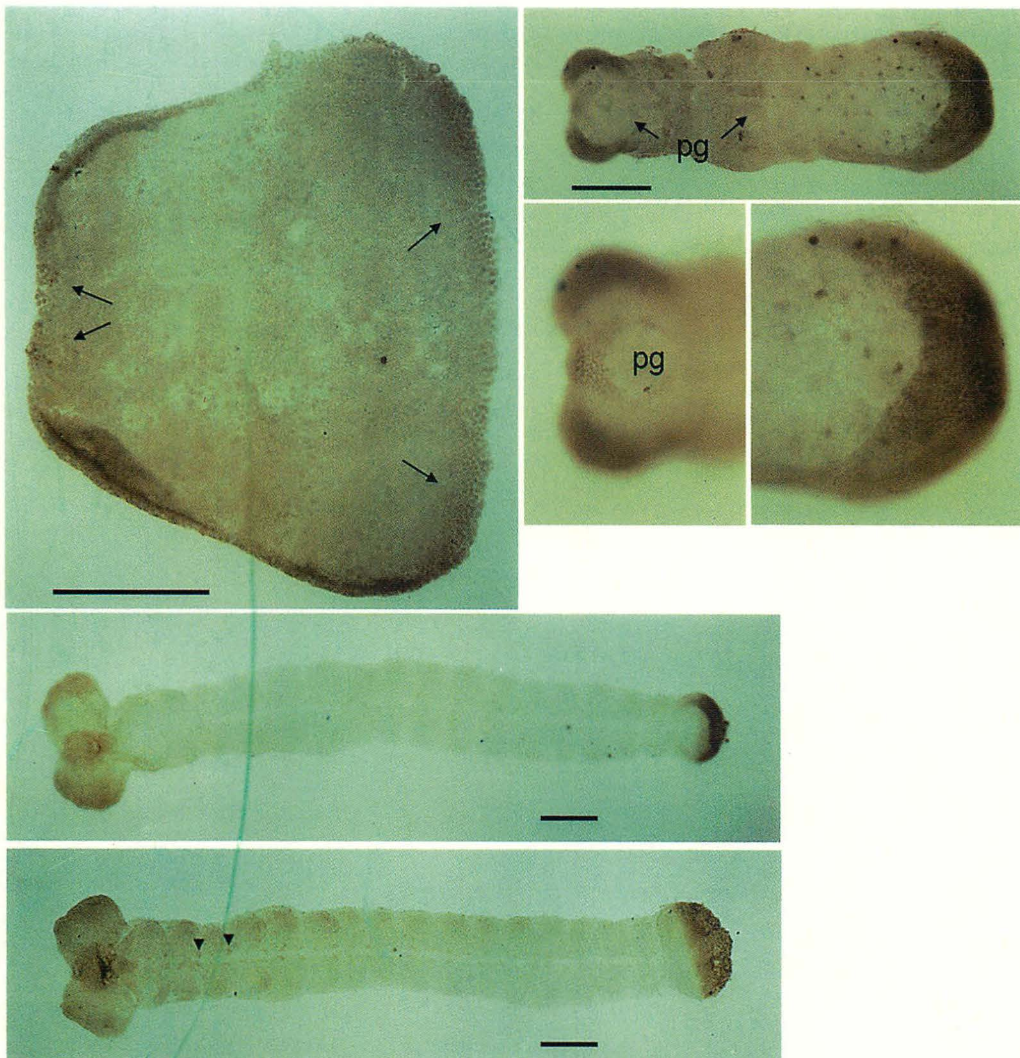
NATIONAL INSTITUTE FOR BASIC BIOLOGY

岡崎国立共同研究機構

基 礎 生 物 学 研 究 所



ANNUAL REPORT

1995

The cover photographs indicate distribution of *Bombyx* Fkh/SGF-1 protein in embryos. For details, see p. 26.

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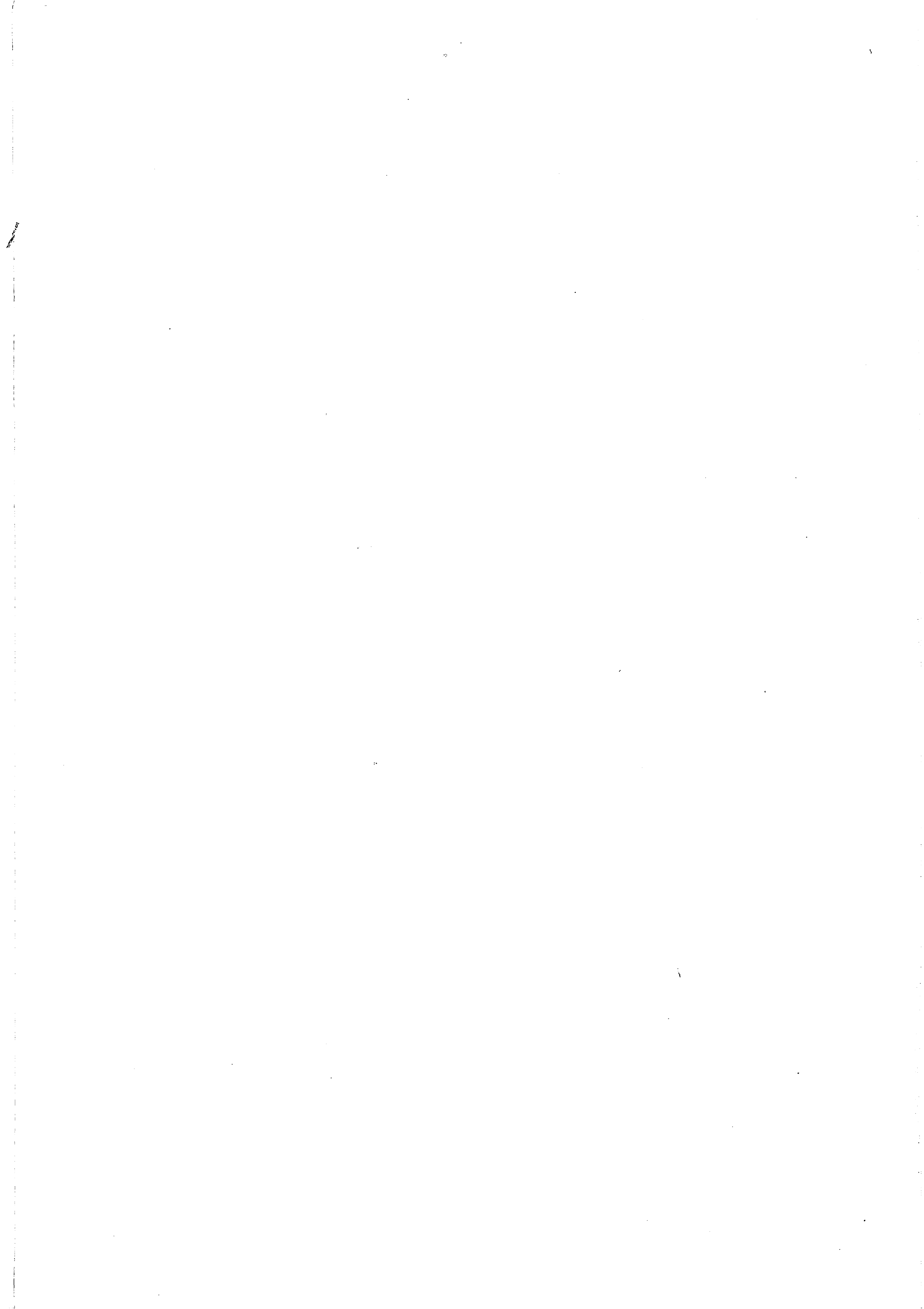

INTRODUCTION

We present here the Annual Report 1995 describing the research activities of the National Institute for Basic Biology (NIBB) during the past year.

The NIBB is a government supported basic research institute in the field of biology. It aims to stimulate and promote studies of biology, by conducting first-rate research as a center of excellence, and by cooperating with other universities and research organizations. The Institute concentrates on the studies of cellular functions, reproduction and development, homeostatic control, and gene expression and regulation in eukaryotic organisms, to elucidate the fundamental mechanisms underlying various biological phenomena at the molecular level.

During the past year, the turnover of personnel has been very high. At the end of March, the former Director-General, Dr. Ikuo Takeuchi retired from the NIBB due to his appointment as the President of the Okazaki National Research Institutes. His position was taken over by Dr. Hideo Mohri from the beginning of April. Professor Yoshihiko Fujita of the Division of Bioenergetics also retired at the end of March and became Emeritus Professor. His successor as well as the new professor of the Division of Gene Expression and Regulation I will be appointed in the coming April. Professor Masaki Iwabuchi (Kyoto University) of the Adjunct Division of Developmental Biology completed his term of office and was followed by Professor Kenzo Nakamura of Nagoya University. Two associate professors, Drs. K. Okada and H. Hayashi, and a research associate, Dr. T. Kondo, moved as full professors to Kyoto University, Ehime University, and Nagoya University, respectively. Drs. M. Yoshikuni, H. Iida and K. Ogawa were appointed as Associate Professor of the Division of Reproductive Biology, the Division of Cellular Regulation and the Radioisotope Facility, respectively. Furthermore, we newly appointed 6 research associates and 8 institute research fellows, while 10 research associates other than Dr. Kondo and two institute research fellows moved to other institutions.

The NIBB plays important roles as a national and international center for the biological research and is responsible for conducting research projects in cooperation with research groups of various universities and institutes. As a part of such cooperative activities, the NIBB carried out Special Programs which are currently directed to "Adaptation and Resistance to Environment" and "Trans-differentiation of Tissue Cells". Based on these programs, the NIBB held the 34th and 35th Conferences entitled as "Responses of the Photosynthetic Apparatus to Environmental Light

H. Mohri

Conditions" (organized by Professor Kimiyuki Satoh) and "Mechanisms of Cell Commitment in Differentiation" (organized by Professor Goro Eguchi), respectively.

In addition, the Institute sponsors symposia and study meetings on current topics at the interdisciplinary level by inviting leading scientists in various related fields, both nationally and internationally. In 1995, the 11th International Symposium in conjunction with the Award of the International Prize for Biology "Dynamics of the Cell" was organized by the NIBB, inviting the laureate of the 1995 Prize, Professor I. R. Gibbons, and many distinguished investigators in the field of cell biology. The NIBB also provided a training course in bioscience for young investigators and participated in the International Science School for high school students of Asian-Pacific countries.

Fortunate enough, the construction of a new building for the Laboratory of Gene Expression and Regulation (about 2500m^2) has been decided by the government, and institution of the new division, Speciation Mechanisms II, is expected in the coming year. At the end of the year, the NIBB had a symposium about the future of its own by inviting authorities in various fields of biology. Discussion at this symposium was quite stimulative and would be utilized for making the future plan of the Institute.

Finally, I would like to congratulate that Emeritus

Professor T. S. Okada, the past President of Okazaki National Research Institutes and the past Director-Genaral of NIBB, was honored as Person of Cultural Merit and Professor G. Eguchi was awarded a Purple Ribbon Medal and Palmes Academique, Chevarier, from the Government of France.

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 16 divisions.

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

Research Support Facility

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

Campus

The Okazaki National Research Institutes covers an area of 150,000 m^2 with four principal buildings. The NIBB's main research building has a floor space of 10,930 m^2. Two-thirds of the space was completed in 1982 and the remaining third in June, 1983. The buildings which house the research support facility were also completed in June, 1983.

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

GRADUATE PROGRAMS

The NIBB carries out two graduate programs.

1. Graduate University for Advanced Studies

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

The Department consists of the following Divisions and Fields:

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

2. Graduate Student Training Program

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

OFFICE OF DIRECTOR

Director-General: Hideo Mohri
Associate Professor: Shigeru Itoh
Research Associates: Mamoru Mimuro
Katsunori Aizawa

The former members of the Division of Bioenergetics have been included in the Office of Director.

Primary processes of light energy conversion

I. Energy transfer processes

Mamoru Mimuro

Primary processes in photosynthesis were studied in terms of structure-function relationship.

A primary electron donor in the photosystem (PS) II reaction center (RC), P680, was shown to be a dimer based on the magnetic circular dichroism (MCD) (Nozawa et al., 1995). An MCD signal is inversely proportional to the association state of pigments, even though its theoretical basis is not yet known. This relationship was confirmed on RC and light harvesting II complexes isolated from purple bacteria and artificial aggregates of bacteriochlorophyll (BChl) *c* (Kobayashi et al., in press).

Two types of pheophytin (Pheo) molecules in the PS II RC was spectrally discriminated. The photochemically active Pheo molecule has a lower energy level than that of the photochemically inactive Pheo molecule, which is analogous to the bacterial RC (Mimuro et al., 1995). This confirms an evolutional linkage between bacterial RC and PS II RC in oxygenic photosynthesis.

An antenna system of green photosynthetic bacteria, chlorosomes was studied in term of relationship among morphology, molecular structure and spectral properties (Wang et al., 1995). The hexanol-induced changes on chlorosomes were explained by a direct interaction between BChl *c* and hexanol, which consequently induced a uni-directional elongation of rod elements and of chlorosomes. In addition, we found a new component in chlorosomes which facilitates a rapid energy migration from chlorosomes to the antenna in membranes (Mimuro et al., in press).

A new approach for understanding of photosynthesis was started by a collaboration entitled "Experimental analyses of the origin of chloroplasts and phylogeny in algae".

II. Energy conversion processes

Shigeru Itoh

Photosynthesis in plants and bacteria convert solar energy to electrochemical one in the reaction center (RC) complexes that are the molecular machines made of proteins and prosthetic groups. RC's seem to be established more than 3 billion years ago in the early Earth. We study the function of RC's, electron transfer systems, and their evolution by three approaches: 1) Exchange of the prosthetic groups by artificial compounds, 2) Genetic modification of the proteins, and 3) Exploration of new electron transfer systems. We replaced the phylloquinone (vitamin K_1) in the RC complex of plant photosystem I by artificial quinones. The method enabled us to change the driving force of the reaction and clarified the optimization mechanism of the ultra-rapid electron transfer with a reaction time of 10^{-11} second by laser photolysis. Similar studies were also done in the newly purified RC of green bacteria that is a prototype of the plant RC. Mechanism of quinone reaction was also clarified in the genetically-modified oxidase of bacterial respiratory chain by cryogenic EPR. Surprisingly, we discovered a new organism that does not use Mg-chlorophyll but uses Zn-porphyrin in their photosynthesis. This opened a new stage in the study of photosynthesis that has been believed to be obligatory performed by Mg-chlorophyll's.

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Demidov, A. A. and Mimuro, M. (1995) Deconvolution of C-Phycocyanin ß-84 and ß-155 chromophore absorption and fluorescence spectra of cyanobacterium *Mastigocladus laminosus*. *Biophys. J.* **68**, 1500-1506.

Ikegami, I., Itoh, S.and Iwaki, M. (1995) Photoactive photosystem I particles with a molar ratio of Chlorophyll a to P700 of 9. *Plant Cell Physiol.* **36**, 857-864.

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Oh-oka, H., Kakutani, S., Kamei, S., Matsubara, H., Iwaki, M. and Itoh, S. (1995) Highly purified photosynthetic reaction center (PscA/Cytochrome $c_{551})_2$ complex of the green sulfur bacterium *Chlorobium limicola*. *Biochemistry* **34**, 13091-

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Oh-oka, H., Kamei, S., Matsubara, H. and Iwaki, M. Itoh, S. (1995) Two cytochrome c heme function as the electron donor to P840 in the reaction center complex of a green sulfur bacterium, *Chlorobium tepidum*. *FEBS Lett.* **365**, 30-34.

Sato-Watanabe, M., Itoh, S., Mogi, T., Matsuura, K., Miyoshi, H. and Anraku, Y. (1995) Stabilization of a semiquinone radical at the high affinity quinone binding site of the *Escherichia coli bo*-type ubiquinone oxidase. *FEBS Lett.* **374**, 265-269.

Sonoike, K., Terashima, I., Iwaki, M. and Itoh, S. (1995) Site of destruction of photosystem I iron-sulfur centers in leaves of *Cucumis sativus* L. by weak illumination at chilling temperatures is A_1, the phylloquinone acceptor. *FEBS Lett.* **362**, 235-238.

Wang, Z.-Y., Marx, G., Umetsu, M., Kobayashi, M., Mimuro, M. and Nozawa, T. (1995) Morphology and spectroscopy of chlorosomes from *Chlorobium tepidum* by alcohol treatments. *Biochim. Biophys. Acta* **1232**, 187-196.

DEPARTMENT OF CELL BIOLOGY

Chairman: Goro Eguchi

This department is composed of two regular divisions and three adjunct divisions, and conducts researches on the fundamentals of cell structures and functions in plants and animals at molecular level utilizing modern technologies including genetic engineering.

DIVISION OF CELL MECHANISMS

Professor: Mikio Nishimura
Research Associates: Makoto Hayashi
Ikuko Hara-Nishimura
Tomoo Shimada
NIBB Postdoctral Fellow: Akira Kato
Graduate Students: Tetsu Kinoshita
Nagako Hiraiwa
Shouji Mano
Masahiro Aoki [1)]
Daigo Takemoto [2)]
Technical Staffs: Maki Kondo
Katsushi Yamaguchi
Yasuko Koumoto
Visiting Scientists: Kyoko Hatano [3)]

[1)] *from Shinshu University*
[2)] *from Nagoya University*
[3)] *from Kyoto Univerisity*

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, and other organelles may degenerate. Because the dynamic transformation of organellar function (differentiation of organelles) is responsible for the flexibility of differentiation events in higher plant cells, the elucidation of regulatory mechanisms underlying organelle transformation are currently being studied in this division.

I. Development of microbody membrane proteins during the microbody transition

Dramatic metabolic changes which underlie the shift from heterotrophic to autotrophic growth occur during the in greening of seeds 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. To clarify the molecular mechanisms underlying the microbody transition, the change with development in microbody membrane proteins during transformation of glyoxysomal to leaf peroxisomes was characterized. Two proteins in glyoxysome membranes, with molecular masses of 31 and 28 kDa, were purified and characterized. The 31-kDa membrane protein was found to be a novel isoenzyme of ascorbate peroxidase. Intact glyoxysomes and leaf peroxisomes had no latent peroxidase activity, an indication that the active site of the ascorbate peroxidase was exposed to the cytosol and that the peroxidase would scavenge hydrogen peroxide leaked from microbodies (Fig. 1).

