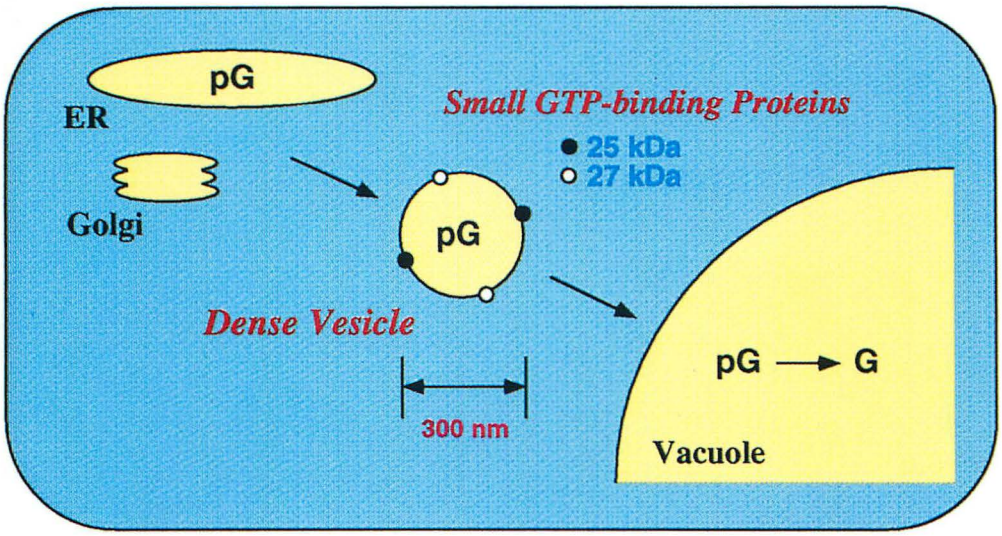
NATIONAL INSTITUTE FOR BASIC BIOLOGY

岡崎国立共同研究機構

基　礎　生　物　学　研　究　所



ANNUAL REPORT

1994

INTRODUCTION

The National Institute for Basic Biology (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 on its own, and by cooperating with other universities and research organizations. The Institute concentrates on the studies of cellular functions, growth and development, homeostatic control, and gene expression and regulation in eukaryotic organisms, to elucidate the fundamental mechanisms underlying various living phenomena at the molecular level.

That the Institute has been active in science was evidenced by the fact that citation indices of papers published in 1981–1991 by the NIBB was highest among Japanese universities and institutes and comparable to those of leading universities in the United States (*Science*, **258**, 562). We are justly proud of this and would like to maintain a high level of research activity at the Institute.

A new professor joined the NIBB in the past year. Prof. Tetsuo Yamamori (former Sub-Teamleader of the Riken) filled the position of Professor of Speciation Mechanisms. His research is aimed at an understanding of the molecular mechanisms for the evolution of information processing by the nervous system. During the past year, the Institute continued to be active in exchange of personnel: we newly appointed 9 research associates from other universities, while three research associates were promoted to associate professors at other universities.

The NIBB is an inter-university research institute and plays many roles as a national and international center for the study of biology. The Institute is responsible for conducting research projects in cooperation with research groups in different universities and institutes. As a part of such cooperative activities, the NIBB carries out Special Programs which are currently directed to "Adaptation and Resistance to Environment" and "Transdifferentiation of Tissue Cells". Based on such programs, the NIBB held the 32nd and 33rd Conferences in 1994, entitled "Stress-induced genes of plants with emphasis on their functions" (organized by Prof. Murata) and "Approaches to the cellular and molecular mechanisms of regeneration" (organized by Prof. 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. The NIBB also shares its research resources to make them available to biologists both from Japan and around the world. Through such activities, the NIBB is and will continue to be a national and international center to promote basic research in the biological sciences.

This report describes an outline of recent research activities of the NIBB and contains a list of papers published by the members of the Institute in 1994.

Ikuo Takeuchi, Ph.D. & 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 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 and Culture.

Research

The Institute conducts its research programs through three departments and one laboratory organized into 16 divisions.

	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

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 staffs.

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 cover 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.

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

Publication List:

Saran, S., Nakao, H., Tasaka, M., Iida, H., Tsuji, F. I., Nanjundiah, V. and Takeuchi, I. (1994) Intracellular free calcium level and its response to cAMP stimulation in developing *Dictyostelium* cells transformed with jellyfish apoaequorin cDNA. *FEBS Letters* **337**, 43–47.

Araki, T., Nakao, H., Takeuchi, I. and Maeda, Y. (1994) Cell-cycle-dependent sorting in the development of *Dictyostelium* cells. *Develop. Biol.* **162**, 221–228.

Traynor, D., Tasaka, M., Takeuchi, I. and Williams, J. (1994) Aberrant pattern formation in myosin heavy chain mutants of *Dictyostelium*. *Development* **120**, 591–601.

Morio, T., Takeuchi, I. and Tasaka, M. (1994) Cooperation of positively and negatively acting promoter elements determines prespore-specific transcription of Dp87 gene in *Dictyostelium*. *Mechanisms of Development* **45**, 59–72.

Nakao, H., Yamamoto, A., Takeuchi, I. and Tasaka, M. (1994) *Dictyostelium* prespore-specific gene Dp87 encodes a sorus matrix protein. *J. Cell Sci.* **107**, 397–403.

Takeuchi, I., Tasaka, M., Okamoto, K. and Maeda, Y. (1994) Regulation of cell differentiation and pattern formation in *Dictyostelium* development. *Int. J. Dev. Biol.* **38**, 311–319.

DEPARTMENT OF CELL BIOLOGY

Chairman: Yoshihiko Fujita

The 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: Kazuo Ogawa
Makoto Hayashi
Ikuko Hara-Nishimura
Graduate Students: Kaori Inoue
Akira Kato
Tomoo Shimada
Tetsu Kinoshita
Nagako Hiraiwa
Masahiro Aoki[1)]
Technical Staff: Maki Kondo
Katsushi Yamaguchi
Yasuko Koumoto
Visiting Scientists: Kyoko Hatano[2)]
Gerhard Bytof[3)]

([1)]from Shinshu University)
([2)]from Kyoto University)
([3)]from Technischen Universitat Braunschweig, Germany)

The cells of higher plants cells contain several distinct organelles that play vital roles in cellular physiology. During the proliferation and differentiation of these cells, the organelles often undergo dynamic changes. The biogenesis of new organelles may occur, existing organelles may undergo a change in function, which other organelles may degenerate. The dynamic transformation of organellar functions (differentiation of organelles) is responsible for the flexibility of differentiation events in higher plant cells. Therefore, the efforts of this division are focussed on the elucidation of regulatory mechanisms that underlie such transformation.

I. Development of microbody membrane proteins during the microbody transition.

Dramatic metabolic changes that underlie the shift from heterotrophic to autotrophic growth occur during the greening associated with the germination of seeds. As these metabolic changes take place, many constitutive organelles are functionally transformed. For example, etioplasts differentiate into chloroplasts and mitochondria acquire the ability to oxidize glycine. Glyoxysomes, which are microbodies engaged in the degradation of reserve oil via β-oxidation and the glyoxylate cycle, are transformed into the leaf peroxisomes that function at several crucial steps in photorespiration. After the functional transition of glyoxysomes to leaf peroxisomes during the greening of pumpkin cotyledons, the reverse transition of leaf peroxisomes to glyoxysomes occurs during senescence. As part of our attempts to clarify the molecular mechanisms that underlie the microbody transition, that changes with development in microbody membrane proteins during the transformation of glyoxysomes to leaf peroxisomes were characterized. Two proteins in glyoxysome membranes, with molecular masses of 31 kDa and 28 kDa respectively, were purified and polyclonal antibodies were raised against the each protein. Analysis of these membrane proteins during development revealed that the amounts of these proteins decreased during the microbody transition. The larger one was retained in leaf peroxisomes, whereas the small one could not be found in leaf peroxisomes after completion of the microbody transition. The results clearly showed that membrane proteins in glyoxysomes change dramatically during the microbody transition, as do the enzymes in the matrix.

II. Characterization of the aconitase that participates in the glyoxylate cycle.

Aconitase catalyzes the reversible interconversion of citrate, isocitrate and *cis*-aconitate. This enzyme is thought to function as a component of the glyoxylate cycle in glyoxysomes, as well as the Krebs cycle in mitochondria. It was reported, however, that less than 0.5% of

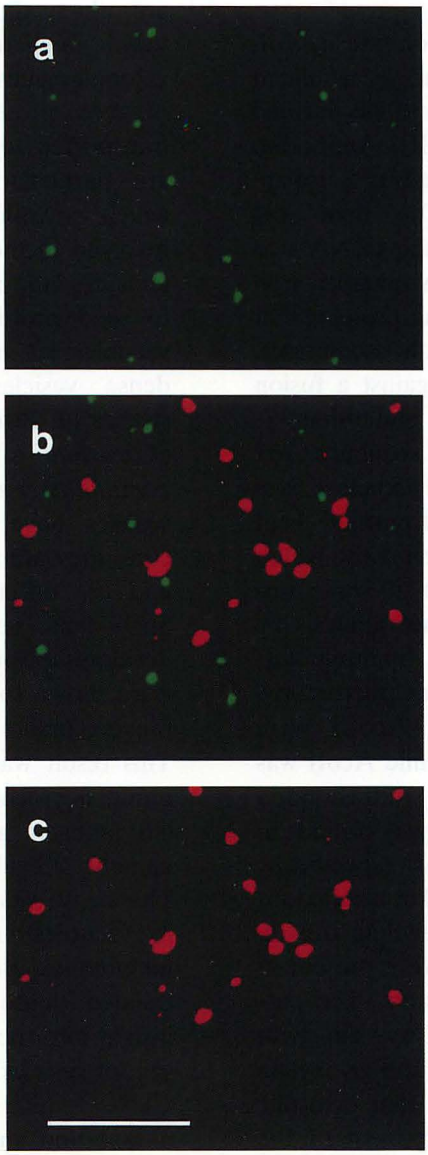


Fig. 1. Localization of aconitase and isocitrate lyase within a cell from a pumpkin cotyledon, as analyzed by double immunofluorescence staining. Pumpkin seedlings were grown for 5 days in darkness. A thin section of a cotyledon was double-stained first with aconitase-specific antibodies which were visualized by use of FITC-conjugated second antibody, and then with isocitrate lyase-specific antibodies that were visualized by use of rhodamine-conjugated second antibody. (a) Immunofluorescent image due to FITC. (b) Immunofluorescent images in a and c are superimposed. (c) Immunofluorescent image due to rhodamine of the same field as in (a). Bar in panel c indicates 10μm. Magnification in a, b and c is the same.

the total aconitase activity could be recovered in glyoxysomal fractions after sucrose gradient centrifugation, with most of the activity being found in mitochondria and the cytosol. It remained to be confirmed whether mitochondria and glyoxysomes contain different and specific isoenzymes of

aconitase.

