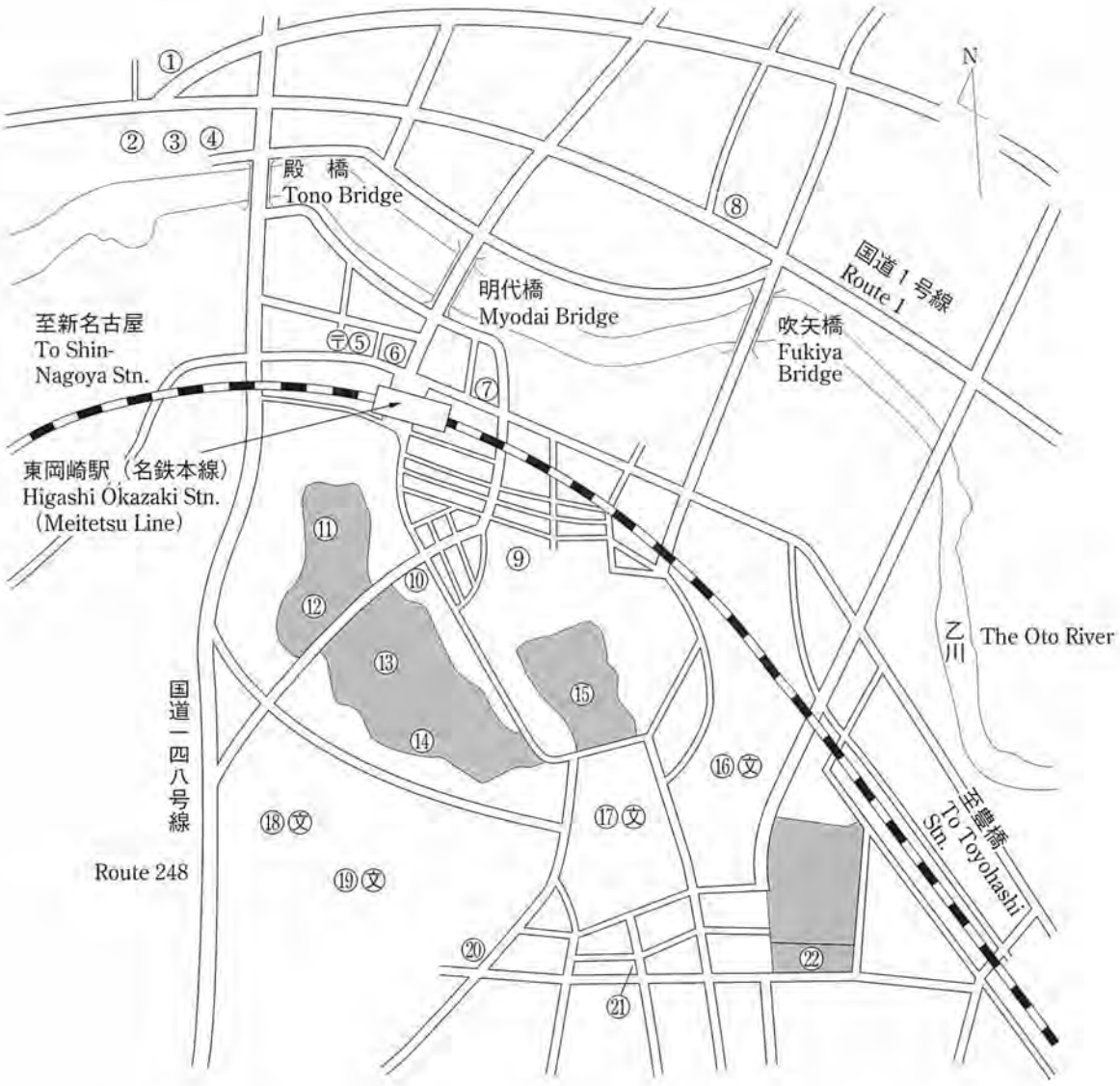
The Center serves for amino acid sequence analysis, and chemical syntheses of peptides and nucleotids to support researchers in NIBB and NIPS. Newly installed instruments in 1999 are Biomek 2000 Laboratory Automation System and Automatic Nucleic Acid Isolation System. Instruments of the Center can be 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 II)
Electrophoresis Imaging Systems (PDI Discovery Series, BIOIMAGE)
Microscopes (Carl Zeiss Axiophot, Axiovert)
Microscope Photometer (Carl Zeiss MPM 03-FL)



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 山手スーパーマーケット
Yamate Supermarket

㉑ 薬局
Pharmacy

㉒ 山手ロッジ
Yamate Lodge

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