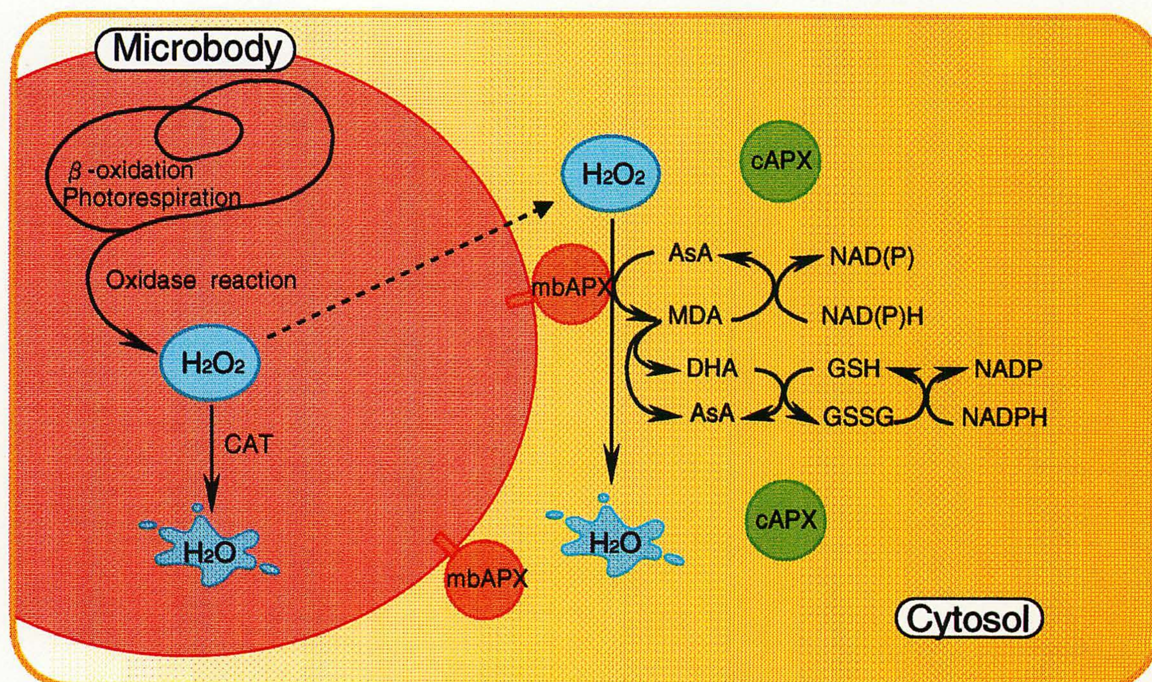

Fig. 1. Possible function of the ascorbate peroxidase localized on microbody membranes. mbAPX, microbody ascorbate peroxidase; cAPX, cytosolic ascorbate peroxidase; CAT, catalase; AsA, ascorbate; DHA, dehydroascorbate; MDA, monodehydroascorbate.

Analysis of these membrane proteins during development revealed that the amounts of these membrane proteins decreased during the microbody transition and that the larger one was retained in leaf peroxisomes, whereas the smaller one could not be found in leaf peroxisomes after completion of the microbody transition. These results clearly showed that membrane proteins in glyoxysomes change dramatically during the microbody transition, as do the enzymes in the matrix.

II. Characterization of citrate synthase responsible for the glyoxylate cycle

Citrate synthase catalyzes the conversion of oxaloacetate to citrate. In plant cells, there are two isozymes of citrate synthase, one is an enzyme involved in the glyoxylate cycle in glyoxysomes (gCS) and the other is an enzyme involved in the TCA cycle in mitochondria. To study the structural characteristics and development of gCS during the microbody transition at the molecular level, we cloned cDNA for pumpkin gCS. The deduced amino acid sequence of gCS did not have a typical peroxisomal targeting signal at its carboxyl terminus. A study of the *in vitro* expression of the cDNA and an analysis of the amino-terminal sequence of the citrate synthase indicated that gCS is synthesized as a larger precursor that has a cleavable amino-terminal presequence of 43 amino acids. The predicted amino-terminal sequence of pumpkin gCS was highly homologous to those of other microbody enzymes, such as 3-ketoacyl-CoA thiolase of rat and malate dehydrogenase of watermelon that are also synthesized as precursors of higher molecular mass. An immunoblot analysis showed that the level of gCS protein increased markedly during germination and decreased rapidly during the light-induced transition of microbodies from glyoxysomes to leaf peroxisomes. By contrast, the level of mRNA for gCS reached a maximum earlier than that of the protein and declined even in darkness. The level of the mRNA was low during the microbody transition. These results indicate that the accumulation of the gCS protein does not correspond to that of the mRNA and that degradation of gCS is induced during the microbody transition, suggesting that post-transcriptional regulation plays an important role in the microbody transition.

III. Membrane proteins of protein bodies and the developmental change during transformation between protein bodies and vacuoles

During seed maturation, protein-storage vacuoles are converted to protein bodies that are found in dry seeds. In contrast, during the postgermination growth of seeds, protein bodies fuse with one another and are converted to a central vacuole. To investigate this transition, we prepared protein-body membranes from dry seeds of pumpkin (*Cucurbita* sp.) and characterized their protein components. Five major proteins (designated MP23, MP27, MP28, MP32 and MP73) were detected in the protein-body membranes. We have isolated cDNAs for MP23, MP27, MP28 and MP32 and characterized them.

Among the five membrane proteins, MP27 and MP32 disappeared most rapidly during seedling growth. Both MP27 and MP32 were encoded by a single cDNA. The deduced precursor polypeptide was composed of a hydrophobic signal sequence, MP27 and MP32, in that order. A putative site of cleavage between MP27 and MP32 was located on the COOH-terminal side of asparagine 278, an indication that the post-translational cleavage may occur by the action of a vacuolar processing enzyme (VPE) that converts proprotein precursors of seed proteins into the mature forms. Immunoelectron microscopic analysis showed that MP27 and MP32 was associated with the protein-body membrane of dry pumpkin seeds. The degradation of MP27 and MP32 starts just after seed germination and proceeds in parallel with the transformation of the protein bodies into vacuoles.

Molecular characterization revealed that both MP28 and MP23 belong to the seed TIP (tonoplast intrinsic protein) subfamily. TIP is an integral membrane protein that was originally found in plant seeds and belongs to the MIP (major intrinsic protein) family, the members of which are widely distributed in bacteria, animals and plants. The TIP of plant seeds is abundant and is conserved among both monocots and dicots. The predicted 29-kDa precursor to MP23 includes six putative membrane-spanning domains, and the first loop between the first and second transmembrane domains is larger than that of MP28. The N-terminal sequence of the mature MP23 starts from residue 66 in the first loop, indicating that an N-terminal 7-kDa fragment that contains one transmembrane domain is post-translationally removed. During maturation of pumpkin seeds, mRNAs for MP28 and MP23 became detectable in cotyledons at the early stage, and their levels increased slightly until a rapid decrease occurred at the late stage. This is consistent with the accumulation of the 29-kDa precursor and MP28 in the cotyledons at the early stage. By contrast, MP23 appeared at the late stage simultaneously with the disappearance of the 29-kDa precursor. Thus, it seems possible that the conversion of the 29-kDa precursor to the mature MP23 might occur in the vacuoles after the middle stage of seed maturation. Both proteins were localized immunocytochemically on the membranes of the vacuoles at the middle stage and the protein bodies at the late stage. These results suggest that both MP28 and the precursor to MP23 accumulate on vacuolar membranes before the deposition of storage proteins, and then the precursor is converted to the mature MP23 at the late stage. These two TIPs might have a specific function during the maturation of pumpkin

seeds.

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

Processing enzymes responsible for the maturation of seed proteins belong to a novel group of cysteine proteinases with molecular masses of 37 to 39 kDa. However, the processing enzyme activity can be found not only in seeds but also in vegetative tissues such as hypocotyls, roots and mature leaves. Thus, we designated the enzyme as a vacuolar processing enzyme (VPE). The molecular characterization of all the members of the VPE family in *Arabidopsis* is required if we are to elucidate the mechanisms of regulation of genes for VPE homologues and the physiological functions of these proteins in protein-storage vacuoles and vegetative vacuoles.

Southern blot analysis showed that a family of genes for VPEs in *Arabidopsis thaliana* was composed of three genes, for α-VPE, β-VPE and γ-VPE. We isolated the three genes of VPEs from a genomic library of *Arabidopsis.* The positions of eight introns were fully conserved among the three genes, with the exception that the α-VPE gene was missing the fifth intron found in the β-VPE and γ-VPE genes.

Northern blot analysis revealed that α-VPE was expressed in rosette leaves, cauline leaves and stems of *Arabidopsis*, while β-VPE was predominantly expressed in the flowers and buds. The γ-VPE gene was expressed predominantly in the stems, with a lower level of expression in rosette and cauline leaves. However, the expression was not detected in roots, flowers plus buds, or green siliques. This result strongly suggests that the α-VPE and γ-VPE genes encode isozymes of VPE that are specific to vegetative organs. To demonstrate temporal and spacial expression of the promoters of the vegetative VPE gene, we transformed tobacco plants with a reporter gene containing the promoter of the γ-VPE gene and the coding region of β-glucuronidase (GUS). The GUS activity was predominantly expressed in the senescing tissues (Fig. 2).

Members of the VPE family can be separated into two subfamilies, one that is specific to seeds and another that is specific to vegetative organs, such as leaves and stems. The members of the seed subfamily might function in the protein-storage vacuoles of seeds, while those of the vegetative subfamily might function in the lytic vacuoles of non-storage organs. The VPE cleaved a peptide bond on the carbonyl side of an exposed asparagine residue on the molecular surface of proprotein precursors to generate the mature forms of seed proteins. A similar type of post-translational processing has been reported in the case of maturation of vacuolar proteins in vegetative tissues, such as the proteinase inhibitors of tomato leaves and tobacco stigmas, and the chitinase of tobacco leaves and cultured cells. These observations suggested that VPEs are widely distributed in plant tissues and play crucial roles in the maturation of a variety of proteins in plant vacuoles. This suggestion is supported by the report that VPE activity can be detected not only in seeds but also in vegetative organs and, moreover, that two of the VPE genes of *Arabidopsis* are specifically expressed in vegetative organs.

Publication List:

De Bellis, L., Hayashi, M., Nishimura, M. and Alpi, A. (1995) Subcellular distribution of aconitase isoforms in pumpkin cotyledons. *Planta* **195**, 464-

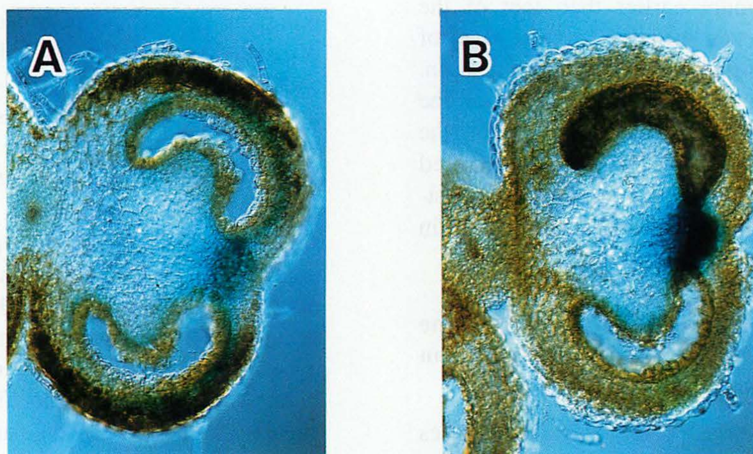

Fig. 2. Histochemical localization of GUS activity in tobacco plants transformed with the γ-VPE promoter-*gus* fusion gene. Tobacco was transformed with a reporter gene containing the promoter of the γ-VPE gene and the coding region of β-glucuronidase (GUS). Transverse sections of the anthers of the transgenic plants were subjected to GUS staining. GUS activity was found in the in endothelium, connective and circular cell cluster in the anther at developmetanl stage 1 (A). The predominant expression was observed in the circular cell cluster at developmental stage 3 (B).

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Takeda, S., Kowyama, Y., Takeuchi, Y., Matsuoka, K., Nishimura, M. and Nakamura, K. (1995) Spatial patterns of sucrose-inducible and polygalacturonic acid-inducible expression of genes that encode sporamin and b-amylase in sweet potato. *Plant Cell Physiol.* **36**, 321-333.

Yamaguchi, K., Mori, H. and Nishimura, M. (1995) A novel isoenzyme of ascorbate peroxidase localized on glyoxysomal and leaf peroxisomal membranes in pumpkin. *Plant Cell Physiol.* **36**, 1157-1162.

Yamaguchi, K., Takeuchi, Y., Mori, H. and Nishimura, M. (1995) Development of microbody membrane proteins during the transformation of glyoxysomes to leaf peroxisomes in pumpkin cotyledons. *Plant Cell Physiol.* **36**, 455-464.

DIVISION OF BIOENERGETICS

The Division has been closed upon Professor Yoshihiko Fujita's retirement on March 31, 1995, and will be re-intiated in 1996 on new projects.

Publication List:

Fujita, Y., Murakami, A., and Aizawa, K. (1995) The accumulation of protochlorophilide in cells of *Synechocystis* PCC 6714 with a low PSI/PSII stoichiometry. *Plant Cell Physiol.* **36**, 575-582.

Ohki, K. and Fujita, Y. (1995) Intracellular location of cytochrome oxidase active *in vivo* in the cyanophytes, *Synechocystis sp.* PCC 6714 and *Anacystis nidulans* Tx20 and R2, grown under various conditions. *Protoplasma* **188**, 70-77.

DIVISION OF CELL PROLIFERATION (ADJUNCT)

Professor: Masayuki Yamamoto
Research Associate: Masuo Goto
NIBB Postdoctoral Fellow: Takashi Kuromori
Graduate Students: Satsuki Okamoto (from Nara Women's University)
Fumihiko Hakuno (from The University of Tokyo)
Chikako Kitayama (from The University of Tokyo)

This Division aims to explore the regulation of meiosis in higher organisms. Meiosis is a crucial step in gamete formation and is essential for sexual reproduction. Meiotic steps are highly conserved among eukaryotic species. The major strategy taken by us to isolate genes that may be relevant to the regulation of meiosis in animals or plants depends upon trans-complementation between heterologous organisms. In the fission yeast *Schizosaccharomyces pombe*, which is a unicellular eukaryotic microorganism, genes involved in control of meiosis have been well characterized and many of them are cloned (reviewed in Yamamoto (1996) Trends Biochem. Sci. 21, 18-22). Mutants defective in these genes, isolated either by classical genetics or by gene disruption and chromosome manipulation, are available. We have thus set out to isolate homologs of these *S. pombe* genes from animals and a plant, mainly by using functional complementation of the mutants. To facilitate this strategy, we also paid efforts to elucidation of the regulatory mechanisms of meiosis in the fission yeast.

I. Animal and plant genes that trans-complement meiotic defects in the fission yeast

Using cDNA libraries prepared from mouse testis, *Xenopus* oocyte and *Arabidopsis thaliana*, we screened extensively for genes that can rescue loss of function of the following three genes, which are involved in the regulation of sexual development in *S. pombe*: The *sme2* gene, which encodes an RNA product essential for the promotion of meiosis I; the *pde1* gene, which encodes cAMP phosphodiesterase; and the *mes1* gene, which is required for the promotion of meiosis II. Genes encoding putative kinases, transcription factors, RNA-binding proteins and others have been isolated in these screenings, and their possible roles in the regulation of meiosis are currently under investigation. In particular, we found that various genes encoding cytoskeletal proteins can suppress the *mes1* defect, suggesting that the regulation of meiosis II is closely related to modification or reorganization of the cytoskeleton.

II. The requirement of cell cycle regulatory genes for meiosis in the fission yeast

The *cdc2* gene of *S. pombe* encodes a serine/threonine protein kinase, which plays key roles in the progression of both G1 and G2 phases in the mitotic cell cycle. This kinase regulates G2/M transition in cooperation with a B-type cyclin encoded by *cdc13*, forming a complex called MPF (M-phase promoting factor). The *cdc25* gene encodes a tyrosine protein phosphatase that activates *cdc2* kinase by dephosphorylation. These three *cdc* genes are required for mitosis, and cells carrying a temperature-sensitive mutation in either of these genes frequently produce two-spored asci at the semi-restrictive temperature, apparently not undergoing meiosis II. Cells carrying a mutant allele of *cdc2*, originally named *tws1*, have no obvious defect in the mitotic cell cycle but produce two-spored asci skipping meiosis II. These previous observations suggest that these three genes are essential for meiosis II. However, the involvement of these genes in meiosis I has been left unclear. We carefully investigated this problem in three different experimental systems and concluded that the function of *cdc2* is essential for premeiotic DNA synthesis, and that *cdc13* and *cdc25* are essential for meiosis I. We could not conclude whether *cdc2* is essential for meiosis I, due to experimental difficulties, although it appears likely. The necessity of *cdc2* function for premeiotic DNA synthesis in *S. pombe* contrasts to the previous report by others that *CDC28*, the *S. cerevisiae* homolog of *cdc2*, is dispensable for it.

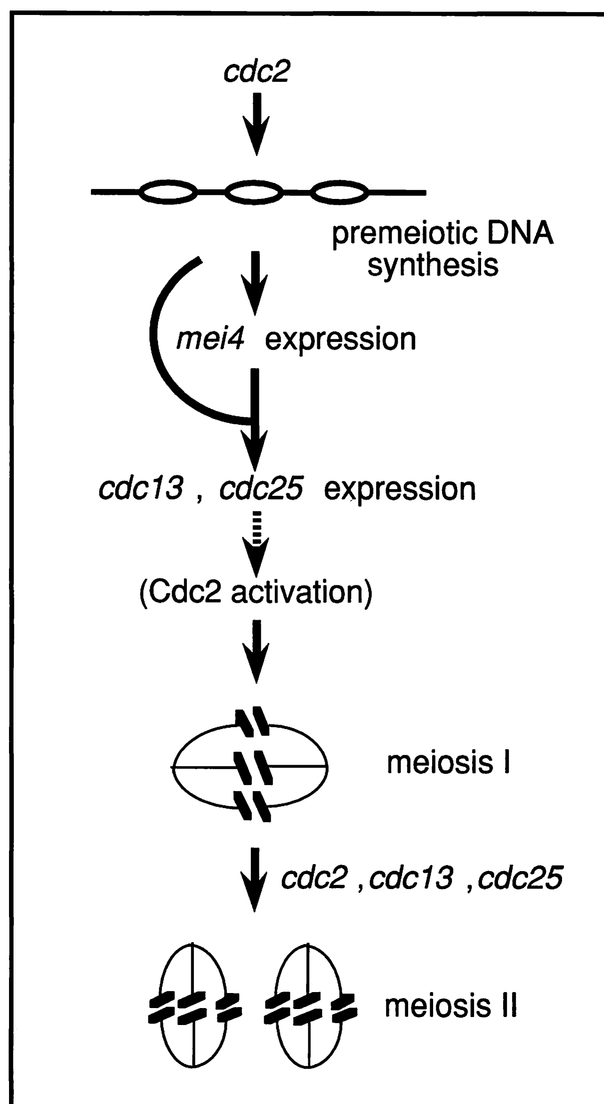

Fig. 1. The function of cell cycle genes in the regulation of meiosis in fission yeast. See Iino et al. (1995) for more details.