Three isoforms of aconitase (AcoI, AcoII, AcoIII) are found in etiolated cotyledons of pumpkin, and we purified two of them (AcoI, AcoII). Antibodies raised against AcoI allowed us to isolate a cDNA for aconitase. The amino acid sequence deduced from the cDNA was very similar to those of mammalian iron responsive element-binding proteins that are also known as cytosolic aconitases. Antibodies were raised against a fusion protein that consisted of glutathione-S-transferase and the partial sequence (162 amino acids) of the cloned aconitase. Not only the antibodies against purified AcoI but also the antibodies against the fusion protein cross-reacted with all three isoforms of aconitase. Further analysis by subcellular fractionation, immunoblotting and immunofluorescene microscopy, revealed that AcoI and AcoIII were localized in the cytosol, while AcoII was localized exclusively in mitochondria (Fig. 1). No aconitase was found in glyoxysomes. AcoI was specifically found in etiolated pumpkin cotyledons during the early stage of seedling growth, while AcoII and AcoIII were present in all tissues of pumpkin plants. The pattern of expression of AcoI was similar to those of other enzymes of the glyoxylate cycle. These data suggest that cytosolic AcoI functions as a component of the glyoxylate cycle despite the fact that all other enzymes of the glyoxylate cycle appear to be localized in glyoxysomes.

III. Mechanisms for vesicular transport of seed proteins.

i) Dense vesicles responsible for the transport of precursors to seed proteins.

Most vacuolar proteins are synthesized on the rough endoplasmic reticulum and are then delivered to vacuoles via vesicle-mediated transport systems. Developing seeds, in which large amounts of seed proteins are synthesized and transported to protein-storage vacuoles, are particularly useful materials with which to explore the cellular machinery involved in the sorting of vacuolar proteins in plant cells. Proprotein precursors to seed proteins are delivered to the vacuoles via unique vesicles, known as dense vesicles. Electron microscopic studies of developing seeds of pumpkin (*Cucurbita* sp.) and castor bean (*Ricinus communis*) have shown that the dense vesicles have a highly electron-dense core; they are about 300 nm in diameter and are different from Golgi vesicles. Such dense vesicles were isolated from developing pumpkin cotyledons and were shown to contain large amounts of the precursors to various seed proteins. This result was supported by results of immunocytochemical analysis with specific antisera against two seed proteins, namely, 2S albumin and 11S globulin. The dense vesicles are directly targeted to vacuoles and fuse to the vacuolar membranes. Biochemical analysis of the isolated dense vesicles should provide insight into the specific targeting and fusion of vesicles to vacuolar membranes.

ii) Isolation and characterization of the dense vesicles; association of small GTP-binding proteins on the membrane of dense vesicles.

To date, a vesicle-mediated transport system through secretory pathway has been investigated exclusively in yeast. Distinct members of the ras superfamily of small GTP-binding proteins facilitate the targeting of vesicles with the appropriate membranes in a GTP-dependent manner to regulate specific steps in the

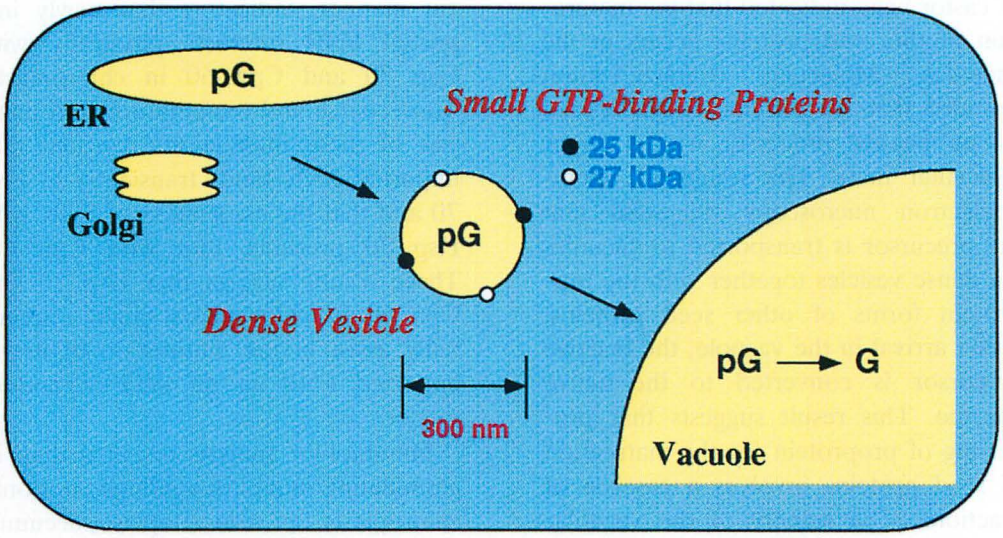


Fig. 2. Dense vesicles mediate the final step in the delivery of seed proteins to vacuoles in developing pumpkin (*Cucurbita* sp.) cotyledons. Two different, small GTP-binding proteins of 25 kDa and 27 kDa might function in the targeting and/or fusion of the vesicles to the vacuolar membranes or in the budding of the vesicles.

secretion pathway in yeast. However, the mechanism of the vesicular transport that is targeted to vacuoles is still obscure.

Isolation of these vesicles allows us to analyze the components responsible for the specific targeting of vesicles to vacuoles and for the fusion between vesicles and vacuoles. To explore the vesicle-mediated transport system that is targeted to vacuoles in plant cells, we isolated dense vesicles and examined them for the presence of guanine nucleotide-binding proteins. GTP-binding proteins of 25 kDa and 27 kDa were detected on the dense vesicles isolated from developing pumpkin cotyledons. The two different, small GTP-binding proteins might function in the targeting and/or fusion of the vesicles to the vacuolar membranes or in the budding of the vesicles (Fig. 2).

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

Proprotein precursors of vacuolar components are transported from the endoplasmic reticulum to the dense vesicles and then they are targeted to the vacuoles. In the vacuoles, the proproteins are processed proteolytically to their mature forms by a unique vacuolar processing enzyme. A vacuolar processing enzyme responsible for the maturation of seed proteins was isolated from castor bean and soybean. The processing enzyme is a novel cysteine proteinase with a molecular mass of 37 kDa (castor bean) or 39 kDa (soybean). The enzyme splits a peptide bond on the C-terminal side of an exposed asparagine residue of a proprotein precursor to produce a mature seed protein such as 11S globulin and 2S albumin. The immunocytochemical localization of the enzyme in the vacuolar matrix of the maturing endosperm

of castor bean indicated that the maturation of the seed proteins occurs in the vacuoles. Molecular characterization revealed that the enzyme is synthesized as an inactive precursor with a larger molecular mass. The results of immunoelectron microscopy suggested that the precursor is transported to vacuoles via dense vesicles together with the proprotein forms of other seed proteins. Upon arrival in the vacuole, the inactive precursor is converted to the active enzyme. This result suggests that processing of proprotein for the maturation of seed proteins involves a cascade of reactions. The activity of the vacuolar processing enzyme was found in various plant tissues and several cDNAs corresponding to homologues of the enzyme were isolated from different plants. Thus, a similar processing enzyme appears to be widely distributed in plant tissues and to play a crucial role in the maturation of a variety of proteins in plant vacuoles.

V. Role of molecular chaperones in the translocation of proteins into chloroplasts.

Molecular chaperones are cellular proteins that function in the folding and assembly into oligomeric structures of certain other polypeptides but that are not, themselves, components of the final oligomeric structure. To clarify the roles of chaperonin (Cpn) and Hsp 70 in the translocation of chloroplast proteins, we examined the interaction of Cpn and Hsp 70 with chloroplast proteins during their import into chloroplasts. Ferredoxin $NADP^+$ reductase (FNR), imported into pea chloroplasts *in vitro*, was immunoprecipitated not only with antisera raised against Hsp 70, but also with against Cpn 60 in an ATP-dependent manner, indicating that newly imported FNR interacts physically with Hsp 70 and Cpn 60 in chloroplasts. Time-course and temperature-shift analysis of these interactions revealed that imported FNR binds transiently to Hsp 70 and that the association of FNR with Hsp 70 precedes that with Cpn 60. These results suggest that Hsp 70 and Cpn 60 in chloroplasts might sequentially assist in the maturation of newly imported FNR in an ATP-dependent matter.

Immunoblot analysis revealed that, in germinating pumpkin seedlings, not only chloroplasts but also etioplasts accumulate heat shock-induced Hsp 70 proteins. Moreover, cDNAs for homologues of Cpn60 and Cpn10 have been isolated and characterized. Further investigations on the function of Cpn10 in the transport of proteins into chloroplasts are in progress.

Publication List:

De Bellis, L., M. Hayashi, I. Hara-Nishimura, A. Alpi and M. Nishimura (1994) Immunological analysis of aconitase in pumpkin cotyledons: The absence of aconitase in glyoxysomes. *Physiol. Plant.* **90**, 757–762.

Kinoshita, T., I. Hara-Nishimura, H. Shiraishi, K. Okada, Y. Shimura and M. Nishimura (1994) Nucleotide sequence of a transmembrane protein (TMP-C) cDNA in *Arabidopsis thaliana. Plant Physiol.* **105**, 1441–1442.

Maeshima, M., I. Hara-Nishimura, Y. Takeuchi and M. Nishimura (1994) Accumulation of vacuolar H^+-pyrophosphatase and H^+-ATPase during reformation of the central vacuole in germinating pumpkin seeds. *Plant Physiol.* **106**, 61–69.

Ozaki, K., A. Terakita, M. Ozaki, R. Hara, T. Hara, I. Hara-Nishimura, H. Mori and M. Nishimura (1994) Molecular characterization and functional expression of squid retinal-binding protein: A novel species of hydrophobic ligand-binding protein. *J. Biol. Chem.* **269**, 3838–3845.

Shigemori, Y., J. Inagaki, H. Mori, M. Nishimura, S. Takahashi and Y. Yamamoto (1994) The presence of the precursor to the nucleus-encoded 30-kDa protein of photosystem II in *Euglena gracilis* Z includes two hydrophobic domains. *Plant Mol. Biol.* **24**, 209–215.

Shimada, T., N. Hiraiwa, M. Nishimura and I. Hara-Nishimura (1994) Vacuolar processing enzyme of soybean that converts proproteins to the corresponding mature forms. *Plant Cell Physiol.* **35**, 713–718.

Shimada, T., M. Nishimura and I. Hara-Nishimura (1994) Small GTP-binding proteins are associated with the vesicles targeting to vacuoles in developing pumpkin cotyledons. *Plant Cell Physiol.* **35**, 995–1001.

Strzalka, K., R. Tsugeki and M. Nishimura (1994) Heat shock induces synthesis of plastid-associated hsp 70 in etiolated and greening pumpkin cotyledons. *Folia Histochem. Cytobiol.* **32**, 45–49.

DIVISION OF BIOENERGETICS

Professor: Yoshihiko Fujita
Associate Professor: Sigeru Itoh
Research Associates: Mamoru Mimuro
Katsunori Aizawa
JSPS-Post-doctral Fellow: Tohru Ikeya
NIBB Visiting Scientist: Sung-Jun Kim[1)]
Technical Staff: Akio Murakami

([1)]from Kyngpook National University)

Primary processes of photosynthesis have been studied. A special attention has been paid for the regulation of PSI/PSII stoichiometry in thylakoid electron transport system (ETS) in response to photosynthetic environments. Biophysical studies of the excitation energy transfer in light-harvesting system and the electron transfer in photosynthetic reaction center complexes have also been carried out.