Publication List:

Iino, Y., Hiramine, Y. and Yamamoto, M. (1995) The role of *cdc2* and other genes in meiosis in *Schizosaccharomyces pombe*. *Genetics* **140**, 1235-1245.

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DIVISION OF CELLULAR COMMUNICATION (ADJUNCT)

Professor: Yoshiki Hotta
Associate Professor: Hitoshi Okamoto
Research Associates: Mika Tokumoto
Shin-ichi Higashijima
Institute Research Fellow: Nobuyoshi Shimoda

Brain can be seen as an integrated circuit where neurons of various identities are interconnected in a highly ordered manner by their axons. We have been interested in how individual neurons aquire their own identities and how their axons find their own pathways and finally recognize their proper targets. Using zebrafish (*Danio rerio*), which is suitable for genetic analysis and gene manipulation, we are trying to address these questions both at the molecular and cellular levels.

I. Differential expression of islet-1 homologs during specification of primary motoneuron

Islet-1 (Isl-1) is a LIM domain/homeodomain-type transcription regulator originally identified as an insulin gene enhancer binding protein. Isl-1 is considered to be involved in the differentiation of the neuronal cells. We previously cloned a *isl-1* homolog from zebrafish cDNA library, named it zebrafish*isl-1*. Recently, we have isolated two novel *isl-1* homologs from zebrafish cDNA library, named them zebrafish*isl-2* and *3*.

We examined the mRNA expression pattern of each homolog using in situ hybridization to whole-mount embryos. All three homologs are expressed in Rohon-Beard neurons. However the expression in primary motoneurons diverged. Zebrafish*isl-1* mRNA is expressed in the rostral primary motoneuron (RoP). *isl-2* mRNA is expressed in the caudal primary motoneuron (CaP) and its variant sibling (VaP). *isl-3* mRNA is expressed in the ventral region of the myotome but not in the primary motoneurons. The ventral myotome is the region that the axon of the CaP extend into. *isl-3* mRNA is also expressed throughout the developing eye and tectal region of the midbrain, the target for the retial axons. These results raise possibilities that the *isl-1* homologs may be involved in the specification and/or target recognition by the primary motoneurons.

II. Cloning and expression of AN34/F-Spondin family in zebrafish

F-Spondin is a secreted protein expressed at high levels in the floor plate. The C-terminal half of the protein contains six TSRs (Thrombospondin Type 1 repeats), while the N-terminal half exhibited no homology to other proteins. Functions of F-Spondin in vivo remain largely unknown. AN34, expressed in a subset of muscle cells in *Drosophila*, was cloned and found to encode a secreted protein sharing high degree of homology in two parts (H1 and H2) with the N-terminal half of F-Spondin (personal communication). This suggests that the homologous reigions are novel domains important for neuronal development. Thus, we searched for AN34/F-Spondin family from zebrafish (*Danio rerio*). By PCR screening and subsequent cDNA screening, we identified two novel genes (AN1 and AN2) in addition to zebrafish F-Spondin. AN1 and AN2 consist of H1, H2 and one TSR. The overall structure are highly similar to AN34 and more related to AN34 than F-Spondin, suggesing that AN1, AN2 and Drosophila AN34 constitute a novel subfamily. AN1 is expressed weakly in the floor plate and a small subset of neuronal cells at 22-26 hr. AN2 expression is first observed broadly around the axial mesoderm (notochord) at 10-12 hr. Then, the expression becomes restricted to the floor plate and hypochord at 14 hr and continues at high levels up to 26 hr. F-Spondin is expressed in the floor plate as seen in rat. These findings suggest that the AN34/F-Spondin family play important roles in the midline development in the vertebrate.

III. Two families of transposon in zebrafish

We have discovered two families of short interspersed repetitive elements (SINEs) in the zebrafish genome. The two families are marked by their wide distribution: one family, designated *mermaid*, is also present in the genomes of other fish, amphibian and primates, but absent in the mouse genome. Some members of the *mermaid* family were found in transposon-like repetitive elements including Tc1-like elements which are also distributed in the genomes of fish and amphibian. This raises the possibility of horizontal transfer of the *mermaid* family between vertebrates via DNA-mediated transposition. The *mermaid* family is distinctive in each species except for a conserved region of approximately 80 bp. The zebrafish *mermaid* sequence is about 400 bp long and has a typical SINE structure: split promoter of RNA polymerase III at the 5' end, tandem repeats of short oligonucleotide (AATG)n at the 3' end and target site duplications.

The zebrafish *mermaid* were estimated to be 12,000 copies per haploid genome and highly polymorphic between AB comp and Darjeeling strains. We also found that oligonucleotides directed to the conserved region of the *mermaid* family can be used to recover zebrafish specific DNA from zebrafish-mouse cell hybrids by PCR (*mermaid* PCR). Thus, the *mermaid* sequence serves as a valuable genetic tool for the zebrafish genome mapping.

Another SINE family is distributed not only in other Cypriniformes, such as silver carp and grass carp but also in medaka, at least. The family was named *angel* since some DNA fragments cloned from medaka which harbor the entire region of the

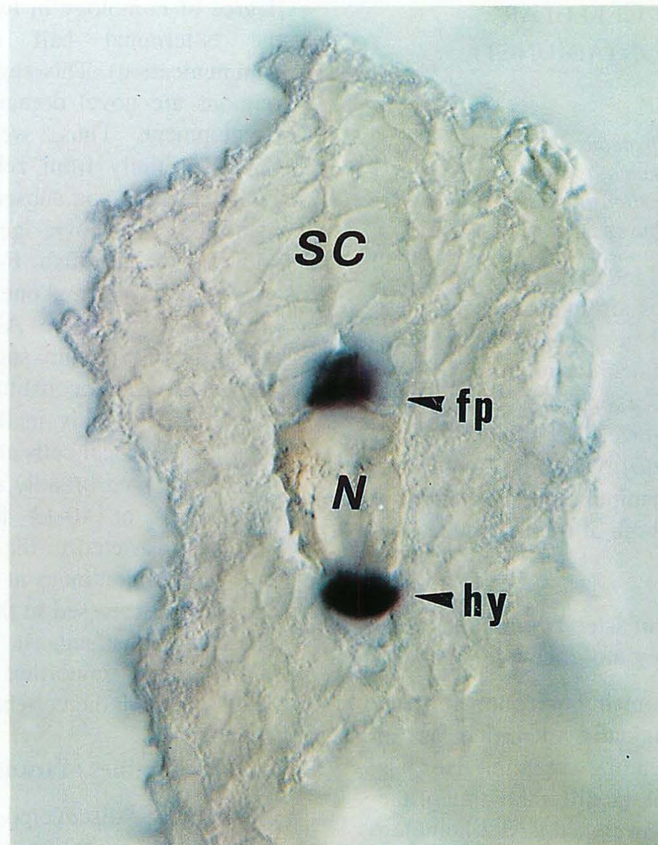

Fig. 1. Expression of *AN2* mRNA in the developing zebrafish spinal cord.
AN2 mRNA is specifically expressed in the floor plate (fp) and hypochord (hy). SC, spinal cord; N, notochord.

SINE migrated *an*omalously on *gel*s. The anomaly was temperature-dependent, suggesting a sequence-induced unusual structure of DNA. The *angel* family belongs to a new class of elements referred to as miniature inverted repeat transposable elements (MITEs) or inverted repeat SINEs. The *angel* sequence has relatively long terminal inverted repeats (TIRs) that flank short non-coding DNA. The TIRs of *angel* begin with the sequence TTAAAGGRR, known as the T2 motif first identified in other inverted repeat SINEs in *Xenopus*.

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Hosoya, T., Takizawa, K., Nitta, K. and Hotta, Y. (1995). *glial cells missing*: A binary switch between neuronal and glial determination in *Drosophila*. *Cell* **82**, 1025-1036.

Tokumoto, M., Gong, Z., Tsubokawa, T., Hew, C. L., Uyemura, K., Hotta, Y. and Okamoto, H. (1995). Molecular heterogeneity among primary motoneurons and within myotomes revealed by the differential mRNA expression of novel Islet-1 homologs in embryonic zebrafish. *Dev. Biol.* **171**, 578-589.

DIVISION OF CELL FUSION (ADJUNCT)

Professor: Hitoshi Sakano
Research Associate: Masahiro Ishiura (until March)
Fumikiyo Nagawa (until April)
Kanae Muraiso (on leave)
Akio Tsuboi
Institute Research Fellow: Hiroaki Kasai
Graduate Student: Setsuyuki Aoki (from Kyoto University)
Nika Yamazaki (from Tokyo Institute of Technology)

Our research interest has been focused on somatic DNA changes in the immune system. In lymphocytes, DNA recombination and gene conversion play important roles in the expression of antigen receptor genes. Gene rearrangement, known as V-(D)-J joining, not only generates a vast diversity in the receptor genes, but also insures the activation of a particular member of the multigene family.

Recently, hundreds of odorant receptor (OR) genes have been reported in the olfactory system, although it is yet to be studied how this gene system is regulated for expression. Each OR gene is expressed in one of the four topographically distinct zones, where olfactory neurons expressing a given OR gene are randomly distributed. It is assumed that only a limited number of the OR genes (possibly one) are activated in each olfactory neuron.

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

Developmental analyses have revealed that MOR10-expressing neurons come out earlier than MOR28-expressing ones during mouse embryogenesis. However, the number of MOR28 neurons overcomes that of MOR10 neurons after birth; the ratio of cells expressing MOR28 vs. MOR10 reaches 2.5 : 1 in the adult. These results suggest that individual olfactory neurons may activate OR genes through a stochastic mechanism even between two neighboring and closely related OR genes; yet this selection appears to be biased in both the onset and levels of expression.

In order to study whether the projection of axons to the olfactory bulb exerts influences on the OR gene expression, we have characterized *pdn/pdn* mutant mice in collaboration with Dr. Naruse at Aichi prefectural colony. The homozygous mice show a number of developmental abnormalities including polydactyly and gross malformations of the brain; they also lack olfactory bulbs. *In situ* hybridization has revealed that expression patterns of the MOR10 and MOR28 genes in the *pdn/pdn* embryos are comparable to those found in the wild-type, suggesting that the zonal expression of OR genes in the olfactory epithelium is regulated independently of influences from the olfactory bulb.

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

When the olfactory neurons are regenerated, they send axons to specific sets of glomeruli in the olfactory bulb. It is amazing that axons expressing a given OR gene are able to find their target among two thousand pairs of glomeruli. We hope that our transgenic approach mentioned above will become a useful clue to study the target specificity and selectivity in the synapse formation.

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Liu, Y., Golden, S. S., Kondo, T., Ishiura, M. and Johnson, C. H. (1995) Bacterial luciferase as a reporter of circadian gene expression in cyanobacteria. *J. Bacteriol.* **177**, 2080-2086.

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Moriya, H., Kasai, H. and Isono, K. (1995) Cloning and characterization of the *hrpA* gene in the *terC* region of *Escherichia coli* that is highly similar to the DEAH family RNA helicase genes of *Saccharomyces cerevisiae*. *Nucleic Acids Res.*, **23**, 595-598.

DEPARTMENT OF DEVELOPMENTAL BIOLOGY

Chairman: Yoshiaki Suzuki

Three regular divisions and one adjunct division that has been reinitiated on new projects during 1995, construct the Department. The department members conduct molecular analysis on various aspects of developmental phenomena; differentiation and maturation of the germ cells, molecular basis of the body plans, and gene regulation in different phases of the cell cycle, in the differentiating tissues, and during changes of the differentiated states.

DIVISION OF REPRODUCTIVE BIOLOGY

Professor: Yoshitaka Nagahama
Associate Professor: Michiyasu Yoshikuni
Research Associates: Minoru Tanaka
Tohru Kobayashi
JSPS Postdoctoral Fellow: Yoshinao Katsu
Postdoctoral Fellows: Wei Ge
Ahn Ryun-Sap
Kazumasa Suzuki
Takashi Todo
Graduate Students: Shinji Onoe (Graduate University for Advanced Studies)
Xiao-Tian Chang (Graduate University for Advanced Studies)
Daisuke Kobayashi (Graduate University for Advanced Studies)
Yuichi Ohba (Graduate University for Advanced Studies)
Jun Ding (Graduate University for Advanced Studies)
Masatada Watanabe (Graduate University for Advanced Studies)
Shigenari Ijiri (Hokkaido University)
Visiting Scientists: Joel Sohn (California Institute of Technology)
Craig Morrey (University of Hawaii)
Jian-Quiao Jiang (University of Wuhan)

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

I. Endocrine regulation of oocyte growth and maturation

Our research effort in previous years concentrated on the identification and characterization of the molecules (pituitary gonadotropins and gonadal steroid hormones) that stimulate and control germ cell growth and maturation. It was in 1985 that we identified, for the first time in any vertebrate, 17α, 20β-dihydroxy-4-pregnen-3-one (17α, 20β-DP) as the maturation-inducing hormone of amago salmon (*Oncorhynchus rhodurus*). Along with estradiol-17β, which was identified as the major mediator of oocyte growth, we now have two known biologically important mediators of oocyte growth and maturation in female salmonid fishes. It is established that the granulosa cells are the site of production of these two mediators, but their production by the ovarian follicle depends on the provision of precursor steroids by the thecal cell (two-cell type model). A dramatic switch in the steroidogenic pathway from estradiol-17β to 17α, 20β-DP occurs in ovarian follicle cells immediately prior to oocyte maturation. This switch is a prerequisite step for the growing oocyte to enter the maturation phase, and requires a complex and integrated network of gene regulation involving cell-specificity, hormonal regulation, and developmental patterning.

We have isolated and characterized cDNAs encoding several ovarian steroidogenic enzymes of rainbow trout (*Oncorhynchus mykiss*), medaka (*Oryzias latipes*), tilapia (*Oreochromis niloticus*), and ayu (*Plecoglossus altivelis*) which are responsible for estradiol-17β and 17α, 20β-DP biosynthesis: cholesterol side-chain cleavage cytochrome P450 (P450scc), 3β-hydroxysteroid dehydrogenase (3β-HSD), 17α-hydroxylase/C17,20-lyase cytochrome P450 (P450c17), cytochrome P450 aromatase (P450arom) and 20β-hydroxysteroid dehydrogenase (20β-HSD). We also isolated the structural genes encoding P450c17 and P450arom from medaka. The medaka P450arom gene consists of nine exons, but spans only 2.6 kb, being much smaller than the human P450arom gene (at least 70 kb), as the result of extremely small introns.

Northern blots using a tilapia P450arom cDNA fragment and Western blots using an antiserum against a tilapia P450arom oligopeptide fragment revealed a single P450arom mRNA (2.6 kb) and a single protein (59 kDa) in tilapia ovarian tissue, respectively. These analyses also reveal that the levels of both P450arom mRNA and protein are low in early vitellogenic follicles, increase in mid-vitellogenic follicles, and decline to non-detectable levels in post-vitellogenic follicles. Changes in the ability of follicles to convert exogenous testosterone to estrogens (aromatase activity) are similar to those of P450arom mRNA and protein. These observations indicate that the capacity of tilapia ovarian follicles to synthesize estradiol-17β is closely related to the contents of P450arom mRNA and protein within them.

In rainbow trout and medaka, P450scc and P450c17 (also 3β-HSD in rainbow trout) mRNA transcripts increase in follicles towards the end of oocyte growth phase and during oocyte maturation. Furthermore, incubations of isolated thecal layers with gonadotropin result in the elevation of P450scc mRNA. The effect of gonadotropin becomes more dramatic when the expression of P450scc mRNA is examined in granulosa cells. P450scc mRNA is not detected in the absence of gonadotropin, but markedly expresses in the presence of gonadotropin. The increase in the amount of P450scc, 3β-HSD and P450c17 transcripts provide an explanation for the dramatic increase in 17α, 20β-DP production in follicles during naturally- and gonadotropin-induced oocyte maturation. In contrast, levels of mRNA for P450arom are high during oocyte growth, but rapidly decrease during oocyte maturation. This decrease in P450arom mRNA levels appears to be correlated with the decreased ability of maturing follicles to produce estradiol-17β.

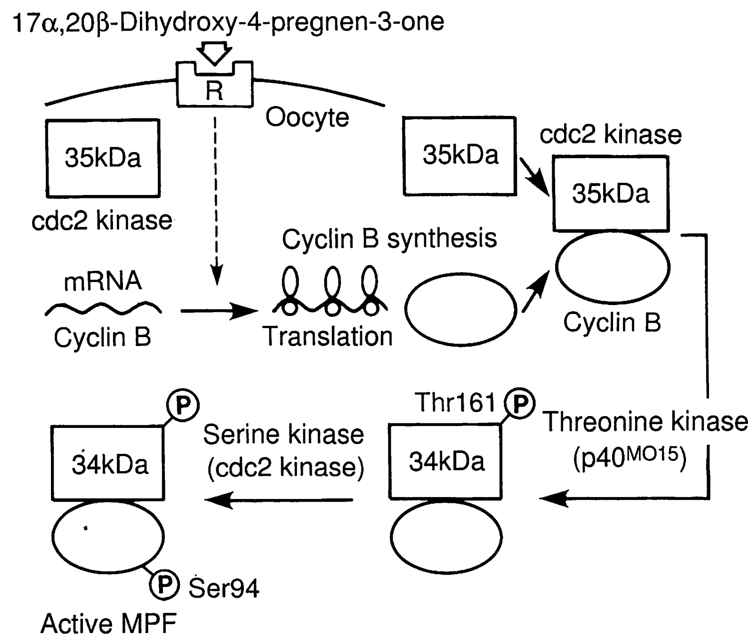

Fig. 1. Molecular mechanisms of 17α, 20β-dihydroxy-4-pregnen-3-one-induced maturation-promoting factor (MPF) activation in goldfish oocytes.

17α, 20β-DP acts via a receptor on the plasma membrane of oocytes. We have identified and characterized a specific 17α, 20β-DP receptor from defolliculated oocytes of several fish species. 17α,

20β-DP receptor concentrations increase during oocyte maturation. Pertussis toxin (PT) catalyzed the ADP ribosylation of a 40 kDa protein in crude membranes from rainbow trout oocytes. The 40 kDa protein is recognized by an antibody against a subunit of inhibitory G-protein. Treatment of the membrane fraction with 17α, 20β-DP decreases the PT-catalyzed ADP ribosylation of the 40 kDa protein. The specific binding of 17α, 20β-DP is decreased by PT. We conclude that the PT-sensitive Gi is involved in the signal transduction pathway of 17α, 20β-DP in fish oocytes.

The early steps following 17α, 20β-DP action involve the formation of the major mediator of this steroid, maturation-promoing factor or metaphase-promoting factor (MPF). MPF activity cycles during 17α, 20β-DP. induced oocyte maturation with the highest activity occurring at the first and second meiotic metaphase. Studies from our laboratory and others have shown that MPF activity is not species-specific and can be detected in both meiotic and mitotic cells of various organisms, from yeast to mammals. Fish MPF, like that of amphibians, consists of two components, catalytic cdc2 kinase (34 kDa) and regulatory cyclin B (46 to 48 kDa).

Immature goldfish oocytes contain only monomeric 35 kDa cdc2. Although immature oocytes contain mRNA for cyclin B, they do not contain cyclin B protein. 17α, 20β-DP induces oocytes to synthesize cyclin B. The preexisting 35 kDa inactive cdc2 kinase binds to *de novo* synthesized cyclin B at first, and then is rapidly converted into the 34 kDa active form by a threonine (Thr) kinase, $p40^{MO15}$ (Fig.1).

Introduction of a bacterially-produced goldfish cyclin B into immature goldfish oocyte extracts induces cdc2 kinase activation. Phosphoamino acid analysis shows that Thr phosphorylation of the 34 kDa cdc2 kinase is associated with the activation. The site of Thr phosphorylation on cdc2 kinase was mapped to residue Thr 161. Since goldfish cyclin B mRNA contains four copies of the usual cytoplasmic polyadenylation element in the 3'UTR, the initiation of its synthesis during oocyte maturation may be controlled by the elongation of poly (A) tail, as suggested in other systems. We exmined the polyadenylation state of cyclin B mRNA during goldfish oocyte maturation by means of a PCR poly (A) test, and found that cyclin B mRNA is polyadenylated during oocyte maturation.

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

digestion by 26S proteasome and the degradation in *Xenopus* egg extracts. These findings strongly suggest the involvement of 26S proteasome in cyclin degradation through the first cleave on its N-terminus.

II. Endocrine regulation of male germ cell development and maturation

We have identified two steroidal mediators of male germ cell development in salmonid fishes (11-ketotestosterone for spermatogenesis and 17α, 20β-DP for sperm maturation). 11-ketotestosterone can also induce the entire process of spermatogenesis in an organ culture system developed for eel testes from premitotic spermatogonia to spermatozoa within 21 days. A steroidogenic switch, from 11-ketotestosterone to 17α, 20β-DP, occurs in salmonid testes around the onset of final maturation. *In vitro* incubation studies using different testicular preparations have revealed that the site of 17α, 20β-DP production is in the sperm, but its production depends on the provision of precursor steroids by somatic cells. The site of 11-ketotestosterone production is in the testicular somatic cells.

In the cultivated male Japanese eel (*Auguilla japonica*), spermatogonia are the only germ cells present in the testis. Subtractive hybridization was used to identify genes that are expressed differentially in eel testes in the first 24 hr after HCG treatment *in vivo*, which ultimately induces spermatogenesis. One up-regulated cDNA was isolated from subtractive cDNA libraries derived from mRNA extracted from control testes and testes one day after a single injection of HCG. From its deduced amino acid sequence, this clone was identified as coding for the activin βB subunit. The HCG-dependent activin βB mRNA expression in the testes was restricted to Sertoli cells in testes treated with HCG for one to three days. A marked stimulation of activin B production was observed in testes after HCG and 11-ketotestosterone treatment. Addition of recombinant human activin B induced spermatogonial proliferation *in vitro*. Taken together, these findings suggest the following sequence of the hormonal induction of spermatogenesis in the eel. Gonadotropin stimulates the Leydig cells to produce 11-ketotestosterone, which, in turn, activates the Sertoli cells to produce activin B. Activin B then acts on spermatogonia to induce mitosis leading to the formation of spermatocytes.

In salmonid fishes, spermatozoa taken from the testes are immotile, but acquire motility during their passage through the sperm duct. Using male masu salmon (*Oncorhynchus masou*), we found that gonadotropin-induced testicular production of 17α, 20β-DP is responsible for the acquisition of sperm motility; 17α, 20β-DP acts to increase sperm duct pH, which in turn increases the cAMP content of sperm, allowing the acquisition of motility.

III. Endocrine regulation of gonadal sex differentiation

In tilapia, a pair of gonadal primordia formed on both sides of the intestine at 15 days after hatching. The gonadal anlage consists of several roundish germ cells surrounded by a few stromal cells. At 20 days after hatching, prior to the period of sex differentiation, positive immunostaining for P450scc, 3β-HSD, P450c17, and P450arom antibodies becomes evident for the first time in gonads. Immunostained stromal cells are observed in the vicinity of blood vessels (Fig. 2).

From 23-26 days after hatching, morphological gonadal sex differentiation begins to be recognized. Initial ovarian differentiation is marked by the appearance of a narrow space in the stromal tissue, representing the formatoin of the ovarian cavity. On the other hand, initial testicular differentiation is characterized by the appearance of a narrow space in the stromal tissue, representing the efferent duct construction. At this stage, germ cells in testes and ovaries remain in the gonial stage. In ovaries, positive immunostaining for four kinds of steroidogenic enzymes is recognized in large stromal cells located in the vicinity of blood vessels. In contrast, no immunoreaction is evident in testes during sex differentiation. This situation continues until testes initiate spermatogenesis. Thus, tilapia ovaries express the steroidogenic enzymes required for estradiol-17β biosynthesis from cholesterol before sexual differentiation, which is consistent with the concept that estrogen biosynthesis is essential for sexual differentiation of female phenotype during early development.

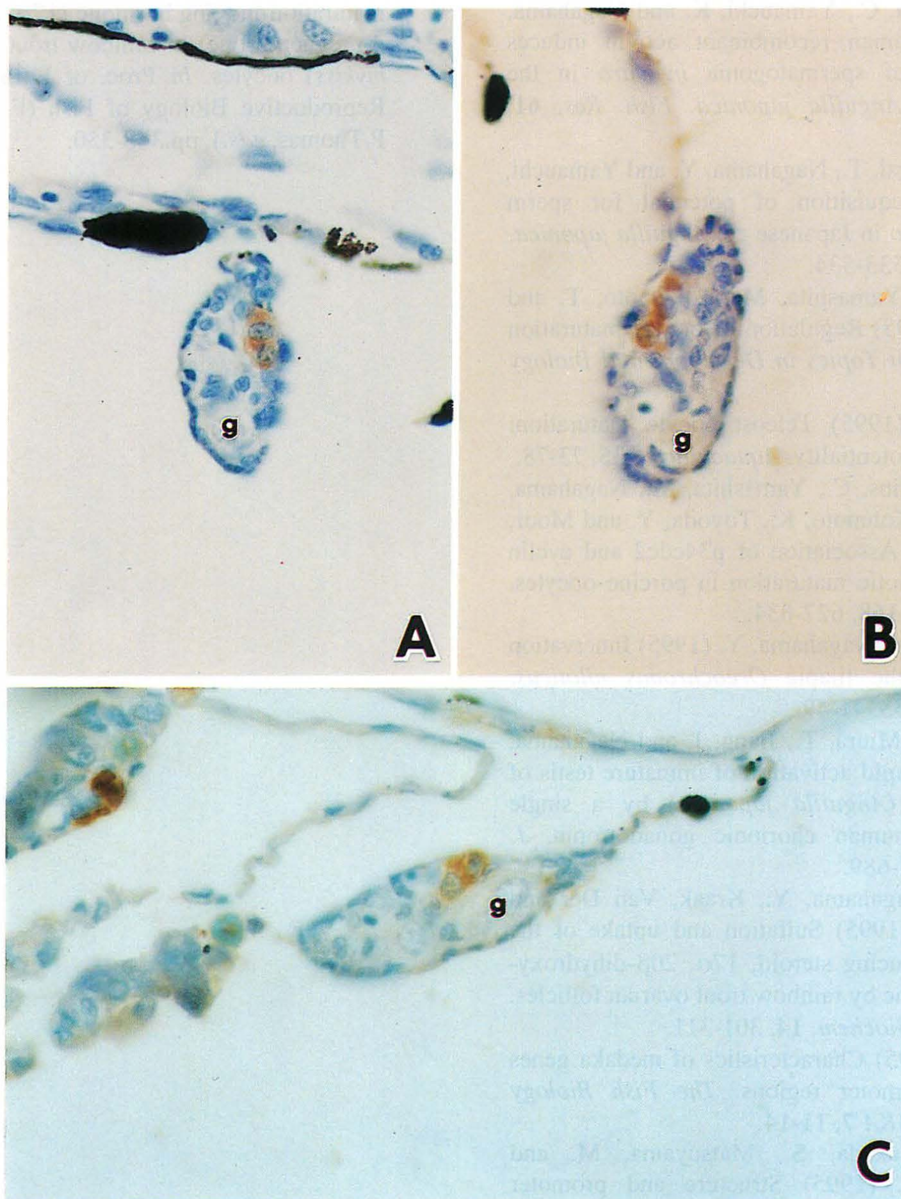

Fig 2. Immunocytochemical localization of cholesterol side-chain cleavage cytochrome P450 (A), 3β-hydroxysteroid dehydrogenase (B) and cytochrome P450 aromatase (C) in tilapia gonads collected at 20 days after hatching (prior to sexual differentiation). g, gonium. X800.

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DIVISION OF CELL DIFFERENTIATION

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Hiroki Kokubo
JSPS Postdoctoral Fellow: Václav Mach
Graduate Students: Katsuyoshi Matsunami (Graduate University for Advanced Studies)
Yoshinori Ueno (Graduate University for Advanced Studies)
Technical Staffs: Miyuki Ohkubo (deceased on January 12, 1995)
Chikako Inoue

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

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

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

Previously we described cloning and labial segment-specific expression of the *Bombyx Scr.* Silk gland specific transcription factor-1 (SGF-1) interacts with the SA site of the sericin-1 gene and FA and FB sites of the fibroin gene. Previously, we purified and sequenced the SGF-1 40 kDa protein, and found it to be a new member of the *fork head/HNF-3* family. Taking advantage of the immediate availability of *Bombyx fork head (fkh)* cDNA in this laboratory, we have analyzed the expression patterns of *SGF-1/fkh* mRNA and protein in developing embryos (see Fig. 1 and H. Kokubo et al. (1996). *Development Genes Evolution,* in press). At the time of embryo retraction (stage 20), the transcripts and protein were detected in the invaginating silk glands. Interestingly, preceding the appearance of the *Bombyx* Fkh protein in the invaginating silk glands, *Bombyx* Scr disappeared from the spots. This observation suggests the possibility that the *Bombyx* Scr is necessary to determine the nature of the labial segment and induce the silk gland invagination accompanied by the *Bombyx fkh* expression but the Scr protein is probably not necessary for the direct induction of *Bombyx fkh* expression in the invagination spots.

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

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

Among many factors proposed to bind and control the fibroin and sericin-1 genes, the POU-M1 that accommodates a POU-domain identical to *Drosophila* Cf1a was cloned and characterized previously. The POU-M1 binds to the SC site of the sericin-1 gene and is assumed to enhance the transcription. A function assay of the POU-M1 is being pursued in cell-free transcription system. This protein also binds to the PB element of the *POU-M1* gene and suppresses the transcription. The expression of the *POU-M1* gene has been analyzed in *Bombyx* embryos by *in situ* hybridization and immunohistochemistry. The gene was expressed specifically for the first time at stage 18-19 in a pair of restricted sites of the labial segment where a pair of prothoracic glands is going to be formed by invagination (previously we have mistaken these as the invagination sites of the silk glands). After the silk gland invagination, the *POU-M1* expression was detected in the developing silk gland and confined to the middle portion of the silk gland by late embryonic stages.

Cloning of a few other transcription factors of the fibroin gene is being continued.

With an intention to study the regulator-target relationship between genes, we have begun to use an antibody-selection method of fixed chromatin in embryonic stages. Screening of one of such chromatin libraries with a POU-M1 antibody has revealed a high selection of the sericin-1 promoter covering SB and SC sites. This result indicates that the sericin-1 promoter region is one of the POU-M1 targets and this method should be useful characterizing other regulator-target relationships.

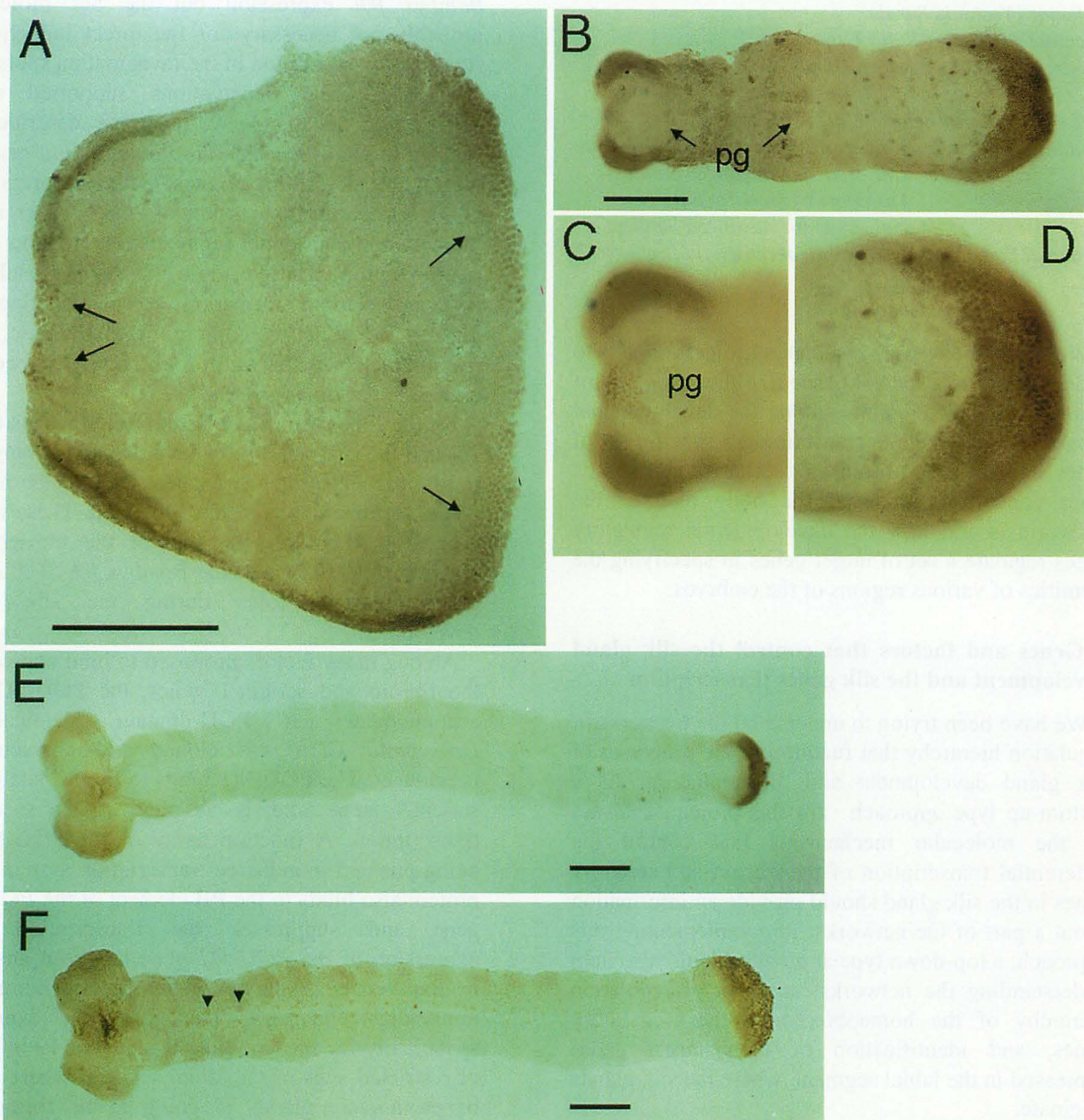

Fig. 1. Distribution of *Bombyx* Fkh/SGF-1 protein in embryos. **A** A germ anlage at 24 hrs after egg deposition. Two terminal regions reveal weak signals (arrows). **B** A germ anlage at 36 hrs. pg indicates the primitive groove. **C** and **D** Magnified anterior and posterior regions of **B**. Some dots in **B** and **D** are the yolk nuclei. **E** and **F** Embryos at 48 and 60 hrs. The triangles indicate primodia of central nervous system. All scale bars represent 100 μm. Revised from H. Kokubo et al. (1996). Develop. Genes Evol., in press.

II. Other genes of developmental interest

In continuation of the studies of abdominal segment identification, we have concentrated on the study of morphogenesis of embryonic abdominal legs. Previously we analyzed proteins in the wild type embryos, and found that two high molecular weight proteins of 260 and 270 kDa (p260/270) are expressed specifically in a restricted region of the abdominal legs. These proteins are not detectable in the E^{Ca}/E^{Ca} embryo that lacks *Bombyx abd-A* gene as reported previously, suggesting a control under the *Bombyx abd-A*.

We have screened an embryonic cDNA library with an antibody against the p260/270 and obtained one type of cDNA. This cDNA encodes a long open reading frame. Amino acid sequences from the N-terminal and two other regions of the p270 matched with those of the deduced sequence. Northern blot analysis revealed the *p270* transcripts from embryonic stage 19-20, whereas the *Bombyx abd-A* transcripts were observed from stage 17-18. From these results we speculate that the *Bombyx abd-A* may directly regulate the expression of the *p270*.