I. Regulation of PSI/PSII stoichiometry in oxygenic photo-synthesis.

In our previous study, we proposed a hypothesis that (1) stoichiometry between the two photosystems is regulated in response to light regime for photosynthesis so as to maintain the efficiency of light-energy conversion in photosynthesis, and that (2) the stoichiometry is adjusted by regulation of PSI abundance, mainly regulation of PSI formation; redox state of electron component(s), probably cytochrome b_6-f complex, is monitored, and PSI formation is regulated by the signal from monitoring system (cf. Fig. 1). As one of trials for evaluating this hypothesis, the identification of the step to be regulated in PSI formation was carried out. Assay of mRNA activity for synthesis of PsaA/B polypeptides by the *in vitro* translation showed that the mRNA abundance remains constant irrespective of the PSI/PSII regulation while rates of PsaA/B polypeptides sysnthesis varies by a factor of 2. Sensitivity of PsaA/B synthesis to the inhibitors for peptide elongation was different in differently regulated synthesis; the synthesis was less sensitive when the synthetic rate remained at low level under the light mainly exciting PSI, but the sensitivity became high when the synthesis was accelerated under the light mainly for PSII excitation. Results indicated that the apoprotein synthesis is regulated at the step of translation, and further suggest that the translation is suppressed under PSI light and is released from the suppression under PSII light. Since apoprotein synthesis depends on Chl a supply, we also analyzed Chl a biosynthesis. The step before Chl *a* synthetase was not regulated. However, a marked accumulation of Pchlide upon suppression of PSI synthesis suggested a possibility for regulation of Chl *a* supply to each photosystem assembly. We suspect that the step(s) for formation of ribosome-mRNA complex or installation of Chl a into peptides is the target(s) for the regulation.

In the cyanophyte *Synechocystis* PCC 6714, NaCl- and CO_2-stresses for autotrophic growth were found to induce variation of PSI/PSII stoichiometry besides light regime. Again, PSI abundance varied in these cases. Further, the activity of cytochrome *c* oxidase, another terminal of ETS in thyalkoids (Ohki and Fujita (1995) Protoplasma in press), varied simultaneously in the same direction to PSI variation in all cases. Since these stresses affect the steady state of thylakoid ETS in a similar manner to the light regime, we suspect that the mechanism is common among regulations of PSI/PSII stoichiometry in response to three different environmental stresses, and further that cytochrome *c* oxidase activity is also regulated under the same

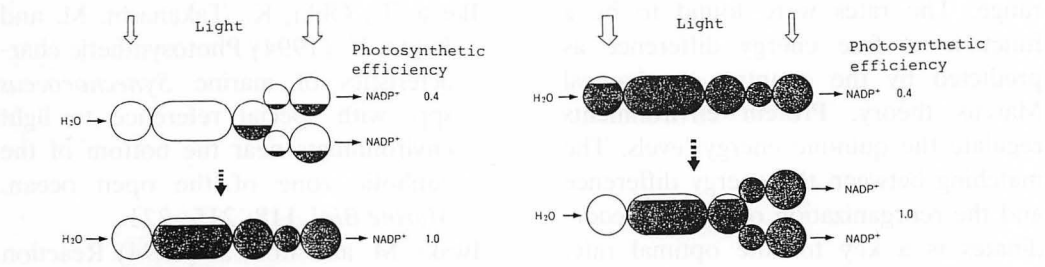


Fig. 1. Schematic presentation of regulation of PSI/PSII stoichiometry and electron transport state. Shaded area in each circle indicates level of reduced state of each component. Reduced PSII and oxidized PSI are photochemically inactive, respectively, and large occurrence of such states causes low efficiency of photosynthesis. PQ, plastoquinone; Cyt b_6-f, cytochrome b_6-f complex; PC, plastocyanin.

mechanism.

II. Energy transfer in light-harvesting pigment system.

Energy transfer processes in photosynthetic pigment systems have been studied using steady-state and time-resolved spectroscopy. Carotenoid-containing pigment systems in algae and chlorosomes of photosynthetic green bacteria were studied. A new method for spectral analysis was also introduced to biological study.

Dynamics of excited state of β-carotene in *n*-hexane were analyzed in subpico second time-range. Decay kinetics of the S_2 state were single-exponential with a lifetime of 195 fs $\pm$ 10 fs, and a dynamic stokes shift was not observed in a 50-fs time range. Spectral analysis was made for peridinine-Chl *a*-protein complex newly isolated from the dinoflagellate *Alexandorium cohorticula.* Magnetic circular dichroism indicated that magnetic mixing of excited states of peridinine, S_1 and S_2, did not occur, and Chl *a* did not interact with other pigments. In the light-harvesting pigment-protein complex II of the siphonous green alga *Bryposis maxima,* siphonein and siphonaxanthin were found to transfer the energy directly to Chl *a.* The feature is the same as the energy transfer from fucoxanthin to Chl *a* in the fucoxanthin-containing pigment protein complex of brown algae, suggesting a general property of the energy transfer in carotenoid-Chl pigment-protein complex.

Based on the *in vitro* self assembly of Bchl *c* and the *in vivo* time-resolved fluorescence polarization spectrum in the ps time-range, a model was proposed for molecular structure of Bchl *c* in chlorosomes.

A new method for spectral analysis, principal multi-component spectral estimation method, was found to valid in application to biological system. The method is advantageous in that the analysis does not require the assumption on number of components and spectral shape.

III. Electron transfer in reaction center complexes.

The study has been focussed on the electron transfer in photosystem I complex. Phylloquinone in PSI complex functions in the electron transfer from Chl *a* to iron-sulfur centers. After replacing phylloquinone with various artificial quinones, rates of the electron transfer from Chl *a* to respective quinones were determined in a pstime-

range. The rates were found to be a function of free energy difference as predicted by the quantum mechanical Marcus theory. Protein environments regulate the quinone energy levels. The matching between the energy difference and the reorganization of reaction coordinates is a key for the optimal rate. Photodamage of PSI *in vivo* was also studied.

Reaction center complexes in the anaerobic green sulfur bacteria were also studied. The highly purified complex from *Chlorobium limicola* consisted of only one kind of polypeptide and forms a "homo-dimer complex". The feature may be characteristic to primitive photosynthetic system in these organisms.

Publication List:

Abe, S., Murakami, A., Ohki, K., Aruga, Y. and Fujita, Y. (1994) Changes in stoichiometry among PSI, PSII and Cyt b_6-f complexes in response to chromatic light for cell growth observed with the red alga *Prophyra yezoensis. Plant Cell Physiol.* **35**:901–906.

Fujita, Y., Murakami, A., Aizawa, K. and Ohki, K. (1994) Short-term and long-term adaptation of the photosynthetic apparatus: Homeostatic properties of thylakoids. *In* The Molecular Biology of Cyanobactria (D.A.Bryant ed.) Kluwer Academic Publishers, Dordrecht, pp.677–692.

Gu, T.-Q., Iwama, Y., Murakami, A., Adhikary, S.P. and Fujita, Y. (1994) Changes in the cytochrome *c* oxidase activity in response to light regime for photosynthesis observed with the cyanophyte *Synechocystis* PCC 6714. *Plant Cell Physiol.* **35**:1135–1140.

Ikeya, T., Ohki, K., Takahashi, M. and Fujita, Y. (1994) Photosynthetic characteristics of marine *Synechococcus* spp. with special reference to light environments near the bottom of the euphotic zone of the open ocean. *Marine Biol.* **118**: 215–221.

Iwaki, M. and Itoh, S. (1994) Reaction of reconstituted acceptor quinone and dynamic equilibration of electron transfer in the photosystem I reaction center. *Plant Cell Physiol.* **35**: 983–993.

Kandori, H., Sasabe, H. and Mimuro, M. (1994) Direct determination of a lifetime of the S_2 state of β-carotene by femtosecond time-resolved fluorescence spectroscopy. *J. Am. Chem. Soc.* **116**: 2671–2672.

Kumazaki, I., Iwaki, M., Ikegami, I., Kandori, H., Yoshihara, K. and Itoh, S. (1994) Rates of primary electron transfer reactions in the photosystem I reaction center reconstituted with different quinones as the secondary acceptor. *J. Chem. Phys.* **98**: 11220–11225.

Mimuro, M., Hirota, M., Nishimura, Y., Moriyama, T., Yamazaki, I., Shimada, K. and Matsuura, K. (1994) Molecular organization of bacteriochlorophylls in chlorosomes of the green photosynthetic bacterium *Chloroflexus aurantiacus:* Studies of fluorescence depolarization accompanied with the energy transfer processes. *Photosyn. Res.* **41**: 181–191.

Mimuro, M., Nozawa, T., Tamai, N., Nishimura, Y. and Yamazaki, I. (1994) Presence and significance of minor antenna components in the energy transfer sequence of a green photosynthetic bacterium *Chloroflexus aurantiacus. FEBS Lett.* **340**: 167–172.

Nakayama, K. and Mimuro, M. (1994) Chlorophyll forms and light energy transfer in light-harvesting chlorophyll *a/b* protein complexes from the siphonous green alga *Bryopsis maxima*. *Biochim. Biophys. Acta* **1184**: 103–110.

Nakayama, K., Mimuro, M., Nishimura, Y., Yamazaki, I. and Okada, M. (1994) Kinetic analysis of energy transfer processes in LHCII isolated from a siphonous green alga *Bryopsis maxima* with use of a picosecond fluorescence spectroscopy. *Biochim. Biophys. Acta* **1188**: 117–124.

Ogata, T., Kodama, M., Nomura, S., Kobayashi, M., Nozawa, T., Katoh, T. and Mimuro, M. (1994) A novel peridinine-chlorophyll a protein (PCP) isolated from the dinoflagellate *Alexandorium cohorticula:* A higher pigment content and plural spectral forms of peridinine and chl *a*. *FEBS Lett.* **356**: 367–371.

Uehara, K., Mimuro, M., Ozaki, Y. and Olson, J.M. (1994) The formation and characterization of the in vitro polymeric aggregates of bacteriochlorophyll *c* homologs from *Chlorobium limicola* in aqueous solution in the presence of monogalactosyl diglyceride. *Photosyn Res.* **41**: 235–243.

Yamazaki, T., Nishimura, Y., Yamazaki, I., Hirano, M., Matsuura, K., Shimada, K. and Mimuro, M. (1994) Energy migration in a allophycocyanin B trimer with linker: Analysis by principal multicomponent spectral separation method. *FEBS Lett.* **353**: 43–47.

DIVISION OF CELL PROLIFERATION (ADJUNCT)

Professor: Masayuki Yamamoto
Research Associates: Hidetoshi Iida
Yoshiyuki Imai
NIBB Post-doctoral Fellows:
Masuo Goto
Makiko Kawagishi-Kobayashi
Graduate Student: Tomoko Ono (from The University of Tokyo)
Visiting Scientist: Makiko S. Okumura

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. 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, by using either similarity in nucleotide sequences or 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. In addition to the above, a project to characterize the effect of calcium ion on cell proliferation of the budding yeast has been performed.