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DIVISION OF MORPHOGENESIS

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Jatupol Kositsawat (from School of Medicine, Universiy of Tokyo)
Technical Staff: Chiyo Takagi

In the newt and some other limited animal species, the lens and neural retina can be completely regenerated through transdifferentiation of pigmented epithelial cells (PECs). Such a phenomenon, transdifferentiation, as observed in regeneration of ocular tissues seems to be a highly powerful model for studying stability and instability in differentiation of tissue cells. From this view point, lens transdifferentiation of PECs of vertebrate has been studied *in vivo* and *in vitro* systems, and our *in vitro* studies have revealed that dormant potential to transdifferentiate into lens cells is widely conserved throughout vertebrate species, and that the cell type-specific genes, are completely inactivated in the multipotent (at least bipotent) dedifferentiated cells originated from pigmented epithelial cells.

Our studies have been conducted to clarify the molecular mechanism controlling the lens transdifferentiation in vertebrate PECs and also to search the reason why the pigmented epithelia of species other than the newt and so forth never regenerate the lens in the *in situ* eyes. Based on findings accumulated up to the last year, we have conducted analysis of the lens transdifferentiation of PECs *in vivo* and *in vitro* and the following results have been established.

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

The *mi* gene was first isolated as a mouse gene in the *microphthalmia (mi)* locus and shown to encode a basic-helix-loop-helix-leucine zipper (bHLHzip) protein. The product of the gene was suggested to transactivate the pigment cell specific genes, tyrosinase and TRP-1, and to have a critical role in development of the melanocytes and the PECs. To reveal the role of the *mi* gene in differentiation and transdifferentiation of the PECs, we isolated the cDNA clones for the chicken *mi* and characterized the expression in PECs *in vivo* and *in vitro*.

The *mi* gene was expressed by the embryonic PECs in tissue specific manner and was inactivated in the dedifferentiated cells during the *in vitro* transdifferentiation process. The *mi* protein was first detected in the whole optic vesicle of 2-day-old embryo. At later stages the expression was maintained only in the outer layer of the optic cup which was a precursor of the pigmented epithelium (Fig.1).

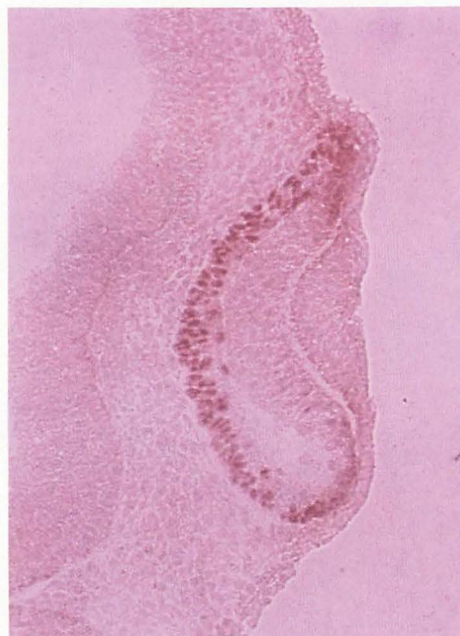

Fig.1. Expression of the *mi* protein in the optic cup. The *mi* protein is specifically localized to the nuclei of the cells constituting the outerlayer of optic cup of a 3-day-old chicken embryo.

To know the functional role of the *mi* gene in the PEC development, the antisense oligonucleotide was added to the culture of the optic cups. The differentiation of the PEC from the optic cup cells was inhibited by the antisense oligonucleotide suggesting an essential role of the *mi* product in the process of the PEC differentiation.

A part of the retinal PECs in quail embryos homozygous in the *silver* mutation start to proliferate in the 5-day-old embryo and make an ectopic neural retina through the transdifferentiation process. The *silver* mutant shared some phenotypes with the mouse *mi* mutant. A comparison of the sequences of the *mi* cDNAs from the wild and the *silver* quails revealed the presence of a frame shift mutation in the *silver* mutant. This result suggests the possibility that the mutation in the *mi* gene may inhibit the default differentiation of the PECs and may induce the transdifferentiation into the neural retina cells.

II. The role of integrin in stabilization and instabilization of pigmented epithelial cells

The extracellular matrix (ECM) has been suggested to play essential roles in transdifferentiation of PECs through extensive cell culture studies of lens transdifferentiation of PECs. For example, collagen matrix inhibits transdifferentiation of PECs dissociated from older chick embryos to lens cells and stabilizes their differentiated phenotype. The pigmented epithelium in the eye *in situ* is supported and stabilized by the underlying ECM consisting of basal lamina and fibrous collagenous matrix as major components. We examined changes in adhesiveness of PECs to various substrates such as fibronectin, collagen type I and IV, and so forth during dedifferentiation of these cells *in vitro*, to approach to the mechanisms involved in the stabilization and instabilization of differentiated state of PECs.

It has been well demonstrated that the dedifferentiated PECs (dePECs) are much less adhesive to ECM components except fibronectin than well-differentiated PECs and also that the differentiated PECs tightly adhere to ECM by focal contacts, which has been thought to be mediated by integrins as receptors of ECM components. Based on these observations, we isolated cDNAs for α3, α6, α8, αv, β1 and β5 integrins from PECs and found that all of these classes of integrin genes were expressed at similar levels in both PECs and dePECs. Although the amount of β1 integrin also did not change during dedifferentiation, focal contact was not observed in dePECs. When β1 integrin-mediated adhesion was blocked by anti-β1 integrin antibody, the cell morphology and gene expression pattern came to resemble those of dePECs. In contrast, dePECs were hardly affected by anti-β1 integrin antibody. In addition, it was clearly demonstrated that such a loss of function of β1 integrin in dePECs was due to phosphorylation of β1 integrin molecules. These findings are strongly suggesting that β1 integrin does not function as an adhesion molecule in dePECs and plays an essential role in maintenance of the differentiated state of the PECs.

III. Comparative analysis of crystallin gene expression between development and regeneration of the lens in newts

The spatio-temporal expression of three major crystallin genes (αA, βB1 and γ3) in developing and regenerating lenses of the newt were analyzed by *in situ* hybridization in order to compare the mode of cell type-specific gene expression in normal lens development with that in lens regeneration.

In normal lens development, at the stage immediately before the onset of primary lens fiber differentiation at the posterior wall of the lens vesicle, all three crystallin gene transcripts were first detected simultaneously in the epithelial cells constituting the posterior region of the lens vesicle. The expression of the crystallin genes was found to continue during lens fiber differentiation of the posterior cells to the primarily lens fiber cells. At later stages, the expression of these three genes was restricted to the secondary lens fibers. αA- and γ3-crystallin transcripts were not detected in lens epithelium at any stage, while βB1-crystallin transcript was first detected in the lens epithelia at the stage when the primary lens fiber mass was formed. In lens regeneration, the three crystallin transcripts were first detected simultaneously at the ventro-posterior region of the regenerating lens rudiment corresponding to the lens vesicle. Although the expression pattern of βB1- and γ3-crystallin transcripts were almost the same as those in normal lens development, αA-crystallin gene was found to be expressed even by lens epithelial cells in addition to lens fiber cells, showing clear contrast to the lens development, in which αA-crystallin was never expressed by the lens epithelial cells.

These results suggest that newts possesses some unique gene expression programme for lens regeneration and also that the regeneration of lens must not be a simple repetition of the development of this tissue.

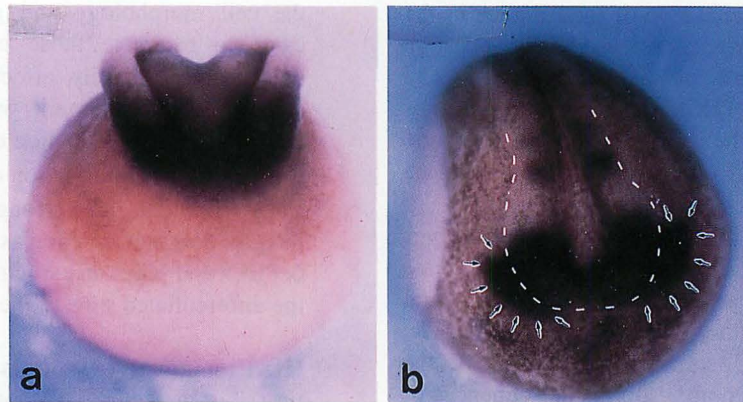

Fig. 2. Expression of the *Pax-6* gene in the newt (A) and *Xenopus* (B) embryos at the mid-neurula stage of development. In the newt embryo, the expression of *Pax-6* gene is restricted within the neural plate and never observed in the head ectoderm (a), but in the *Xenopus* embryo (b) *Pax-6* gene is strongly expressed in a fairly large area of the head ectoderm including the presumptive lens region (arrows). The broken line indicates the margin of the anterior half of the closing neural plate (b).

IV. Comparative analysis of *Pax-6* gene expression during eye development in the newt and *Xenopus*

As the first step of comparative analysis of lens development and regeneration in the newt in which lens can be regenerated from the iris epithelium, and in the *Xenopus*, in which lens can be regenerated from the corneal epithelium in its larval stages, the expression pattern of *Pax-6* gene thought to be an essential gene for organogenesis of the eye, was studied by *in situ* hybridization using *Pax-6* cDNA homologues cloned in both newt and *Xenopus* as probes.

In *Xenopus*, *Pax-6* gene expression can be detected in the head ectoderm including presumptive lens forming region in addition to eye forming region of the neural plate as early as the mid-neural plate stage. The expression of this gene in the head ectoderm is then gradually restricted to the lens forming region and eventually concentrated to the lens placode facing the optic vesicle. Contrary to this, in newts, *Pax-6* gene expression can never be detected in the head surface ectoderm until the optic vesicle underlies the ectoderm (Fig. 2).

These observations are strongly suggesting that the regulatory mechanisms of *Pax-6* gene expression in newts must be quite different from that in *Xenopus* and that the lens development from the ectoderm in the newt must be much more dependent on the inductive effect of the optic vesicle than in *Xenopus*. Based on these results we are extending our studies of this line to a more critical analysis of *Pax-6* gene expression and regulation.

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DIVISION OF DEVELOPMENTAL BIOLOGY (ADJUNCT)

Professor: Kenzo Nakamura

In this laboratory which started in its new form in October of 1995, research attention will be focused on the control of plant development by the changes in metabolic activity of the plant body. In particular, mechanisms involved in the regulation of gene expression by the carbohydrate metabolic signal and the role of sugar-regulation of gene expression in the growth and development of plants will be studied. For this purpose, isolation and characterization of mutants of *Arabidopsis thaliana* which show altered patterns of the sugar-regulated gene expression and defects in growth and development will be pursued. These genetic approaches will be complemented with the identification and characterization of transcription factors and calcium-dependent protein kinase which may participate in the sugar-regulated gene expression.

Publication List:
(from previous projects by Professor Iwabuchi's group)

Mikami, K., Katsura, M., Ito, T., Okada, K., Shimura, Y. and Iwabuchi, M. (1995) Developmental and ktissue-specific regulation of the gene for the wheat basic/leucine zipper protein HBP-1a (17) in transgenic *Arabidopsis* plants. *Mol. Gen. Genet.* **248**,573-582.

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DEPARTMENT OF REGULATION BIOLOGY

Chairman: *Norio Murata*

The Department is composed of two regular divisions and two adjunct divisions. The study of this department is focused on the molecular mechanisms for the response of organisms including tissues and cells toward external and internal environments, such as light, temperature, hormones, signal-transducing substances.

DIVISION OF MOLECULAR NEUROBIOLOGY

Professor: Masaharu Noda
Research Associates:Nobuaki Maeda
Masahito Yamagata
Eiji Watanabe
Institute Research Fellow:Masakazu Takahashi
Graduate Students:Hiroki Hamanaka
Junichi Yuasa
Takafumi Shintani
Taeko Nishiwaki
*Tatsunori Yamamoto**
Technical Staff:Akiko Kawai
Shigemi Ohsugi
*(*from Nagoya University)*

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

I. Topographic and laminar connection in the chick retinotectal system

Neural connection in the vertebrate brain is selective in two ways, which we refer to as topographic and laminar. Topographic specificity determines the orderly maps of connectivity, in which presynaptic neurons arrayed along spatial axes project to corresponding arrays of target cells in the target area. By contrast, laminar specificity determines the local circuitry, in which presynaptic neurons form synapses on the particular neurons and portions of the neuron's surface. We have been studying the retinotectal system in chickens to reveal the crucial factors that determine the development of these two classes of neural connection.

Historically, most intensively studied in this system has been the retinotopic map, where retinal ganglion cells from various parts of the retina project topographically onto the tectal surface. Namely, retinal axons from the temporal (posterior) retina connect to the anterior part of the optic tectum, and nasal (anterior) retinal axons connect to the posterior part, thereby establishing a point-to-point projection map. Many works, since the formulation of 'chemoaffinity theory' by Sperry, have supported the involvement of gradient or position-specific molecules in this topographically organized connection. Subscribing to this model, we applied a subtractive hybridization technique, and found several novel or identified genes to be topographically expressed along the anterior-posterior axis in the chick retina. Among these position-specific molecules, two molecules which belong to a same family of transcription factors are found to be expressed in a mutually exclusive manner in either the nasal or temporal part of the retina. Starting with these molecules, we hope to understand the molecular mechanism by which retinotectal topographical connection is established.

Less well studied, but striking, is a laminar selectivity of retinotectal connection in the orthogonal direction. Retinal axons enter the tectum through the most superficial of its 15 different laminae. Once axons reach defined loci on the tectal surface, they branch inwards and make connections in only 3-4 of the 15 laminae. Moreover, each axon confines most of its synapses to one of these 'retinoreceptive' laminae, in that neurochemically-distinguishable subsets of the ganglion cells connect to distinct laminae to establish a functional network. The laminar selectivity in this system may require an axon to recognize particular target cells (cellular specificity) and particular portions of the target cell's surface (subcellular specificity). To identify the cellular and molecular mechanism underlying these synaptic specificities, we are taking two interrelated approaches. First, we are trying to establish culture systems to analyze the recognition cues between presynaptic axons and their target cells *in vitro*. The second approach is to use molecular techniques, particularly by developing useful probes. We have already obtained a panel of novel monoclonal antibodies that label selectively one of 3-4 retinoreceptive layers of the tectum during the onset of the retinotectal projection. We hope that further characterization of these lamina-specific epitopes will provide a clue to the mechanism that underlies the lamina-specific synapse formation.

II. Proteoglycan, protein-tyrosine phosphatase and brain development

Developmental processes of the brain requires various cell-cell and cell-extracellular matrix interactions. In order to find key molecules in the morphogenesis of the brain, we are focusing our efforts on brain-specific proteoglycans, because proteoglycans are major extracellular matrix components in the developing brain. Previously, we identified a PBS-soluble chondroitin sulfate proteoglycan with a 300-kDa core protein, named 6B4 proteoglycan. Expression of 6B4 proteoglycan is dynamically regulated during development in various brain regions.

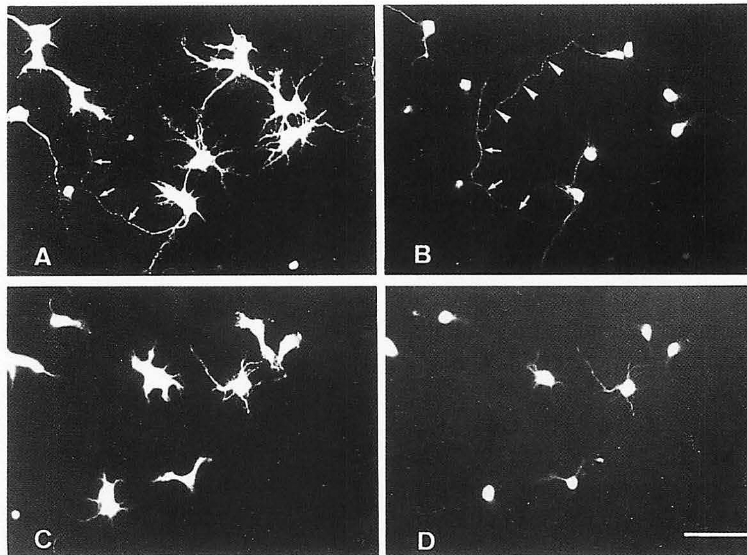

Fig 1. 6B4 proteoglycan promotes differentiation of neurons. Cortical neurons were cultured on the coverslips coated (A, B) or uncoated (C, D) with 6B4 proteoglycan. After 2 days *in vitro*, the neurons were double-immunostained with dendrite-specific (anti-MAP2; A, C) and axon-specific (anti-NFH; B, D) antibodies. Arrowheads indicate an NFH-positive and MAP2-negative axon. Arrows indicate an NFH- and MAP2-positive neurites. Scale bar; 50μm.

Recently, we cloned cDNA for 6B4 proteoglycan, and revealed that the core protein structure corresponds precisely to the extracellular region of a receptor-like protein tyrosine phosphatase, PTPζ (RPTPβ). 6B4 proteoglycan is characterized by the presence of the N-terminal carbonic anhydrase-like domain, a fibronectin type III region, and long serine, glycine-rich domain which is thought to be the chondroitin sulfate attachment domain. We showed that the transmembrane form, PTPζ (RPTPβ), is also present as a membrane-bound chondroitin sulfate proteoglycan in rat brain. This finding suggests that these brain-specific chondroitin sulfate proteoglycans have a role as cell signaling molecules by binding to neural cell adhesion and extracellular matrix molecules, in the case of 6B4 proteglycan, possibly also by competing for ligands of the phosphatase.

In order to examine functional roles of 6B4 proteoglycan, we studied the influence of this proteoglycan on cell adhesion and neurite outgrowth *in vitro* using dissociated neurons from the cerebral cortex and thalamus. 6B4 proteoglycan adsorbed onto plastic tissue-culture dishes did not support neuronal cell adhesion, but rather exerted repulsive effects on the cortical and thalamic neurons. When neurons were densely seeded on patterned substrata consisting of a grid-like structure of alternating poly-L-lysine and 6B4 proteoglycan-coated poly-L-lysine domains, they were concentrated only on the poly-L-lysine domains.

However, 6B4 proteoglycan did not retard the differentiation of neurons but rather promoted neurite outgrowth and development of the dendrites of cortical neurons, when neurons were sparsely seeded on poly-L-lysine-conditioned coverslips continuously coated with 6B4 proteoglycan (Fig 1.).

This effect of 6B4 proteoglycan on the neurite outgrowth of cortical neurons was apparent even on coverslips co-coated with fibronectin or tenascin. By contrast, the neurite extension of thalamic neurons was not modified by 6B4 proteoglycan. Chondroitinase ABC or keratanase digestion of 6B4 proteoglycan did not affect its neurite outgrowth promoting activity, suggesting that a protein moiety is responsible for the activity. 6B4 proteoglycan transiently promoted tyrosine phosphorylation of an 85-kDa protein in the cortical neurons but not in the thalamic neurons, which correlated with the induction of neurite outgrowth. These results suggest that 6B4 proteoglycan modulates morphogenesis and differentiation of neurons spatiotemporally depending on cell types.