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 for genes that can rescue loss of function of the *S. pombe sme2* gene, which encodes an RNA product essential for the promotion of meiosis I (see section II). Several genes have been isolated from mouse and *Arabidopsis* in this screening. Some of the mouse genes thus obtained apparently encode RNA binding proteins, and one of the *Arabidopsis* genes encodes a putative single strand DNA-binding protein. We also characterized three *Arabidopsis* cDNA clones that could rescue the meiosis-deficient phenotype of the *S. pombe pde1* mutant, which lacks cAMP phosphodiesterase. The first clone apparently encodes protein phosphatase 2C (PP2C). The second encodes a homolog of human Dr1. Dr1 is a protein that interacts with TATA-binding protein (TBP) and blocks its ability to activate transcription. The third encodes *Arabidopsis* TBP itself. Furthermore, we obtained mouse and *Xenopus* cDNA clones that can suppress a defect in meiosis II in the fission yeast. Further characterization of these isolated genes is in progress and their relevance to the regulation of meiosis in the original organisms is to be examined.

II. Identification of an RNA-protein complex as a critical regulator of meiosis in the fission yeast

S. pombe mei2 has been shown to be essential for the initiation of premeiotic DNA synthesis. We demonstrated that a temperature-sensitive *mei2* mutant performs premeiotic DNA synthesis but does not undergo meiotic divisions,

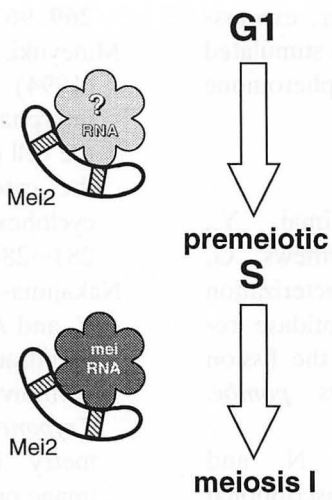


Figure 1. A model for the regulation of meiosis by Mei2-RNA complexes in the fission yeast
The function of Mei2 is required both prior to premeiotic DNA synthesis and prior to meiosis I. Mei2 complexes with meiRNA to promote meiosis I. This RNA is essential for the promotion, but it is not required for premeiotic DNA synthesis. Mei2 is likely to couple with another RNA partner to fulfill its function prior to premeiotic DNA synthesis, because the RNA-binding ability of Mei2 is indispensable to perform this function. (Adopted from ref. Watanabe and Yamamoto, 1994)

suggesting that Mei2 is required also for meiosis I. We screened for high copy suppressors of this *mei2* ts defect, and isolated a gene as an efficient suppressor. Surprisingly, this gene, named *sme2*, does not encode a protein product. It encodes a novel, polyadenylated RNA species of 0.5-kb in length (meiRNA), which specifically binds to Mei2 both *in vivo* and *in vitro*. Cells in which the *sme2* gene is deleted perform premeiotic DNA synthesis but cannot undergo meiosis I. Therefore the meiRNA-Mei2 complex is apparently required for promotion of meiosis I but not for premeiotic DNA synthesis. However, mutations introduced in the RNA-binding motifs in Mei2 blocked premeiotic DNA synthesis. This suggests that Mei2 is likely to couple with another RNA species to promote premeiotic DNA synthesis (Figure 1).

III. Analysis of budding yeast genes involved in Ca^{2+} signaling

The *mid1* and *mid2* mutants were isolated as those defective in Ca^{2+} signaling, on the basis that Ca^{2+} is essential for maintaining viability in *Saccharomyces cerevisiae* cells which are exposed to the mating pheromone released from the mating partner. The *MID1* and *MID2* genes were cloned and their nucleotide sequences determined. The deduced *MID1* gene product is a protein of 548 amino acid residues, which is localized in the plasma membrane. This protein is essential for Ca^{2+} influx and mating but not for vegetative cell growth. The *MID2* gene product is a putative integral membrane protein of 376 amino acids residues. It carries a potential transmembrane domain and a conserved Ca^{2+}-binding domain in its C-terminal half. This protein is not essential for vegetative cell growth but is required to keep viability of the cell in the presence of the

mating pheromone. Moreover, expression of *MID2* was found to be stimulated three fold by the mating pheromone signaling.

Publication List:

Davey, J., Davis, K., Imai, Y., Yamamoto, M. and Matthews, G. (1994) Isolation and characterization of krp, a dibasic endopeptidase required for cell viability in the fission yeast *Schizosaccharomyces pombe*. *EMBO J.* **13**, 5910–5921.

Hughes, D. A., Yabana, N. and Yamamoto, M. (1994) Transcriptional regulation of a Ras nucleotide-exchange factor gene by extracellular signals in fission yeast. *J. Cell Sci.* **107**, 3635–3642.

Iida, H., Nakamura, H., Ono, T., Okumura, M. S. and Anraku, Y. (1994) *MID1*, a novel *Saccharomyces cerevisiae* gene encoding a plasma membrane protein, is required for Ca^{2}+ influx and mating. *Mol Cell Biol.* **14**, 8259–8271.

Imai, Y. and Yamamoto, M. (1994) The fission yeast mating pheromone P-factor: Its molecular structure, gene structure, and ability to induce gene expression and G1 arrest in the mating partner. *Genes Dev.* **8**, 328–338.

Kuromori, T. and Yamamoto, M. (1994) Cloning of cDNAs from *Arabidopsis thaliana* that encode putative protein phosphatase 2C and a human Dr1-like protein by transformation of a fission yeast mutant. *Nucl. Acids Res.* **22**, 5296–5301.

Maeda, T., Watanabe, Y., Kunitomo, H. and Yamamoto, M. (1994) Cloning of the *pka1* gene encoding the catalytic subunit of the cAMP-dependent protein kinase in *Schizosaccharomyces pombe*. *J. Biol. Chem.* **269**, 9632–9637.

Mineyuki, Y., Iida, H. and Anraku, Y. (1994) Loss of microtubules in the interphase cells of onion root tips from the cell cortex and their appearance in the cytoplasm after treatment with cycloheximide. *Plant Physiol.* **104**, 281–284.

Nakajima-Shimada, J., Iida, H., Anraku, Y. and Aoki, T. (1994) Determination of intracellular calcium concentration of individual host cells infected with *Trypanosoma cruzi* using microfluorimetry in conjunction with digital image processing. *Jpn. J. Parasitol.* **43**, 136–139.

Nocero, M., Isshiki, T., Yamamoto, M. and Hoffman, C. S. (1994) Glucose repression of *fbp1* transcription in *Schizosaccharomyces pombe* is partially regulated by adenylate cyclase activation by a G protein α subunit encoded by *gpa2 (git8)*. *Genetics* **138**, 39–45.

Ono, T., Suzuki, T., Anraku, Y. and Iida, H. (1994) The *MID2* gene encodes a putative integral membrane protein with a Ca^{2+}-binding domain and shows mating-pheromone stimulated expression in *Saccharomyces cerevisiae*. *Gene* **151**, 203–208.

Saran, S., Nakao, H., Tasaka, M., Iida, H., Tsuji, F. I., Nanjundiah, V. and Takeuchi, I. (1994) Intracellular free calcium level and its response to cAMP stimulation in developing *Dictyostelium* cells transformed with jellyfish apoaequorin cDNA. *FEBS Lett.* **337**, 43–47.

Watanabe, Y. and Yamamoto, M. (1994) *S. pombe* $mei2^{+}$ encodes an RNA-binding protein essential for premeiotic DNA synthesis and meiosis I, which cooperates with a novel RNA species meiRNA. *Cell* **78**, 487–498.

DIVISION OF CELLULAR COMMUNICATION (ADJUNCT)

Professor: Yoshiki Hotta
Associate Professor: Hitoshi Okamoto
Research Associates: Mika Tokumoto
Shin-ichi Higashijima
Institute Research Fellow: Akira Chiba
Nobuyoshi Shimoda
Graduate Students: Keita Koizumi[1]
Yoko Yasuda[2]

([1])from Tokyo University)
([2])from Aichi University of Education)

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 two different animals, zebrafish (*Danio rerio*) and fly (*Drosophila melanogaster*), both of which are 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

The floor plate is a cell group implicated in the control of neural cell pattern and axonal growth in the developing vertebrate CNS. By subtractive hybridization technique, Klar et al. identified F-Spondin, a novel 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. Although F-Spondin was shown to promote neural cell adhesion and neurite extension in vitro, its functions in vivo remain largely unknown.

Nose performed an enhancer trap screen to search for genes expressed by a subset of muscle cells in *Drosophila*. One of such genes, AN34 was cloned and found to encode a secreted protein sharing homology in two parts (H1 and H2) with the N-terminal half of F-Spondin (Nose, personal communication).

We expected the homologous reigions to be novel domains important for

neuronal development, and thus, searched for AN34/F-Spondin family from zebrafish (*Danio rerio*). We identified two novel genes (zfAN1 and zfAN2) in addition to zf-F-Spondin. zfAN1 and zfAN2 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 zfAN1, zfAN2 and AN34 constitute a novel subfamily. zfAN1 is expressed weakly in the floor plate and a small subset of neuronal cells at 24-30 hr. zfAN2 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 30 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. Search for new cell surface recognition molecules expressed in a subset of CNS axons

During neuronal development, growth cones are known to have ability to recognize and extend along specific axonal surfaces. Several lines of evidence suggest that cell surface glycoproteins play important roles in this process. Immunocytochemical studies have demonstrated that the monoclonal antibody HNK-1, originaly raised to a human lymphoblastoma, recognizes a subset of CNS and PNS axons in many vertebrates including zebrafish by binding to a carbohydrate determinant in several glycoproteins. Thus, we started to characterize glycoproteins recognized by HNK-1, hoping to isolate new cell adhesion molecules (CAMs) expressed in a subset of CNS and PNS axons during neuronal development.

Our strategy is as follows: Many brains of adult zebrafish are homogenized, and from this extract, molecules which binds to HNK-1 are intensely enriched by using HNK-1 affinity chromatography. The affinty-purified fractions are used to immunize mice to get a series of monoclonal antibodies (MAbs). The MAbs, thus obtained, are screened with immunohistochemistry to zebrafish tissues. Hopefully, those MAbs which recognize peptides and not carbohydrates are used to isolate and characterize new molecules.

Immunogen which represents several protein families on Western Blotting was prepared. Mice were immunized with it, and hybrydoma cells were generated by cell fusion. A few MAbs each of which recognizes different protein species have been obtained. One of them could recognize a subset of CNS axons in the adult brain. Efforts are currently underway to obtain more MAbs.

IV. Tol1: Tc1-like element of *Oryzias latipes*

The zebrafish and the medaka have the potential to become important organisms for molecular genetic analysis of early vertebrate development. This is because both fishes offer the possibility of combining genetics with excellent embryology. The goal of this research is to use transposon to perform mutant screens in these fishes in order to identify, and subsequently clone genes important in early development, especially in neurogenesis.

A transposon, Tc1, which moves via DNA intermediate has been a useful tool in *C. elegans* genetics. Recently homologs of Tc1 were found in some fishes, raising the possibility that insertional mutagenesis would also be possible in vertebrate.