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DIVISION OF CELLULAR REGULATION

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The research effort of this division is directed toward a full understanding of the molecular mechanisms that allow plants to acclimate to and tolerate extremes of temperature and salinity. In 1995, significant progress was made in the following areas as a result of studies with cyanobacteria and higher plants as experimental materials.

I. Regulation of the expression of genes for desaturases

Living organisms are exposed to changes in ambient temperatures. However, they are able to maintain the appropriate fluidity of their membrane lipids by regulating the extent of unsaturation of their fatty acids. For example, cyanobacterial cells respond to a decrease in temperature by introducing double bonds into the fatty acids of membrane lipids, thereby compensating for the temperature-induced decrease in the molecular motion of membrane lipids. Desaturases catalyze the introduction of these specific double bonds. We have demonstrated that the low temperature-induced desaturation of fatty acids in membrane lipids is regulated at the level of expression of the genes for the desaturases.

We have shown that the levels of the transcripts of the *desA*, *desB*, and *desD* genes (for the Δ12, Δ15 and Δ6 desaturases, respectively) increase about 10-fold after shift in culture temperature from 34°C to 22°C. However, the level of the transcript of the *desC* gene (for the Δ9 desaturase) remains constant after such a temperature shift, suggesting that expression of this gene is constitutive. Sequencing of the nucleotides at the 5' termini of the genes for all four desaturases indicated that the sites of initiation of transcription are quite similar among all four genes. Identification of the regulatory elements of these genes and cloning of the relevant *trans*-acting factors are in progress.

II. Importance of the unsaturation of membrane lipids in the turnover of the D1 protein of the photosystem II complex

As part of our efforts to understand the role of unsaturation of membrane lipids, we disrupted the *desA* and *desD* genes in *Synechocystis* sp. PCC 6803 by inserting antibiotic-resistance gene cartridges. This disruption greatly reduced the extent of unsaturation of the fatty acids of the membrane lipids. In the wild-type strain, the saturated, mono-unsaturated, di-unsaturated, and tri-unsaturated lipid molecules each accounted for 15% of the total membrane lipids. By contrast, in the *desD*$^-$ mutant, the triunsaturated lipid molecules were absent, while in the *desA*$^-$/*desD*$^-$ mutant, 80% of the total membrane lipids were monounsaturated and only 15% were fully saturated. These decreases in the unsaturation of membrane lipids greatly reduced the tolerance of the cyanobacterium to low temperature in the light.

These results, together with other observations, suggest that unsaturated fatty acids play an important role in protection of the photosynthetic machinery against low-temperature photoinhibition. The extent

of photoinhibition in intact cells reflects a balance between the light-induced inactivation of the photosystem II protein complex and the recovery of the complex from the inactivation. Therefore, we were able to separate the two processes using inhibitors of protein synthesis. Our results demonstrated that the unsaturation of membrane lipids had no effect on the light-induced inactivation process but did accelerate the recovery of the photosystem II complex from the photoinactivated state. Further analysis indicated that the unsaturation of membrane lipids accelerated the incorporation of the precursor to the D1 protein (an important protein component of the photosystem II complex) or the processing of the precursor to the D1 protein to generate the mature D1 protein.

III. Heat stability of the photosynthetic machinery

We also focused on the responses of plants to high-temperature stress. Photosynthesis is one of the physiological processes in plants that is most susceptible to heat stress. Thus, stabilization of the photosynthetic machinery against heat helps plants to tolerate higher temperatures.

We have studied the molecular mechanism responsible for the stabilization against heat of oxygen evolution in the cyanobacterium *Synechococcus* sp. PCC 7002. When thylakoid membranes isolated from the cyanobacterial cells were treated with a low concentration of Triton X-100, the stability to heating of the evolution of oxygen decreased markedly. From the extracts obtained by this treatment with Triton X-100, we purified two proteins that were able to restore the heat stability of oxygen evolution. One protein was identified as cytochrome *c*-550, having a low redox potential. The other protein of 13 kDa was identified as a homology of the 9-kDa extrinsic protein of cyanobacterial photosystem II. These results indicate that cytochrome *c*-550 and the 13-kDa protein are involved in the stabilization against heat of the evolution of oxygen and, therefore, in the stability of the photosynthetic machinery at elevated temperatures. We isolated the genes for cytochrome *c*-550 and the 13-kDa protein of *Synechococcus* sp. PCC 7002. Disruption of these genes in *Synechococcus* sp. PCC 7002 is now in progress in our laboratory. We have also studied the effects of elevated temperatures on photosynthesis in cultured soybean cells. The heat stability of oxygen evolution in these cells was enhanced by 3°C upon an increase in the growth temperature from 25°C to 35°C.

IV. Genetic modification of the tolerance of a cyanobacterium to low-temperature stress by introduction of a gene for the synthesis of glycinebetaine

When cultured under saline conditions, some plants produce compatible solutes to avoid the deleterious effects of external salt. Glycinebetaine, a quaternary ammonium compound, is one such solute that is found in halotolerant plants and bacteria. To examine the effect of glycinebetaine on photosynthesis *in vivo*, we transformed the salinity-sensitive cyanobacterium *Synechococcus* sp. PCC 7942 with the *codA* gene for choline oxidase from *Arthrobacter globiformis*, which catalyzes the oxidation of choline to glycinebetaine. The transformed cells accumulated glycinebetaine at intracellular levels of 60-80 mM and simultaneously acquired the ability to tolerate salt stress. We also observed that the transformed cells could grow at 20°C, a temperature that markedly retarded the growth of the wild-type cells. Photosynthesis in the transformed cells was more resistant to photoinhibition at low temperatures than that in wild-type cells. Furthermore, the recovery of photosystem II from photoinhibition at low temperatures was enhanced in the transformed cells. These findings suggest that glycinebetaine enhanced the growth of the transformed cells at 20°C by accelerating the recovery of the photosynthetic machinery from photoinhibition.

V. Genetic modification of the ability of *Arabidopsis* to tolerate salt and low-temperature stress

A higher plant, *Arabidopsis thaliana*, was transformed with the *codA* gene for choline oxidase under control of the 35S promoter of cauliflower mosaic virus. Such transformation allowed the plant to accumulate glycinebetaine and enhanced its ability to tolerate salt and cold stresses. When cultured in the presence of 100 mM NaCl, transformed plants grew well whereas wild-type plants failed to grow. The transformed plants tolerated 200 mM NaCl, which was lethal to wild-type plants. After plants had been incubated with 400 mM NaCl for two days, the photosystem II activity of wild-type plants had almost completely disappeared, whereas that of transformed plants remained at more than 50% of the original level. When exposed to low temperatures in the light, leaves of wild-type plants exhibited symptoms of chlorosis, whereas those of transformed plants did not. Thus, the genetic modification of *Arabidopsis thaliana* that allowed it to accumulate glycinebetaine also enhanced its ability to tolerate both salt and cold stresses.

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DIVISION OF BIOLOGICAL REGULATION AND PHOTOBIOLOGY (ADJUNCT)

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From an energetic point of view, the conversion of light energy into chemical energy in photosynthesis is the most important biological process on earth. The highly efficient energy conversion in this process is ensured by the highly ordered organization of molecules in the photochemical reaction center, in a physical, chemical and biological sense. The project in this division is aiming to elucidate the organization of photosystem II reaction center of oxygenic photosynthesis which has a unique property to generate a strong oxidant for utilizing the water molecule as electron donor.

In the first approach, molecular organization of the photosystem II reaction center, which has been identified in our study, will be analyzed by several methods which include crystallographic analysis, chemical modification & cross-linking analysis and optical & EPR spectroscopies. Structure-functional analysis will also be conducted for the reaction center using random and site-directed mutagenesis for transformable algae, *Synechocystis* PCC 6803 and *Chlamydomonas reinhardtti*. The principal target of these analyses will be the structure and molecular environment of P-680, the primary donor, which determine the redox potential of this unique photosystem.

In the second approach, the effort will be focused on the elucidation of molecular mechanism of light-regulated metabolic turnover of a subunit of the photosystem II reaction center, D1 protein. Some of unique steps are involved in this process; *i.e.*, photo-damage of the photochemical reaction center, specific degradation of the impaired protein subunit, light-regulated gene expression at the translational level, post-translational cleavage of the C-terminal extension and the incorporation of cofactors and subunits into a multi-component pigment-protein complex.

I. Structural organization of photosystem II reaction center

(1) A photochemically inactive pheophytin (Pheo) a molecule in the photosystem II reaction center, which contains 6 chlorophyll a and two β-carotene molecules for every two Pheo a, was identified by fluorescence excitation spectroscopy at 77K, and confirmed by absorption, linear dichroism, and magnetic circular dichroism spectra (in collaboration with Dr. M. Mimuro, Div. of Bioenergetics). When the emission was monitored at 740 nm, photochemically active Pheo a was detected as peaks at 418, 513, 543, and 681 nm. However, when the emission was monitored at 665 nm, two bands were observed at 414 and 537 nm; these bands were insensitive to photochemical reduction of the primary acceptor and thus appear to be due to photochemically inactive Pheo a.

(2) Solvent extraction at ~ 4°C in complete darkness, and subsequent analysis by high-pressure liquid chromatography using an apparatus equipped with a two-dimensional diode-array detector, spectroscopically identified 15-*cis*-β-carotene in the photosystem II reaction center. The result revises a previous conclusion that β-carotene bound to the reaction center takes the all-trans configuration, and generalizes the concept established for purple photosynthetic bacteria that 15-*cis*-carotenoid is naturally selected by the photosynthetic reaction centers (in collaboration with Drs. Y, Koyama et al., Kwansei Gakuin University).

(3) Site-directed modifications of the environment of the primary donor of photosystem II reaction center were attempted, using *Synechocystis* PCC 6803, in order to elucidate molecular interactions responsible for the high redox-potential.

II. Dynamic aspects of the organization of photosystem II reaction center

(1) The D1 subunit of the photosystem II reaction center has a C-terminal extension consisting of 8-16 amino acids. Post-translational removal of this part of the protein is essential for the manifestation of oxygen-evolving function in photosystem II. The enzyme involved in the C-terminal processing of the D1 precursor protein (pD1) of the photosystem II reaction center was purified from extracts of sonicated spinach thylakoids by a method that included chromatography on quaternary aminoethyl anion-exchange, hydroxyl-apatite, copper-chelating affinity and gel-filtration columns. The enzyme was identified, from its chromatographic behavior, to be a monomeric protein of about 45 kDa. The sequence of the amino-terminal 27 amino acids of this protein was determined directly, which exhibited low but appreciable (37%) homology to that deduced from a gene (*ctpA*) in *Synechocystis* PCC 6803 that was proposed recently to encode the processing protease form results of genetic complementation analysis.

Based on the amino acid sequence data of the purified protease, a cDNA clone encoding the enzyme has identified and sequenced, from a spinach green leaf cDNA library. By the analysis, the *full*-length transcript was established to consist of 1906 nucleotides and a poly(A) tail, containing an open reading frame corresponding to a protein with 539 amino acid residues. By comparing the amino acid sequence of the purified protease with that deduced

form nucleotide sequence of the cDNA clones, the enzyme was shown to be furnished with an extra N-terminal extension characteristic of both a transit peptide and a signal sequence. This suggests that the protease is synthesized in the cytosol and translocated into the lumenal space of thylakoids. The mature part of the enzyme consists of 389 amino acid residues and exhibits a significant sequence homology with two groups of proteins as demonstrated by a computer homology search; *i.e.*, (i) the deduced sequence of a protein proposed to be the C-terminal processing protease for pD1 in *Synechocystis* PCC 6803 and (ii) proteases for C-terminal cleavage identified in *Escherichia coli* and *Bartonella bacilliformis.*

(2) The synthesis of D1 precursor protein is regulated by light at the stage of translation. The mechanism of this light regulation was analyzed using isolated pea chloroplasts. Accumulation of the precursor and the mature form of the D1 protein of the photosystem II reaction center in illuminated pea chloroplasts was prevented by the addition of the inhibitors of photosynthetic electron transport, *i.e.*, atrazine, 3-(3,4-dichlorophenyl-1,1-dimethylurea and bromoxynil. Under such conditions, the compensatory accumulation of two translational intermediates of the D1 protein of 22 and 24 kDa was induced by the addition of ATP, as also observed in darkness in the presence of ATP (Taniguchi et al., FEBS Lett. 317, 57-61, 1993), suggesting that the synthesis of the full-length D1 protein requires a factor that is generated by the operation of photosynthetic electron transport. The accumulation of the full-length D1 protein was induced in the light, even in the presence of atrazine, when both 2,6-dichlorophenolindophenol and ascorbate were also present and in darkness upon the addition of dithiothreitol. Moreover, reagents with a relatively low redox potential, namely, duroquinone and methylviologen, prevented the accumulation. These observations suggest that the translation of the D1 protein is regulated at specific steps during the elongation of the polypeptide via a redox change in a component around photosystem I.

(3) Photo-tolerant mutants of *Synechocystis* PCC 6803 were obtained by *in vitro* random mutagenesis of *psbAII* (gene for D1 protein) by PCR under a condition for reduced fidelity of amplification. A preliminary characterization has been carried out for a mutant. The mutant strain (G-3) was shown to have five substitutions in the amino acid in regions other than so-called "PEST-like sequence" in the D1 protein, *i.e.*, Phe-260 and Phe-273 to Ser, Gln-304 and Gln-310 to Arg, and Bal-351 to Ala. The mutant cells were tolerant to high irradiance both in terms of pigmentation and viability(Fig. 1). The functional characterization of photosynthesis in the mutant has been conducted.

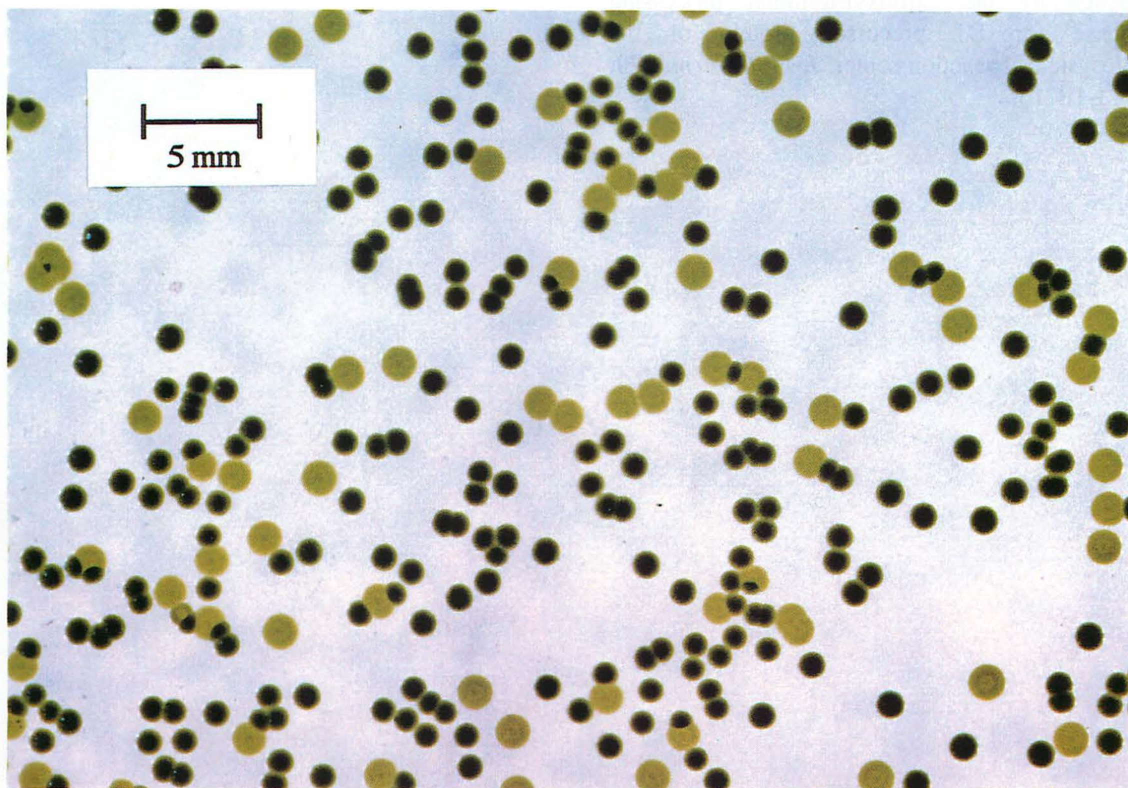

Fig. 1. Mixed culture of G-3 mutant and the control (KC) strains. The mixture of cells were plated on BG-11 agar medium. After colonies were grown under 50 $\mu E \cdot m^{-2} \cdot s^{-1}$ for 10 days, the agar plates were exposed to high photon flux density of 320 $\mu E \cdot m^{-2} \cdot s^{-1}$ for 5 days. Pale green colonies, control; deep green colonies, G-3 mutant (confirmed by mono-culture experiments).

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DIVISION OF BEHAVIOR AND NEUROBIOLOGY (ADJUNCT)

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Takeshi Umemiya (from Kyoto University)

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

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

I. Connectin can function as an attractive target recognition molecule

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

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

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

We are conducting molecular and genetic analysis of other enhancer trap lines that are expressed in small subsets of muscles and/or neurons. One of them, P750 expresses ß-gal in subsets of ventral and dorsal muscles and in a small number of neurons in the central nervous system (CNS). The cDNA cloning and sequencing revealed that P750 encodes a novel transmembrane protein that belongs to LRR family. It is interesting that three of the five muscle enhancer trap lines that we have thus far characterized contain LRRs. It may point to the important role of this motif in the development of muscles and/or neuromuscular specificity. Within the LRR family, P750 was found to be most related to the *Drosophila tartan* gene that have been implicated in neural and muscular development. To study the function of P750, we have isolated the loss-of-function mutants, and are currently characterizing the phenotype.

III. F-spondin/AN34 family of secreted proteins in *Drosophila*

Another enhancer trap line, AN34 expresses ß-gal in a single muscle fiber (#18) per hemisegment (Fig.1A). The remarkable specificity in its expression pattern (one out of 30 muscle fibers) makes it a good candidate for the muscle target recognition molecule. AN34 was found to encode a secreted protein with extensive amino acid similarity to rat F-spondin. F-spondin is a secreted molecule expressed at high levels in the floor plate and has been shown to promote neural cell adhesion and neurite extension in vitro. These results strongly suggest that AN34 is involed in motoneuronal gidance and/or targeting.

There are three regions that are highly conserved between AN34 and F-spondin. One of them (domain III) is a known repeating motif called thrombospondin type I repeats (TSRs). The other two (domain I and II) are novel conserved sequences that we identified. The amino acid identity in domain II is particularly high suggesting its functional importance. We thus searched for novel genes in *Drosophila* that contain this domain by using PCR, and succeeded in cloning two new genes. Similarly, Higashijima et al. (Div. of Cellular Communication) cloned two novel genes in addition to F-spondin in the zebrafish. All of these newly cloned genes share similar overall structure with AN34 and F-spondin in that they possessed domain I, II and one to six TSRs.

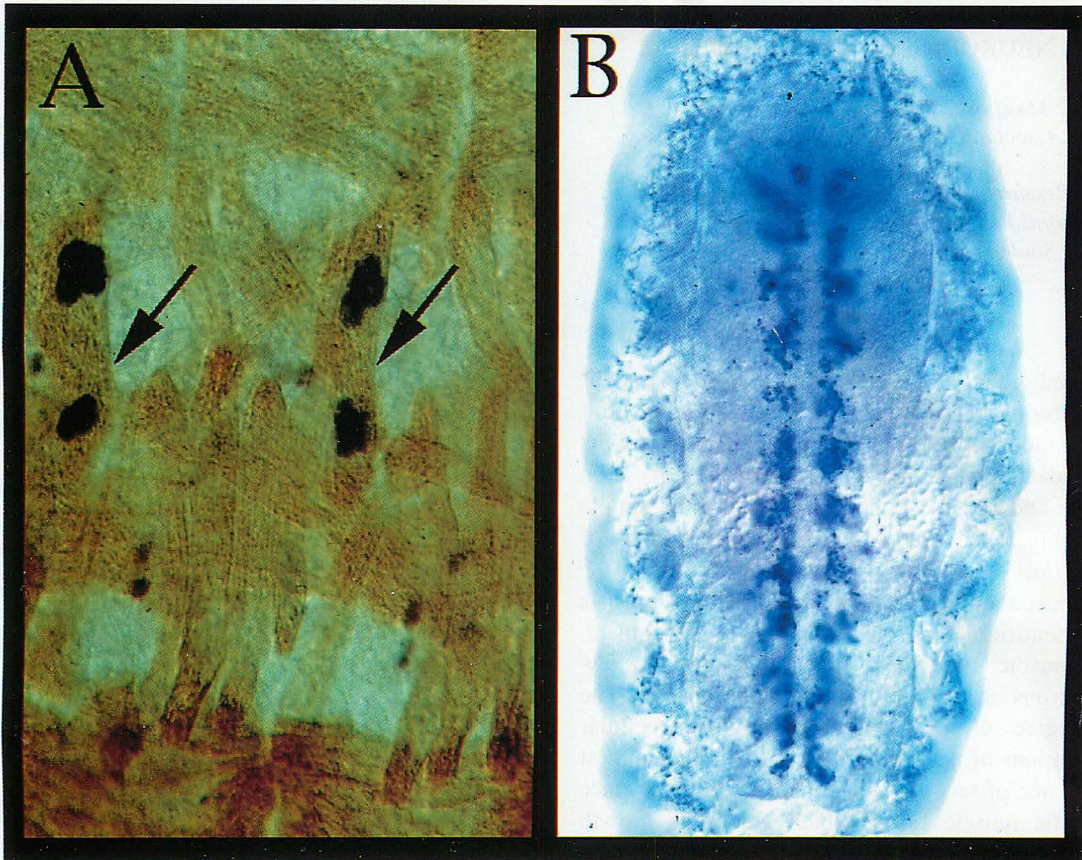

Fig. 1. Expression of the members of AN34/F-spondin family in *Drosophila*.
(A) AN34 enhancer trap line express ß-galactosidase (ß-gal) in the nuclei of single muscle fiber #18 (arrows) in each hemisegment. Anti ß-gal antibody staining in blue and general muscle staining (as visualized with a monoclonal antibody 27D) in brown.
(B) #8 *in situ* hybridization. #8 mRNA is expressed in a small subset of glia along the longitudinal connectives.

They thus constitute a novel gene family of secreted protein with potential roles in neural adhesion.

One of the newly identified genes in *Drosophila* (tentatively called #3) is expressed in a subset of hemocytes, secretory cells of the extracellular matrix (ECM) proteins. Another (called #8) is expressed in a subset of glia that sit along the longitudinal axon tracts in the CNS (Fig.1B).

The specificity of their expression suggest that they might be involved in the guidance of specific axons. We are currently trying to isolate the loss-of-function mutants of these three genes (AN34, #3, #8) as well as the transgenic flies that ectopically express these molecules (as described for Connectin) to study their roles in neural development.

IV. *rH96*, a homeobox containing gene essential for neural and muscular development

Another line rH96 is expressed in the precursors of dorsal muscles. It is also expressed in a subset of neuroblasts and glioblasts in the CNS. In particular, it is expressed in the longitudinal glioblasts (LGB), precursor cells for the longitudinal glia whose lineage is well characterized. rH96 was shown to encode a putative transcription factor with a homeobox. Analysis of the loss-of-function mutants revealed severe defects in the formation of muscles and the CNS. The dorsal muscles that normally express the molecule were often missing totally or failed to form properly. The overall structure of the CNS axon tract was also seriously disrupted. Close examination of the development of the LGB leneage using specific markers showed that the glioblasts fail to divide and migrate properly in the mutants. These results suggested that rH96 play essential roles in the specification and/or differentiation of precursors for neurons, glia and muscles. We are currently trying to ectopically express rH96 to characterize more in detail the function of rH96 in the development of muscles and the nervous system.

Publication List:

Kimura, Y., Matsunami, H., Inoue, T., Shimamura, K., Uchida, N., Ueno, T., Miyazaki, T. and Takeichi, M. (1995) Cadherin-11 expressed in association with mesenchymal morphogenesis in the head, somite, and limb bud of early mouse embryos. *Develop. Biol.* **169**, 347-358.

Matsunami, H. and Takeichi, M. (1995) Fetal brain subdivisions defined by R- and E-cadherin

expressions: evidence for the role of cadherin activity in region-specific, cell-cell adhesion. *Develop. Biol.* **172**, 466-478.

Nakagawa, S. and Takeichi, M. (1995) Neural crest cell-cell adhesion controlled by sequential and subpopulation-specific expression of novel cadherins. *Development* **121**, 1321-1332.

Overduin, M., Harvey, T.S., Bagby, S., Tong, K.I., Yau, P., Takeichi, M. and Ikura, M. (1995) Three-dimensional solution structure and calcium interaction of the epithelial cadherin domain responsible for selective adhesion. *Science* **267**, 386-389.

Shibamoto, S., Hayakawa, M., Takeuchi, K., Hori, T., Miyazawa, K., Kitamura, N., Johnson, K.R., Wheelock, M.J., Matsuyoshi, N., Takeichi, M. and Ito, F. (1995) Association of p120, a tyrosine kinase substrate, with E-cadherin/catenin complexes. *J. Cell Biol.* **128**, 949-957.

Shibuya, Y., Mizoguchi, A., Takeichi, M., Shimada, K. and Ide, C. (1995) Localization of N-cadherin in the normal and regenerating nerve fibers of the chicken peripheral nervous system. *Neuroscience* **67**, 253-261.

Shiozaki, H., Kadowaki, T., Doki, Y., Inoue, M., Tamura, S., Oka, H., Iwazawa, T., Matsui, S., Shimaya, K., Takeichi, M. and Mori, T. (1995) Effect of epidermal growth factor on cadherin-mediated adhesion in a human oesophageal cancer cell line. *British J. Cancer* **71**, 250-258.

Takeichi, M. (1995) Morphogenetic roles of classic cadherins. *Current Opinion Cell Biol.* **7**, 619-627.

LABORATORY OF GENE EXPRESSION AND REGULATION

Head: Yoshitaka Nagahama

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

DIVISION OF GENE EXPRESSION AND REGULATION I

Closed during 1995 and will be reinitiated in 1996 on new projects.

Publication List: (From the previous projects)

Ito, T., Hirano, Y., Shimura, Y. and Okada, K. (1995) Two touch-inducible calmodulin and calmodulin-related genes tandemly located on chromosome of *Arabidopsis thaliana*. *Plant Cell Physiol.* **36**, 1369-1373.

Mikami, K., Katsura, M., Okada, K., Shimura, Y. and Iwabuchi, M. (1995) Developmental and tissue-specific regulation of the gene for the wheat basic/leucine zipper protein HBP-1a(17) in transgenic *Arabidopsis* plants. *Mol. Gen. Genet.* **248,** 573-582.

Okada, K., Ito, T., Sawa, S., Yano, A., Ishiguro, S. and Shimura, Y. (1995) Mutational analysis of flower development in *Arabidopsis thaliana*. *In* "Modification of gene expression and non-Mendelian inheritance" (eds. Oono, K. and Takaiwa, H.) NIAR Japan, pp. 145-156.

DIVISION OF GENE EXPRESSION AND REGULATION II

Professor: Takashi Horiuchi
Research Associate: Masumi Hidaka
Takehiko Kobayashi
Ken-ichi Kodama
Institute Research Fellow: Katsuki Johzuka
Graduate Student: Katsufumi Ohsumi
Keiko Taki
Technical Staff: Kohji Hayashi
Yasushi Takeuchi

Homologous recombination may occur in all organisms. While related functions apparently involve exchange between two parent-derived chromatids and repair of DNA damage incurred by physical and chemical reagents, many questions remain unanswered. As deduced from our analyses of recombinational hotspots of *E. coli* & *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.

I. Analysis of recombinational hot spot in *S. cerevisiae*

HOT1 is a mitotic recombinational hotspot in the yeast *S. cerevisiae* and was first identified by Keil and Roeder. HOT1 stimulates both intra- and inter-chromosomal recombination, and for a precise analysis enhancement of excisional recombination between directly repeated DNAs at its nearby site was investigated. HOT1 was originally cloned on a 4.6 kb BglII fragment which locates in rRNA repeated genes (about 140 copies) on chromosome XII. A single rRNA unit consists of two transcribed 35S and 5S rRNA genes and two non-transcribed regions, NTS1 and NTS2, the former is between 3'-ends of 35S and 5S rRNA genes, and the latter is 5' ends of these two genes. The HOT1 DNA fragment contains the NTS1, 5SRNA gene and NTS2 region but it was later found to be composed of two non-contiguous cis-elements, E and I, located in NTS1 and NTS2, respectively. Because E and I positionally and functionally overlapped with the enhancer and initiator of the 35S rRNA transcription, respectively, Roeder's group suggested that transcription by RNA polymerase I, initiated at the 35S rRNA promoter site may stimulate recombination of the downstream region, thereby revealing Hot1 activity.

The NTS1 has a site at which the replication fork is blocked and we termed this site SOG, but later called RFB (replication fork block). By assaying Rfb activity for various DNA fragments derived from the NTS1 and cloned on plasmids, we determined the minimal region, about 100 bp long, located near the enhancer region of the 35S rRNA transcription and essential for blocking replication fork advancing in a direction opposite that for transcription. The RFB sequence has no homology to any other known sequence and has no characteristic structure such 2-fold symmetry, repeated structure, etc.; hence, *trans*-factor(s) may have a role in blocking the fork. Interestingly, this region is included in one of two *cis*-elements required for a recombinational hotspot, Hot1, activity.

To investigate functional relationships between the fork blocking activity in RFB and the hotspot activity in HOT1, we first isolated mutants defective in Hot1 activity and examined whether these mutations would also affect Rfb activity. Using a colony color sectoring assay method, we isolated 23 Hot defective mutants from approximately 40,000 mutagenized colonies. Among these Hot- mutants, one proved to be a *rad52* mutant; the other 4 mutants lose fork blocking activity (Fig. 1).

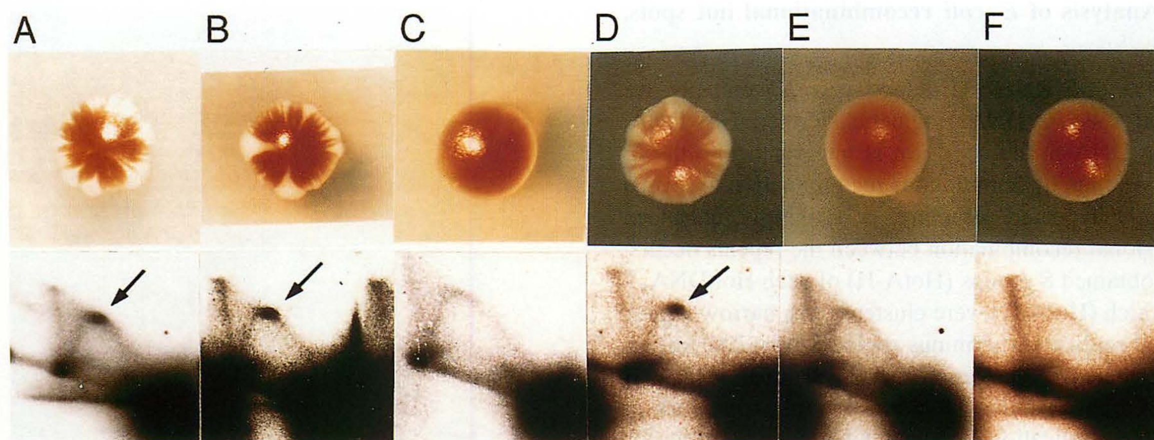

Fig. 1. Correlated relationship between Hot1 and Rfb activities of various yeast strain. Upper panels are colonies and lower panels are the 2D gel analysis pattern. In the upper panels the sectored and the uniform red colonies show Hot1 active and inactive phenotype, respectively. In the lower panels presence (indicated by arrow) and absence of spot on the arc in 2D agarose gel electrophoretic pattern show Rfb active and inactive property, respectively. A; diploid (*FOB1/fob1-4*), B; wild haploid (*FOB1*), C; *fob1-4* mutant, D; *fob1-4* carrying a plasmid with the *FOB1* gene, E; disruptant of *fob1* gene (*fob1::LEU2*), F; diploid (*fob1-4/fob1::LEU2*).

Genetic analysis of these mutants revealed that all four were recessive for the Rfb phenotype and defined one complementation group. This mutation was designated *fob1* (fork blocking function) and one of the *fob1* mutants, *fob1-4*, was further analyzed. First, from yeast cDNA bank, we cloned *FOB1* gene by selecting a DNA fragment which had suppressive activity for Hot deficiency of the mutant. The minimal *FOB1* plasmid was shown to complement both the Hot$^-$ and Rfb$^-$ phenotype of the *fob1-4* mutant, suggesting that both phenotypes are caused by a mutation in the *FOB1* gene (Fig. 1). DNA sequencing of the *FOB1* gene revealed that the putative Fob1 protein consists of 566 amino acids and has a molecular mass of 65,000 daltons. We have overproduced and purified a protein with this molecular weight and the expected amino acid sequence of N-terminus of Fob1 protein. We are testing whether the Fob1 protein has binding activity to RFB sequence or not. We found the same sequence as that of the *FOB1* gene in sequence of the chromosome IV cosmid 9727. Thus, the *FOB1* gene is apparently not linked with the rRNA gene cluster present in chromosome XII. Sequencing of the *fob1-4* mutant gene revealed two mutational changes in the open reading frame, one is non-sense (amber) and other is a miss-sense mutation. The amber mutation may account for the two defective phenotypes of the *fob1-4* mutant and why it is non-leakyness.

Other workers showed that transcription of the rRNA gene is involved in the Hot1 activity, while the transcription appears not to be responsible for the Rfb activity. Our finding indicates that fork blocking event is required for Hot1 activity and seems not to be responsible for rRNA transcription. Thus, in yeast, both two independent events, one is fork blocking at RFB site and other is transcription of the rRNA gene, are required for activation of Hot1.

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

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

To know how to activate other termination event-independent Hots, especially HotG, D and H, DNA sequences of these Hot fragments were determined and found that all Hot DNAs contained Chi sequence, thereby suggesting that RecBCD enzyme may be involved in these Hot activation such as in HotA. We now examine what event occurs near the Hot DNA sites, which probably provides an entrance site for RecBCD enzyme.

Publication List:

Horiuchi, T. and Fujimura, Y. (1995) Recombinational rescue of the stalled DNA replication fork: a model based on analysis of an *Escherichia coli* strain with a chromosome region difficult to replicate. *J. Bacteriol.* **177**, 783-791.

Horiuchi, T., Nishitani, H. and Kobayashi, T. (1995) A new type of *E. coli* recombinational hotspot which requires for activity both DNA replication termination events and the Chi sequence. *In* "Molecular Mechanism of Genetic Recombination". *Advance Biophysics* vol. **31,** 133-147.

DIVISION OF SPECIATION MECHANISMS I

Professor: Tetsuo Yamamori
Research Associates: Satoshi Koike
Yuriko Komine
Technical Staffs: Hideko Utsumi

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

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

Recently, it has been recognized that cytokines, defined as intercellular mediators in the immune system, have a variety of roles in the nervous system as well. One such a factor, LIF (leukemia inhibitory factor) known also as CDF (Cholinergic Differentiation Factor), is a pleiotropic factor which shows a remarkable repertoire of activities from embryonic stem cells to neurons (Yamamori, T. In Chemical Factors in Neuronal Growth, Degeneration and Regeneration (Ed. by C. Bell), Elsevier, in press). Recent study have revealed that CDF/LIF and its receptors belong to the IL-6 family and the receptor family.

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

II. Gene expression and cerebellar long-term plasticity

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

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

Recently, we have examined Jun-B expression *in vivo* under a conjunctive protocol of AMPA, a pharmacological substitute for parallel fiber stimulation, and climbing fiber stimulation via electric stimulation of Inferior Olive. 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.

We are currently working to identify the molecules that are induced after Jun-B induction and playing roles in cerebellar long-term plasticity.

Publication List:

Yamamori, T. Mikawa, S. and Kado, R. (1995) Jun-B expression in Purkinje cells by conjunctive stimulation of climbing fibre and AMPA. *NeuroReport* **6**, 793-796.

Division of Speciation Mechanisms II

The Division will be initiated in 1996.