We discovered a Tc1-like element in the genome of medaka. Southern hybridization analysis showed that the element, Tol1, exists as multi copy in medaka genome but did not exist in zebrafish genome. Although the element seems to encode a protein similar to the Tc1 family of transposes, the putative transposase is probably not functional due to mutations as other putative transposases encoded by Tc1-like elements in fishes.

V. Fasciclin III as a synaptic recognition molecule in *Drosophila*

The larval neuromuscular system of the *Drosophila* consists of uniquely identified cells, and is a powerful model system for studying selective synapse formation. During synaptogenesis, the cell adhesion molecule Fasciclin III appears in both motoneuron RP3 and its targets, muscle 6 and 7. We have tested whether *fasciclin III* is necessary and/or sufficient for RP3 targe selection using intracellular dye injection.

First, we have found that in the existing *fasciclin III* null mutant RP3 reliably formed synapse with its normal targets. Therefore, *fasciclin III* is either irrelevant for the process, or playing a positive role but its absence can be compensated for another redundant mechanism (*"X"*).

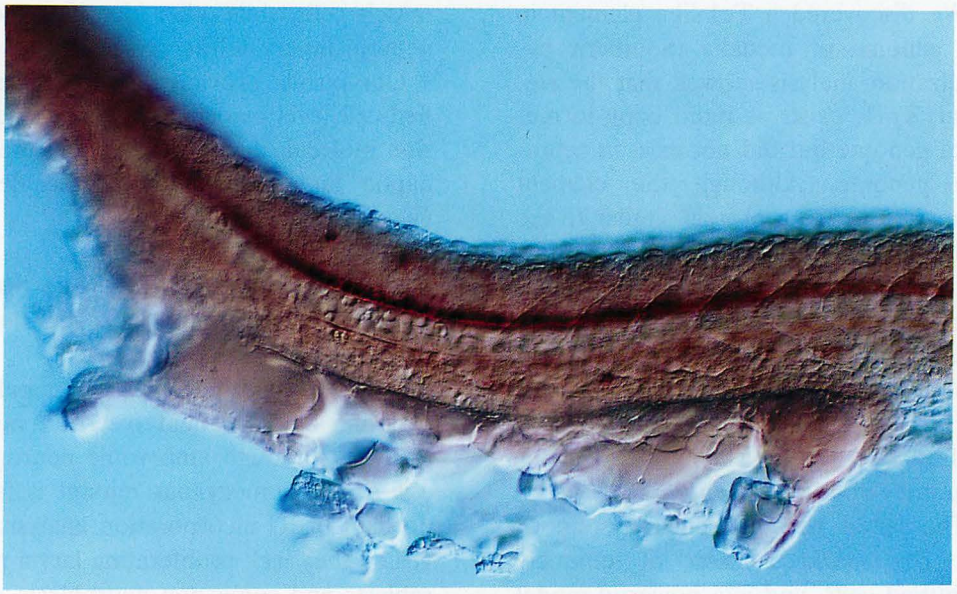
We now have demonstrated that the latter is the case. We generated transgenic flies which misexpresses *fasciclin III* extopically on all skeltal muscles during neuromuscular synaptogenesis. This was accompanied by creating a construct which placed the *fasciclin III* gene under the control of the myosin heavy chain promoter, and introducing this construct into the fly genome by P-element mediated genomic transformation. In these flies, RP3 often innervated non-target muscle cells while other identified motoneurons innervated targets normally.

Our results provide the single identified-cell level evidence that a cell adhesion molecule functions as a specific synaptic target recognition molecule in vivo.

VI. *no optic lobes* (*nol*), a gene that is essential for larval neurogenesis in *Drosophila*

We have identified a larval/pupal lethal line by P-element insertion mutagenesis. Although embryonic neurogenesis of the homozygous mutant is fairly normal, BrdU incorporation analysis revealed they lack proliferating larval neuroblasts and some non-neuroblasts cells (neurons?) start DNA synthesis at late larval stage. On the other hand, imaginal discs differentiate normally, so that rare escapers become normal looking pupae with a small central nervous system. The most characteristic feature is that they have no optic lobes. In such an individual, retinular axons lose their targets and are directly guided into the tiny brain. We conclude that there is an interesting gene named as *no optic lobes* (*nol*) near the P-insertion, which is essential for proliferation and differentiation of larval neuroblasts.

We cloned genomic regions around the P-element to find a transcription unit whose expression is significantly reduced in the homozygotes. The putative *nol* gene encodes a novel protein with a hydrophobic signal sequence. The deduced amino acid sequence has a mucin-like region, but its significance remains to be analyzed.



The expression pattern of F-Spondin mRNA in the spinal cord.
F-Spondin mRNA is expressed in the floor plate cells.

Publication List:

Inoue, A., Hatta, K., Hotta, Y. and Okamoto, H. (1994) Developmental regulation of Islet-1 mRNA expression during neuronal differentiation in embryonic zebrafish. *Devel. Dynam.* **199**, 1–11.

Goto, S., Tanimura, T. and Hotta, Y. (1995) Emhancer trap detection of positional information corresponding to the polar coordinate system in the imainal disc of *Drosophila melanogaster. Roux's Archiv Develop. Biol.* (In the Press).

Chiba, A., Snow, P., Keshishian, H. and Hotta, Y. (1995) Fasciclin III as a synaptic target recognition molecule in *Drosophila. Nature* **374**, 166–168.

DIVISION OF CELL FUSION (ADJUNCT)

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

Our research interest is focused on the regulatory mechanisms of multigene families both in the immune and in the central nervous systems. In the immune system, somatic 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 activates a particular member of the gene family by bringing an enhancer element and promotor element into close proximity.

For the olfactory system, hundreds of odorant receptor genes have been reported, although it is yet to be studied how this multigene family is regulated for expression. It is known that each member of the gene family is expressed in one of the four topographically distinct zones, where olfactory neurons expressing one particular kind of receptor are randomly distributed. Furthermore, it is assumed that a limited number of the receptor genes (possibly one) is activated in each olfactory neuron.

We have been studying how each neuron activates a limited number of the receptor genes keeping the rest of the genes silent. One obvious regulatory mechanism is at the level of transcription. In the mammalian immune system, DNA rearrangement is also utilized to activate one particular member of the receptor genes, and to exclude one of the two alleles (allelic exclusion). It is of interest to study whether such a mechanism is involved in the regulation of the olfactory system.

In order to study selective expression of the odorant receptor genes, we have been characterizing the genomic structure of the murine odorant receptor genes using genomic clones from the P1 phage library. Many P1 clones were found to contain multiple receptor genes, indicating that these genes are linked in tandem on the chromosome. We have extensively analyzed a P1 clone that contains two highly related (92% homologous) receptor genes, No.28 and No.10. It was found that these genes were expressed in the same zone in the olfactory epithelium, yet each gene was expressed in a different olfactory neuron. We are trying to understand the molecular mechanisms for the differential expression of the two highly related receptor genes.

Some odorant receptor genes are known to be expressed also in the testes, suggesting that they may be involved in maturation and/or chemotaxis of sperm cells. In order to elucidate the expression of the odorant receptor genes in the testis, we are currently characterizing a murine olfactory receptor gene which shares a significant homology with the HGMP07J gene isolated from a human testicular cDNA library.

For the study of mutually exclusive expression of the odorant receptor genes, we have generated transgenic mice which are forced to express one particular member of the receptors in every single olfactory neuron. The transgene contains the mouse receptor No.28 gene under the control of OMP promoter.

Since the OMP gene is expressed specifically in the olfactory epithelium, the transgenic receptor gene is expected to be activated in all mature neurons in the transgenic mouse. These studies will give us a new insight into the molecular mechanisms not only of the receptor gene expression, but also of neuronal projection in the olfactory bulb. When the olfactory neurons are regenerated, they send axons to the specific region called glomeruli in the olfactory bulb. It is amazing that axons are able to find a right target glomerulus out of two thousand similar structures. We are hoping that our transgenic approach will become a useful clue to study the target specificity and selectivity in the synapses formation.

Publication List:

Akamatsu, Y., Tsurushita, N., Nagawa, F., Matsuoka, M., Okazaki, K., Imai, M. and Sakano, H. (1994) Essential residues in V(D)J recombination signals. *J. Immunol.*, **153**, 4520–4529.

Heike, T., Nishikomori, R., Kawai, M., Tsuboi, A., Arai, N. and Mikawa, H. (1994) Molecular basis for developmental changes of GM-CSF gene inducibility in embryonal carcinoma cells. *Bioche. Biophys. Res. Comm.* **198**, 473–479.

Heiki, T., Nishikomori, R., Kawai, M., Tsuboi, A., Arai, N. and Mikawa, H. (1994) Developmental changes of GM-CSF gene inducibility in embryonal carcinoma cells. *Mol. Immun.* **31**, 1269–1275.

Kondo, T. and Ishiura, M. (1994) Circadian Rhythms of Cyanobacteria: Monitoring the Biological Clocks of Individual Colonies by Bioluminescence. *J. Bacteriol.* **176**, 1881–1885.

Kondo, T., Tsinoremas, N.F., Golden, S.S., Johnson, C.H., Kutsuna, S. and Ishiura M. (1994) Circadian Clock Mutants of Cyanobacteria. *Science* **266**, 1233–1236.

Matsuda, I., Masuda, E.M., Tsuboi, A., Behnam, S., Arai, N. and Arai, K. (1994) Characterization of NF(P), the nuclear factor that interacts with the regulatory P sequence (5'-CGAAAATTTCC-3') of the human interleukin-4 gene: relationship to NF-κB and NF-AT. *Bioche. Biophys. Res. Comm.* **199**, 439–446.

Tsuboi, A., Matsuda, E.S., Naito, Y., Tokumitsu, H., Arai, K. and Arai, N. (1994) Calcineurin potentiates activation of the granulocyte-macrophage colony-stimulating factor gene in T cells: involvement of the conserved lymphokine element 0. *Mol. Biol. Cell* **5**, 119–128.

Tsuboi, A., Muramatsu, M., Tsutsumi, A., Arai, K. and Arai, N. (1994) Calcineurin activates transcription from the GM-CSF promoter in synergy with either protein kinase C or NF-κB/AP-1 in T cells. *Bioche. Biophys. Res. Comm.* **199**, 1064–1072.

Watanabe, M., Muramatsu, M., Tsuboi, A. and Arai, K. (1994) Differential response of NF-κB1 p105 and NF-κB2 p100 to HTLV-I encoded Tax. *FEBS Letters* **342**, 115–118.

Yoshimatsu, T. and Nagawa, F. (1994) Effect of artificially inserted introns on gene expression in *Sacchromyces cerevisiae*. *DNA Cell Biol.* **13**, 51–58.

DEPARTMENT OF DEVELOPMENTAL BIOLOGY

Chairman: Yoshitaka Nagahama

The Department is composed of three regular divisions and one adjunct division and conducts molecular analysis on various aspects of developmental phenomena; differentiation and maturation of the germ cells, molecular basis of the body plan, and gene regulation in the differentiated tissues, changes of the differentiated states, and different phases of the cell cycle.