RESEARCH SUPPORT

TECHNOLOGY DEPARTMENT

Head:Hiroyuki Hattori

Common Facility Group
Chief:Kazuhiko Furukawa

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

Radioisotope Facility
Yousuke Kato(Subunit Chief)
Yoshimi Matsuda

Center for Analytical Instruments
Akio Murakami(Unit Chief)
Sonoko Ohsawa
Yukiko Kabeya
Yumiko Makino
Takeshi Mizutani

Glassware Washing Facility
(Kazuhiko Furukawa)
(Toshiki Ohkawa)

Research Support Group

Cell Biology Group
Maki Kondo
Katsushi Yamaguchi

Developmental Biology Group
Hiroko Kobayashi(Unit Chief)
Sachiko Fukada
Chikako Inoue
Chiyo Takagi

Regulation Biology Group
Shoichi Higashi
Miki Ida
Akiko Kawai
Shigemi Ohsugi

Gene Expression and Regulation Group
Koji Hayashi
Tomoko Mori
Yasushi Takeuchi
Hideko Utsumi

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

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

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

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

RESEARCH SUPPORT FACILITY

Head of Facility: Mikio Nishimura
Associate Professor: Masakatsu Watanabe
Research Associates: Yoshio Hamada
(Tissue and Cell Culture)
Kenta Nakai (Computer ;-April 30, 1995)
Technical Staff: Mamoru Kubota
Chieko Nanba
Toshiki Ohkawa
Kaoru Sawada
Tomoki Miwa
Hideko Nonaka
Kimiko Yamamiya (-March 31, 1995)
Masayo Iwaki (June 1, 1995-)

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

I. Facilities

1. *The Large Spectrograph Laboratory*

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

A tunable two-wavelength CW laser irradiation system is also available as a complementary light source to OLS to be used in irradiation experiments which specifically require ultra-high fluence rates as well as ultra-high spectral-, time-and spatial-resolutions. It is composed of a high-power Ar-ion laser (Coherent, Innova 20) (336.6-528.7 nm, 20 W output), two CW dye lasers (Coherent, CR599-01) (420-930 nm, 250-1000 mW output),

A/O modulators (up to 40 MHz) to chop the laser beam, a beam expander, and a tracking microbeam irradiator (up to 200$\mu m s^{-1}$ in tracking speed, down to 2μm in beam diameter) with an infrared phase-contrast observation system.

2. *Tissue and Cell Culture Laboratory*

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

3. *Computer Laboratory*

To meet various computational needs and to provide means of electronic communication in this Institute, many kind of computers are equipped: VAX/VMS machines, UNIX engineering workstations (SPARCstations, NEWS machines, DECstations), and some personal computers (Macintosh's and Windows machines). All of these machines are connected each other through the Ethernet, the Fast Ethernet, or the CDDI, which are also linked by optical fibers to the high performance multimedia backbone network of Okazaki National Research Institutes. Since this backbone network, called ORION, is joined to the Internet, the users of these machines can access various services and databases on the Internet. Each laboratory has several computers that are also connected to the network of the Institute. The Computer Laboratory provides various computational services to the Institute members: file servers for Macintosh and NetWare users, some print servers that accept printing requests from PC and UNIX users, a computational server that provides sequence analyses and database retrievals, communication servers to the Internet, and so on. The laboratory also provides an information dispatching service to the Internet using the World Wide Web (URL is http://www.nibb.ac.jp).

4. *Plant Culture Laboratory*

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

5. *Experimental Farm*

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

6. *Plant Cell Laboratory*

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

7. *Laboratory of Stress-Resistant Plants*

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

University (30 km from the National Institute for Basic Biology). The laboratory provides a variety of growth chambers that precisely control the conditions of plant growth and facilities for molecular biological, and physiological evaluations of transgenic plants. The laboratory is also a base of domestic and international collaborations devoted to the topic of stress-resistant transgenic plants.

II. Research activities

1. *Faculty*

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

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

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

(3) Computational Biology: Efforts to develop new methodology for sequence analysis have been continued. A system to predict protein localization sites from the sequences had been developed and is now open to the public through the Internet (http://psort.nibb.ac.jp). Sequence motifs are thought to be good indices of biological functions. Methodology of protein sequence analysis based on sequence motifs has been studied and is implemented as a computer program. The product of computer assistance for sequence analysis is being constructed using the World Wide Web.

Many kind of databases become available in recent years, but almost all of them are those of molecular information such as nucleic acids or amino acids sequences. It is also desired to construct a database for biological functions like networks of the regulation of gene expression. As a model case, Bacillus subtilis genome database is constructed to represent the categorized classification of gene products in collaboration with Dr. N. Ogasawara (NAIST) and Human Genome Center, University of Tokyo. Other kind of biological functional databases including image data are planned to represent the accumulated knowledge of development, differentiation, and morphogenesis.

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

Erata, M., Kubota, M., Takahashi, T., Inouye, I. and Watanabe, M. (1995) Ultrastructure and phototactic action spectra of two genera of cryptophyte flagellate algae, *Cryptomonas* and *Chroomonas*. *Protoplasma,* **188**, 258-266.

Higuchi, M., Kiyama, H., Hayakawa, T., Hamada, Y. and Tsujimoto, Y. (1995) Differential expression of Notch1 and Notch 2 in developing and adult mouse brain, *Mol. Brain Research*, **29**, 263-272.

Nakamura, S., Kawanishi, E., Nakamura, S., Watanabe, M., and Kojima, M. K. (1995) A new paralyzed flagella mutant, OC-10, in *Chlamydomonas reinhardtii* that can be reactivated with ATP. *Phycol. Res.,* **43**, 65-69.

Watanabe, M. (1995) Action spectroscopy : photomovement and photomorphogenesis spectra. *In* "CRC Handbook of Organic Photochemistry and Photobiology", (Edited by B. Horspool and P.-S. Song), CRC Press, Boca Raton, pp. 1276-1288.

II. Cooperative Research Program for the Okazaki Large Spectrograph

Erata, M., Kubota, M., Takahashi, T., Inouye, I. and Watanabe, M. (1995) Ultrastructure and phototactic action spectra of two genera of cryptophyte flagellate algae, *Cryptomonas* and *Chroomonas*. *Protoplasma*, **188**, 258-266.

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RADIOISOTOPE FACILITY
(Managed by NIBB)

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

This Facility consists of a main center where a variety of radioisotopes such as ^{22}Na, ^{125}I, ^{32}P, ^{3}H and ^{14}C are handled as well as various species of γ-ray emitting nuclides and two subcenters, one placed in the NIBB and the other in the NIPS. At the subcenters, only a limited variety of radioisotopes such as ^{35}S and ^{32}P are processed. The members of the Radioisotope Facility are engaged in maintaining and controlling both the center and subcenters, and provide users an appropriate guidance for radioisotope handling.

In 1995, the new system for the going in and out the controlled area was introduced. ID card has been issued to each registered individual and non-registered persons are allowed to enter the controlled area. In this system, when he or she goes in and out the controlled area, his or her names, times, and places are automatically recorded by a computer. Furthermore, the renewal of the radiation monitoring system and the radioactive contamination assessment system in this Facility has been supported by the secondary supplementary budget of the Ministry of Education, Science and Culture and is going on until the end of Mach, 1996. In these refreshed systems, the radioactivities of air, and draining in the controlled area of the three places will be monitored by a computer for 24 hours. The third subcenter of this Facility will open in the next spring (1997) in the building of the Laboratory of Gene Expression and Regulation that is now under construction.

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

The native dyneins are very large. They range in molecular mass up to 1 to $2x10^6$ daltons and they are complex proteins. Each dynein contains two or three heavy chains (HCs) with ATPase activity, which range in molecular mass up to 500 kDa. The motor activity of dynein is associated with these chains. Some functional differences have been reported between HCs of outer-arm dynein. Sea urchin outer-arm dynein is a heterodimer of HCs (α and β) and at least the β-HC is able to induce gliding of microtubules in vitro. The α-HC might amplify the function of β-HC and it has been reported to have no motile activity. After the first cloning of β-HC from sea urchin ciliary axonemes (Gibbons et al., 1991; Ogawa, 1991), the sequences of HCs of axonemal and cytoplasmic dyneins from a variety of organisms were determined in their entirety. Without exception, all the HCs cloned to date contain four P-loop (ATP-binding) sequences in the midregion of the molecule. Thus, they can be classified as a four P-loop family.

The outer-arm dyneins contain two or three proteins that range in molecular mass from 70 to 120 kDa and copurify with HCs. ICs of sea urchin outer-arm dynein are abbreviated as IC1, IC2, and IC3. Those of *Chlamydomonas* are called IC78 and IC69, and ICs of cytoplasmic dynein are called IC74. *Chlamydomonas* IC78 and IC69 were cloned by Wilkerson et al. (1995) and Mitchell and Kang (1991),respectively. The sequences of sea urchin IC2 and IC3 were determined by Ogawa et al. (1995).

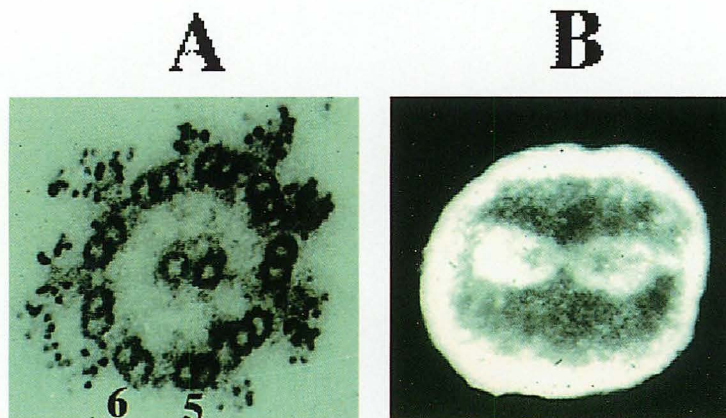

Fig. 1. Localization of dynein in sea urchin sperm axoneme (A) and a cleaving egg (B).

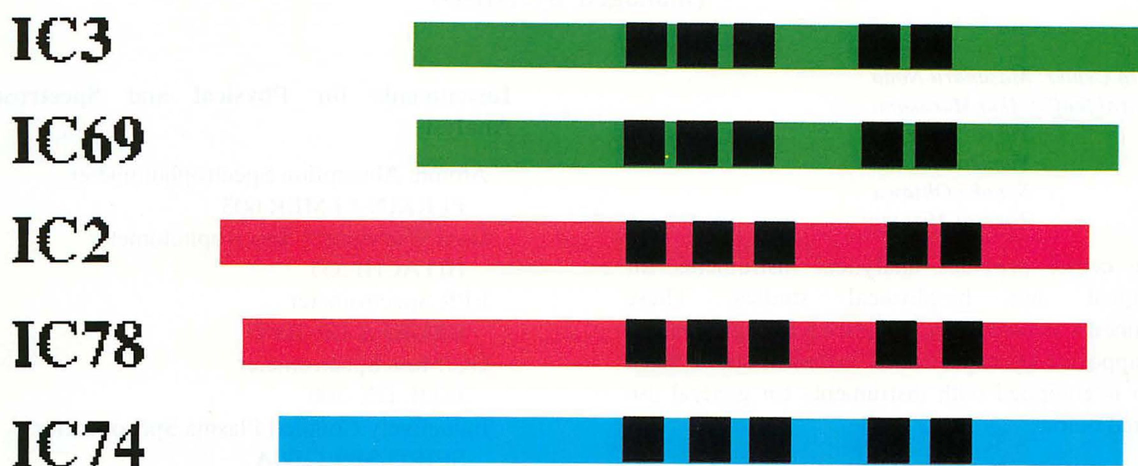

Fig .2. Relative positions of WD repeats (filled boxes) in sea urchin outer-arm dynein IC2 and IC3, *Chlamydomonas* outer-arm dynein IC78 and IC69, and rat cytoplasmic dynein IC74. Colours mean subclass of ICs.

IC1 has recently been cloned (Ogawa, accession number D63884). Rat brain IC74 was cloned by Paschal et al. (1992). Thus, all the ICs found in the axonemal and cytoplasmic dyneins of the model organisms used for studies of dynein function have been completely sequenced. Comparison of amino acid sequences of IC2 and IC3 with those of IC78 and IC69 and with that of IC74 showed that, although all five ICs are homologous, IC2 is much more closely related to IC78, and IC3 is much more closely related to IC69, than either sea urchin chain or either *Chlamydomonas* chain is related to each other. Regions of similarity between all five ICs are limited to the carboxy-terminal halves of the molecules. Similarity are due primarily to conservation of the WD repeats in all of these chains. The WD repeats are involved in protein-protein interactions in a large family of regulatory molecules (Neer et al., 1994). The relative positions of WD repeats in these chains are shown schematically in Fig. 2.

A parsimony tree for these chains (Ogawa et al., 1995) shows that, although the carboxy-terminal halves of all of these chains contain WD repeats, the chains can be divided into three distinct subclasses (IC3 plus IC69, IC2 plus IC78, and IC78). By contrast, sea urchin IC1 is not a member of the WD family. Sequence analysis showed that IC1 consists of a thioredoxin-like (TRXL) sequence and three nucleoside diphosphate kinase-like (NDKL) sequences. IC1 might play a dynamic role in flagellar bending and/or wave propagation.

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CENTER FOR ANALYTICAL INSTRUMENTS (managed by NIBB)

Head of Center: Masaharu Noda
Technical Staffs: Akio Murakami
Yukiko Kabeya
Yumiko Makino
Sonoko Ohsawa
Takeshi Mizutani

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

Instruments for Protein and Gene Analysis

Amino Acid Analyzer
HITACHI 835
Automatic Plasmid Isolation System
KURABO PI-100Σ
DNA Sequencers
ABI 370A, 373A-36
DNA/RNA Synthesizers
ABI 381A, 392
Nucleic Acid Extractor
ABI 340A
Peptide Synthesizers
ABI 430A, 431A, 432A
Protein Sequencers
ABI 470A, 473A, 492

Instruments for Chemical Analysis

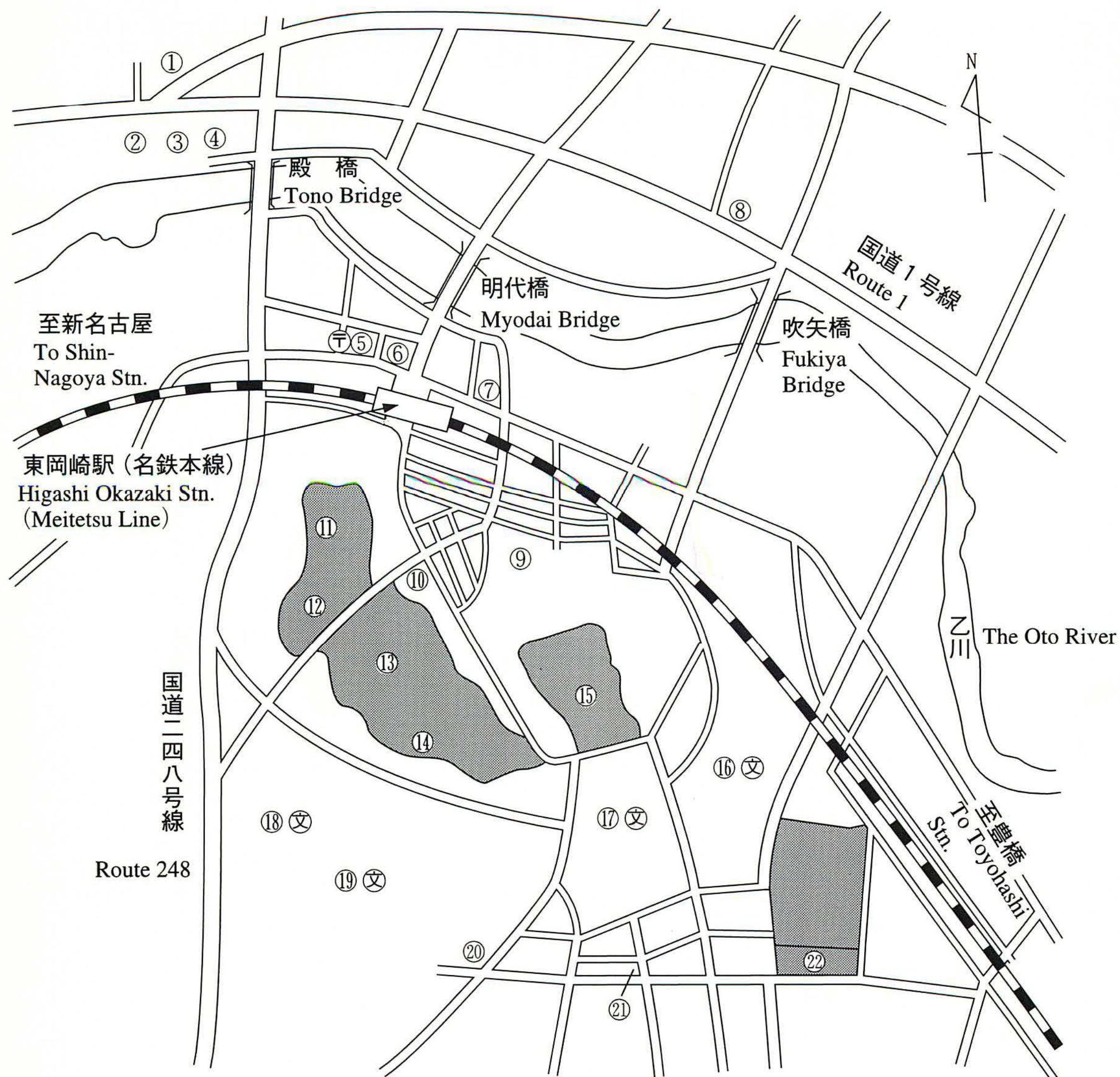
Capillary Electrophoresis
ABI 270A
Gas Chromatographs
SHIMADZU GC-7APTF, GC-14APFSC
Glycoprotein Analysis System
TAKARA Glyco-Tag
High Performance Liquid Chromatographs
SHIMADZU LC-10AD, 6AD
Preparative Electrophoresis System
ABI 230A
Preparative Ultracentrifuges
BECKMAN L8-80, L5-75
Table-top Ultracentrifuges
BECKMAN TL-100
Micro Preparative System
PHARMACIA SMART System

Instruments for Physical and Spectroscopic Analysis

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

Instruments for Microscopic and Image Analysis

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



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Meitetsu Okazaki Hotel

② 岡崎城
Okazaki Castle

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

④ 岡崎グランドホテル
Okazaki Grand Hotel

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）
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National Institute for Basic Biology

⑫ 生理学研究所
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