LABORATORY OF REPRODUCTIVE BIOLOGY

Professor: Yoshitaka Nagahama
Research Associates: Michiyasu Yoshikuni
Minoru Tanaka
Tohru Kobayashi
JSPS Post-doctoral Fellows: Takeshi Miura
Chiemi Miura
Graduate Students: Yoshinao Katsu
Shinji Onoe
Daisuke Kobayashi
Xiao-Tian Chang
Yuichi Ooba
Institute Research Fellows: Akihiko Yamaguchi
Toshinobu Tokumoto
Visiting Scientists: Mishiya Matsuyama[1)]
Jian-quiao Jiang[2)]
Wei Ge[3)]
Technical Staffs: Hiroko Kobayashi
Sachiko Fukada

[1)]from Kyushu University
[2)]from Wuhan University
[3)]from University of Alberta

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

I. Endocrine regulation of oocyte differentiation, growth and maturation

Our research effort in previous years concentrated on the identification and characterization of the molecules (gonadotropin hormones 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 the cDNA encoding several ovarian steroidogenic enzymes of rainbow trout (*Oncorhynchus mykiss*) and medaka (*Oryzias latipes*) 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), P450 aromatase (P450arom) and 20β-hydroxysteroid dehydrogenase (20β-HSD). We also isolated the structural gene encoding P450arom, for the first time from a non-mammalian vertebrate, the medaka. The medaka P450arom gene consisted 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. Genomic Southern blots revealed the presence of a single medaka gene. Promoter analyses indicated two major transcription initiation sites 60 and 61 bp upstream from a putative initiation codon. The promoter region of medaka P450arom gene contain Ad4BP sites and estrogen responsive element half-sites.

The cDNA clones obtained have been

used for Northern and whole mount *in situ* hybridization to investigate the molecular basis of differential production of estradiol-17β and 17α,20β-DP during oocyte growth and maturation in rainbow trout and medaka. In both species, P450scc and P450c17 (also 3β-HSD in rainbow trout) mRNA transcripts were increased in follicles towards the end of oocyte growth phase and during oocyte maturation. Furthermore, incubations of isolated thecal layers with gonadotropin resulted 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 expressed in the presence of gonadotropin. The increases 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 were high during oocyte growth, but rapidly decreased 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).

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. Scatchard analysis reveled two different receptors: a high affinity with a Kd of 18 nM and a Bmax of 0.2 pmoles/mg protein, and a low affinity receptor with a Kd of 0.5 μM and a Bmax of 1 pmole/mg protein. 17α,20β-DP receptor concentrations increase during oocyte maturation. The interaction between

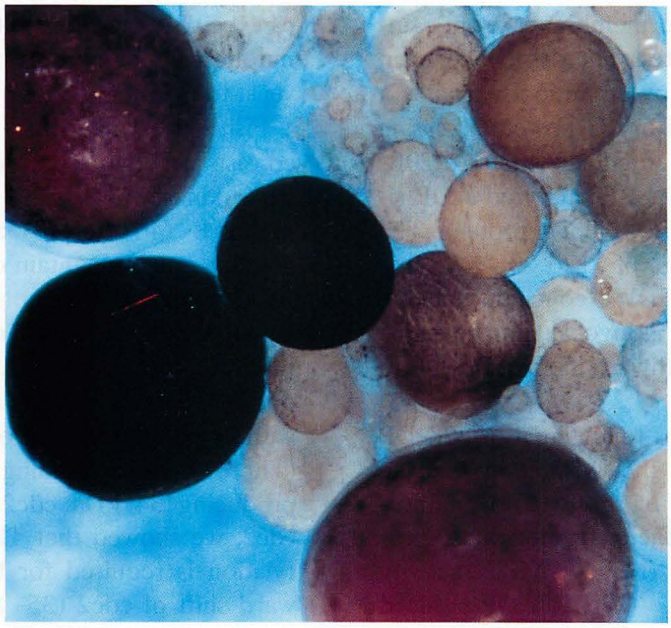


Fig. 1 Whole mount *in situ* hybridization showing P450 aromatase mRNA in medaka ovarian follicles.

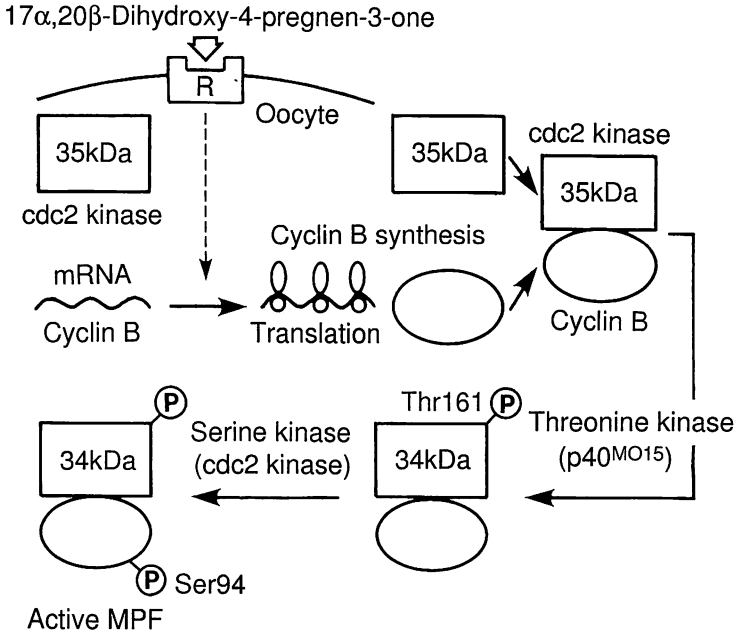


Fig. 2 Molecular mechanisms of 17α,20β-dihydroxy-4-pregnen-3-one induced MPF activation in goldfish oocytes.

17α,20β-DP receptors and G-proteins was examined. Pertussis toxin (PT) catalyzed the ADP ribosylation of a 40 kDa protein in crude membranes from rainbow trout oocytes. The 40 kDa protein was recognized by an antibody against a subunit of inhibitory G-protein. Treating the membrane fraction with 17α,20β-DP decreased the PT-catalyzed ADP ribosylation of the 40 kDa protein. The specific binding of 17α,20β-DP was decreased by PT. We conclude that the PT-sensitive Gi is involved in the signal transduction pathway of 17α,20β-DP in fish oocytes.

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

Fish MPF, like that of amphibians, consists of two components, catalytic cdc2 kinase (34 kDa) and regulatory cyclin B (46- to 48 kDa). Immature goldfish oocytes contain only monomeric 35 kDa cdc2 and do not stockpile cyclin B, although immature oocytes contain mRNA for cyclin B. In maturing oocytes, activation of cdc2 is associated with its phophorylation of threonine 161 (Thr161) after binding to cyclin B, producing 34 kDa cdc2. Using mutant cdc2, we showed that Thr161 phosphorylation is required for both the downward shift of cdc2 (35- to 34 kDa) and the kinase activation. Since thyrosine 15 of cdc2 is not phosphorylated after

binding to cyclin B, it does not require dephosphorylation. This situation is obviously different from that in immature *Xenopus* oocytes, in which the cdc2-cyclin B complex preexists with cdc2 phosphorylated on both Tyr15 and Thr161, thereby requiring Tyr15 dephosphorylation catalyzed by cdc25 phosphatase for MPF activation. Recently we have isolated a cDNA clone encoding a goldfish homolog of $p40^{MO15}$, the catalytic subunit of a protein kinase which activates cdc2 kinase through phosphorylation of Thr161, from a goldfish oocyte cDNA library. Northern and Western blot analyses revealed that both $p40^{MO15}$ mRNA and protein are already present in goldfish immature oocytes and do not exhibit any changes during hormonally induced 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, which was transiently detected at the initial phase of the normal egg activation. The 42 kDa cyclin B, as well as full-length one, was degraded in *Xenopus* egg extracts, but a mutation on K57 (K57R, the 26S proteasome-catalyzed digestion site K57 was substituted with arginine) 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). A steroidogenic switch, from 11-ketotestosterone to 17α,20β-DP, occurs in salmonid testes around the onset of final maturation. *In vitro* incubation studies using different testicular preparations have revealed that the site of 17α,20β-DP production is in the sperm, but its production depends on the provision of precursor steroids by somatic cells. The site of 11-ketotestosterone production is in the testicular somatic cells.

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

We have used subtractive hybridization to identify genes that are expressed differentially in eel testes in the first 24 hr after HCG treatment *in vivo*, which ultimately induces spermatogenesis. One up-regulated cDNA was isolated from subtractive cDNA libraries derived from mRNA extracted from control testes and testes one day after a single injection of HCG. From its deduced amino acid

seqence, this clone was identified as coding for the activin βB subunit. Using Northern blot analysis and *in situ* hybridization techniques, we examined sequential changes in transcripts of testicular activin βB during HCG-induced spermatogenesis. No transcripts for activin βB were found in testes prior to HCG injection. In contrast, 3.3 kb mRNA transcripts were prominent in testes one day after the injection. The transcript concentration began to decrease three days after the injection, followed by a further sharp decrease by nine days. The HCG-dependent activin βB mRNA expression in the testes was confirmed by *in situ* hybridization using a digoxigenin-labelled RNA probe: the signal was restricted to Sertoli cells in testes treated with HCG for one to three days. A marked stimulation of activin B production, but not either activin A or activin AB, was observed in testes after HCG and 11-ketotestosterone treatment. Addition of recombinant human 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.

Publication List:

Fukada, S., Sakai, N., Adachi, S. and Nagahama, Y. (1994) Steroidogenesis in the ovarian follicle of medaka (*Oryzias latipes*, a daily spawner) during oocyte maturation. *Develop. Growth Differ.* **36**, 81–88.

Furukawa, K., Inagaki, H., Naruge, T., Tabata, S., Tomida, T., Yamaguchi, A., Yoshikuni, M., Nagahama, Y. and Hotta, Y. (1994) cDNA cloning and functional characterization of a meiosis-specific protein (NMS1) with apparent nuclear association. *Chromosome Res.* **2**, 99–113.

Iwamatsu, T., Nakashima, S., Onitake, K., Matsuhisa, A. and Nagahama, Y. (1994) Regional differences in granulosa cells of preovulatory medaka follicles. *Zool. Sci.* **11**, 77–82.

Matsuyama, M., Fukada, T., Ikeura, S., Nagahama, Y. and Matsuura, S. (1994) Spawning characteristics and steroid hormone profiles in the wild female Japanese sardine *Sardinops melanostictus*. *Fish. Sci.* **60**, 703–706.

Mita, M., Yoshikuni, M. and Nagahama, Y. (1994) G-proteins and adenylate cyclase in ovarian granulosa cells of amago salmon (*Oncorhynchus rhodurus*). *Mol. Cell. Endocrinol.* **105**, 83–88.

Miura, T., Kobayashi, T. and Nagahama, Y. (1994) Hormonal regulation of spermatogenesis in the Japanese eel (*Anguilla japonica*). *Perspecitive in Comparative Endocrinology*, pp. 631–635.

Nagahama, Y. (1994) Molecular biology of oocyte maturation in fish. *Perspective in Comparative Endocrinology*,

193–198.

Nagahama, Y. (1994) The onset of spermatogenesis in fish. *In* Germline Development, Ciba Foundation Symposium No. 182, 255–270.

Nagahama, Y. (1994) Endocrine regulation of gametogenesis in fish. *Int. J. Develop. Biol.* **38**, 217–229.

Nagahama, Y., Yamashita, M., Tokumoto, T. and Katsu, Y. (1994) Regulation of oocyte maturation in fish. *Current Topics in Developmental Biology*, **30**.

Nagahama, Y., Yoshikuni, M., Yamashita, M. and Nagahama, Y. (1994) Regulation of oocyte maturation in fish. *In* Molecular Endocrinology of Fish, Fish Physiology XIII (N.M. Sherwood and C.L. Hew, eds.), Academic Press, New York, pp.393–439.

Nakamura, M., Mariko, T. and Nagahama, Y. (1994) Ultrastructure and *in vitro* steroidogenesis of the gonads in the protandrous anemonefish *Amphiprion frenatus*. *Japan. J. Ichthyol.* **41**, 47–56.

Sakai, N., Tanaka, M., Takahashi, M., Fukada, S., Mason, J.I. and Nagahama, Y. (1994) Ovarian 3β-hydroxysteroid dehydrogenase/Δ^{5-4}-isomerase of rainbow trout: its cDNA cloning and properties of the enzyme expressed in a mammalian cell. *FEBS Lett.* **350**, 309–313.

Yamashita, M., Kajiura, H., Tanaka, T., Onoe, S. and Nagahama, Y. (1994) Molecular mechanisms of the activation of maturation-promoting factor during goldfish oocyte maturation. *Develop. Biol.* **166** (in press).

Yoshikuni, M. and Nagahama, Y. (1994) Involvement of an inhibitory G-protein in the signal transduction pathway of maturation-inducing hormone (17α,20β-dihydroxy-4-pregnen-3-one) action in rainbow trout (*Oncorhynchus rhodurus*) oocytes. *Develop. Biol.* **166**, 615–622.

Yoshikuni, M., Matsushita, H., Shibata, N. and Nagahama, Y. (1994) Purification and characterization of 17α,20β-dihydroxy-4-pregnen-3-one-binding protein from plasma of rainbow trout, *Oncorhynchus mykiss*. *Gen. Comp. Endocrinol.* **96**, 189–196.

DIVISION OF CELL DIFFERENTIATION

Professor: Yoshiaki Suzuki
Associate Professor: Kohji Ueno
Research Associates: Shigeharu Takiya
Toshiharu Suzuki
Kaoru Ohno
Hiroki Kokubo
JSPS Postdoctoral Fellow: Václav Mach
Visiting Scientists: Pin-Xian Xu
Xin Xu
Graduate Students:
Hiroki Kokubo (Graduate University for Advanced Studies)
Xin Xu (Graduate University for Advanced Studies)
Yoshinori Ueno (Graduate University for Advanced Studies)
Katsuyoshi Matsunami (Graduate University for Advanced Studies)
Technical Staffs: Miyuki Ohkubo
Chikako Inoue

Members of the Division have been involved in two well associated projects. One initiated in 1968 is to understand how a special tissue like the silk gland of *Bombyx mori* is differentiated along the developmental programs and results in transcribing a specific set of genes like the silk fibroin, fibroin L-chain, P_{25}, sericin-1, and sericin-2 genes. The other initiated at the time when the Division was established in 1978 is concerned with how the body plan of the silkworm is controlled 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 gene 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 shed light on 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 genes and other regulatory genes, and identification of their target genes expressed in the labial segment, where the silk gland is originated.

Among many factors proposed to bind and control the fibroin and sericin-1 genes, the POU-M1 which accommodates a POU-domain identical to *Drosophila* Cf1-a was cloned previously and characterized. The POU-M1 binds to the SC site of the sericin-1 gene and is assumed to enhance the transcription. 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 for the first time at stage 18-19 in a restricted site of the labial segment; the primordial cells of prothoracic gland. This location matches with the site where the *Bombyx Scr* expression disappears which was detected in the entire labial segment in the preceding stage. Later the *POU-M1* expression is observed along with developing silk gland and is confined to the anterior and middle portions of the silk gland by late embryonic stages. These observations suggest that the *POU-M1* gene may have multiple functions.

Silk gland specific transcription factor SGF-1 interacts with the SA site of the sericin-1 gene and FA and FB sites of the fibroin gene. Two related SGF-1 polypeptides were purified of observed molecular weights 40 kDa and 41 kDa (V. Mach et al. (1995). *J. Biol. Chem.*,

in press). These proteins interact specifically with the SA site in electrophoretic mobility shift assay and DMS methylation interference assay. The SGF-1 40 kDa protein was partially sequenced and found to be a new member of the fork head/HNF-3 family. This information facilitated cloning of several full length cDNAs coding for the SGF-1 40 kDa and possibly also for the SGF-1 41 kDa proteins (V. Mach et al. (1995). *J. Biol. Chem.*, in press). Sequencing of the full length cDNAs revealed three domains conserved between *Drosophila* Fork head protein and mammalian HNF-3 factors (Fig. 1). *SGF-1* mRNA is expressed consitently with the presumed role of its protein product in the regulation of sericin-1 gene and SGF-1 protein contains putative transactivation domains. We conclude that regulation of the sericin-1 gene transcription via SA site is exerted by the SGF-1 40 and 41 kDa proteins.

The expression patterns of *SGF-1* mRNA and its protein were analyzed in embryos. The transcripts and protein were detected firstly in the most anterior and posterior domains at the time when the germ anlage is formed, and at later stages in the entire region of the foregut, in the most anterior and posterior regions of the elongated midgut, and in the entire region of the hindgut (Fig. 2A). At the time when the embryo retraction is finished (stage 20) the transcripts and protein are also detected in the invaginated silk gland (Fig. 2A). By the time when the blastokinesis is finished (stage 25) the transcripts and protein are restricted to the middle and posterior regions of the silk gland (Fig. 2B). These results suggest that the *Bombyx fork head/SGF-1* might play important roles in the gut and silk gland development.

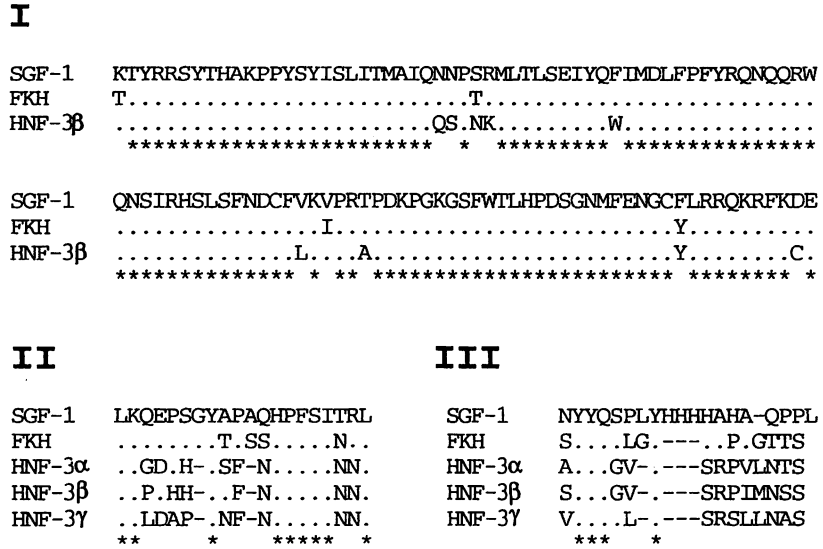


Fig. 1 Comparison of SGF-1 with other members of Fork head/HNF-3 family.
The three domains conserved between *Drosophila* Fork head and mammalian HNF-3 factors are present also in SGF-1. The domain boundaries in SGF-1 are Lys103/Glu212 (I), Leu286/Leu303 (II), and Asp331/Leu349 (III). Domain I is the DNA binding domain, and domains II and III participate in transactivation by HNF-3 beta.

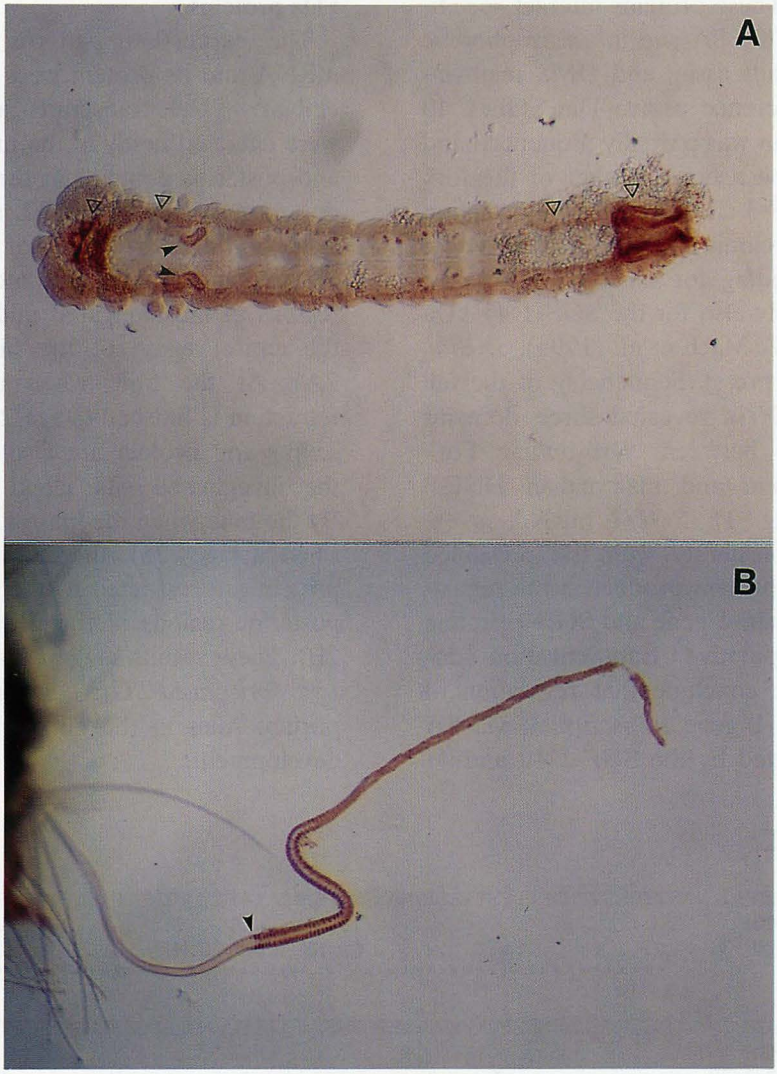


Fig. 2 Expression of *Bombyx* Fork head/SGF-1 protein.
A. An embryo at the embryo retraction stage (stage 20). The triangles from left to right indicate positive signals at the foregut, the anterior most of the midgut, the posterior most of the midgut, and the hindgut, respectively. The arrowheads indicate the signals at the invaginated silk glands.
B. A silk gland dissected out from an embryo of stage 25. The arrowhead indicates the border between the anterior (negative) and the middle (positive) silk gland. The positive signal continues all the way to the end of the posterior silk gland.

Cloning and expression pattern analysis of the *Bombyx Scr* have been described previously. Regulation of the *Bombyx Scr* gene itself in the labial segment and search for the target genes of the *Bombyx Scr* have been intiated.

II. Other genes of developmental interest

Following the previous discovery that the *Bombyx cad* transcripts and protein form concentration gradients at different timing of development than in *Droso-*

phila, our attention has been forwarded to *Bombyx* pair-rule genes. We have cloned the *Bombyx even-skipped* and *Bombyx hunchback* cDNAs. After examining the expression patterns of these genes, we propose a possible molecular explanation for *Bombyx* segmentation mechanism. At the top of interests, our data suggest that *Bombyx even-skipped* might serve a double-segment defining role and determine on the odd-numbered *Bombyx engrailed* stripes.

In continuation of the abdominal segments identification, we have concentrated in the study of morphogenesis of embryonic abdominal legs. We have analyzed proteins in the wild type embryos by SDS-PAGE, and found that two high molecular weight proteins of 270 and 260 kDa (p270/260) are expressed specifically in the abdominal legs. These proteins are not detectable in the E^{Ca}/E^{Ca} embryo which lacks *Bombyx abd-A* gene as reported previously, suggesting a control under the *Bombyx abd-A*. We have purified the p270/260 to greater than 90% homogeniety by ammonium sulfate fractionation, hydroxyapatite column chromatography and gel filtration column chromatography. Since p270 and p260 were coeluted through these procedures, we assume that p270 and p260 participate in the formation of a stable complex.

Immunohistochemical analysis demonstrated that p270/260 was detected in a few cells in each abdominal leg anlage of the first to the eighth abdominal segments at an early developmental stage. The number of positive cells increased and they were integrated into abdominal legs of the third to the sixth abdominal segments in later stages. From these results we speculate that p270/260 may have an important role in the development of abdominal legs.

Publication List:

Amanai, K., Suzuki, Y. and Ohtaki, T. (1994) Involvement of a maternally transcribed lectin gene in the early development of *Bombyx mori*. *Roux's Arch. Dev. Biol.* **203**, 397–401.

Suzuki, Y. (1994) Genes that are involved in *Bombyx* body plan and silk gene regulation. *Int. J. Dev. Biol.* **38**, 231–235.

Xu, P.-X., Fukuta, M., Takiya, S., Matsuno, K., Xu, X. and Suzuki, Y. (1994) Promoter of the *POU-M1/SGF-3* gene involved in the expression of *Bombyx* silk genes. *J. Biol. Chem.* **269**, 2733–2742.

Xu, X., Xu, P.-X. and Suzuki, Y. (1994) A maternal homeobox gene, *Bombyx caudal*, forms both mRNA and protein concentration gradients spanning anteroposterior axis during gastrulation. *Develop.* **120**, 277–285.

DIVISION OF MORPHOGENESIS

Professor: Goro Eguchi
Associate Professor: Ryuji Kodama
Research Associates: Makoto Mochii
Mitsuko Kosaka
Visiting Scientists: Takamasa S. Yamamoto
Kaichiro Sawada
Akio Iio
Toshiyuki Nagamoto
Graduate Students:
Nobuhiko Mizuno (Graduate University for Advanced Studies)
Yuuichi Mazaki (Graduate University for Advanced Studies)
Harutoshi Hayashi (from School of Agriculture, University of Tokyo)
Jatupol Kositsawat (from School of Medicine, University of Tokyo)
Technical Staffs: Chiyo Takagi
Hisae Urai

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. Multipotency conserved in the human PECs

It has been proved that retinal PECs isolated from the eye of 80-year-old human can transdifferentiate into lens cells to construct lentoids when dissociated and cultured under the culture condition established in our laboratory (cf. Itoh and Eguchi, Dev. Biol. 115: 353–362, 1986). Human PECs can readily transdifferentiate and form lentoids, which express major human crystallin genes and ultrastructures characteristic to lens, through the dedifferentiated state in the similar manner as observed in chicken retinal PECs. We have isolated clones from the progeny of dedifferentiated human retinal PECs to establish cell lines. Reaggregates of cells of one of established cell lines were found to express lens specificities when maintained under the culture condition permissive for lens cell differentiation (cf. Itoh and Eguchi, 1986), although these dedifferentiated cells could not develop to lentoid in the monolayer culture. However, when maintained with the ordinary medium, Eagle's minimum essential medium supplemented with 10% of fetal bovine serum, for long time, they express neuronal cell morphology (Fig. 1). Neuronal specificities of these cells with neuronal morphology could be detected by indirect immunofluorescent staining using antibodies against 70, 150 and 200 kd subunits of neurofilament protein. This result is the first evidence that human retinal PECs can express neuronal phenotype by themselves and is strongly

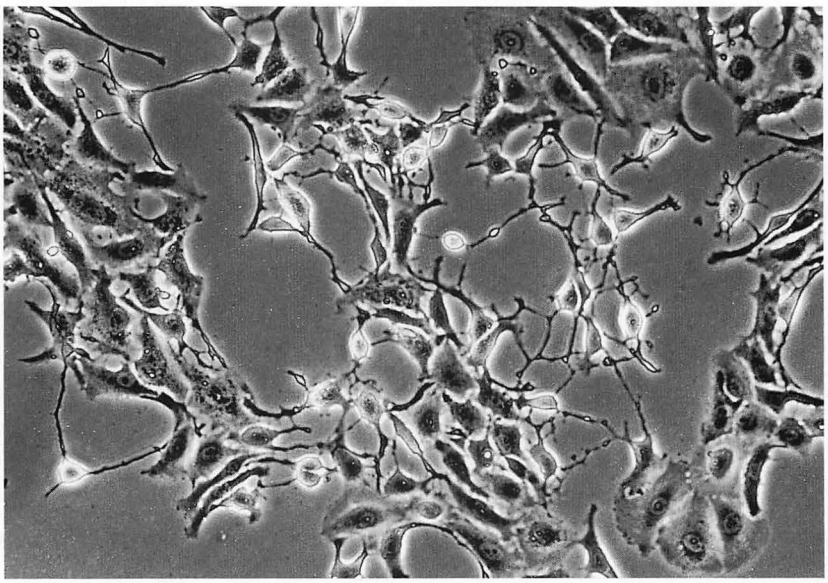


Fig. 1. Human retinal PECs showing a neuron-like morphology. Cells were isolated from the eye of an 80-year-old donor and their growth was enhanced by the EdFPH medium. Cells were then cultured in the standard medium for about 30 days.

suggesting that the human retinal PECs, like the newt PECs, conserve multipotency for differentiation, although neuronal transdifferentiation of human retinal PECs by transfection with H-*ras* proto-oncogene has been reported by Dutt, Scott et al (DNA and Cell Biology 12(8): 667–673, 1993). The human PECs grow well and endure genetic manipulations, such as gene transfection, thus are supposed to be a promising material to study transdifferentiation. We are now extending our studies of transdifferentiation using this cell line established from human retinal PECs.

II. Establishment of *in vitro* model system of lens transdifferentiation using chicken iris PECs

As the main material to study the process of transdifferentiation in our laboratory, we have been utilized retinal PECs isolated from approximately 10 day-old chick embryos, because we can obtain large number of PECs through an easy manipulation, owing to the large size of the eye of the chick embryo. Recently, however, we have come to recognize that cell culture of the retinal PECs has gradually become difficult. Cells are susceptible to the change of culture conditions, such as the product lot of the fetal calf serum or other components of the culture medium, and often stop growing, embedded in irregularly accumulated extracellular matrices. Besides trials to modify culture conditions to enable more efficient culture of the retinal PECs, other sources of PECs were also sought. The iris PECs of the chick has emerged as a promising material as briefly described below.

Because it is the iris PECs that give rise to lens regeneration in the newt, the iris PECs of the chicken was tested for their ability to transdifferentiate *in vitro*. The iris was isolated from newly hatched

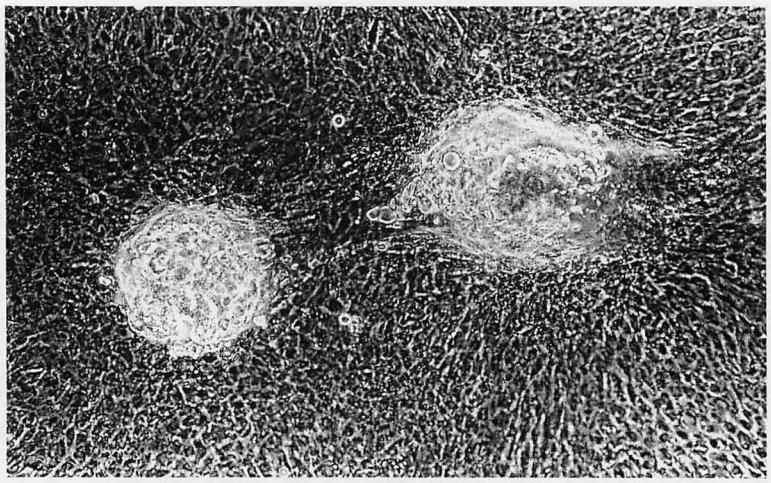


Fig. 2. Lentoids formed in the culture of the iris PECs of newly hatched chicks. Cells passaged in the EdFPH medium for several times were cultured with ascorbic acid for about 30 days.

chicks, and the pigmented epithelium was cleanly separated from the stroma using dispase. The iris PECs slowly grow and stably maintain the phenotype in a standard culture medium. When the EdFPH medium, which is effective in inducing dedifferentiation of retinal PECs (cf. Itoh and Eguchi, 1986), was applied, the iris PECs grow rapidly and dedifferentiate. By further addition of ascorbic acid, such dedifferentiated cells accumulate and form large number of lentoids (Fig. 2). The growth and transdifferentiation of the iris PECs are highly reproducible. Preliminary studies have shown that all the genes we have analyzed in the retinal PECs are expressed in the culture of the iris PECs.

III. Detailed analysis of the gap junctions in the Wolffian lens regeneration

On the basis of the results accumulated through the studies of lens transdifferentiation of the retinal PECs of the chick embryo, detailed studies of the mechanisms regulating the lens regeneration in the newt have become possible. Our recent observation in the chick PECs has shown that the dePECs are devoid of both the structure and conductance of the gap junction. There was also a preliminary study that showed a transient absence of the electric coupling between dorsal iris PECs prior to the lens regeneration. We have started a study on the gap junction between the iris PECs during lens regeneration, and have obtained preliminary results shown below.

A significant decrease in the number of gap junctions was detected in the dorsal iris pigmented epithelium, but not in the ventral, on approximately 10 days after lens removal. This result confirms the preliminary result in the electric coupling, and is in accordance with the result in the chick retinal PECs. However, we also found that, just after the lens removal, there is a period during which gap junctions are increased all over the iris. This increase was accompanied with the increase in the transcript of

the connexin gene, which codes for a major component of the gap junction (R. Kodama, unpublished).

Similar study in the partial hepatectomy showed that, shortly after the hepatectomy, there is a quick decrease in gap junction number, which is then followed by the raise in DNA synthesis and mitoses. Our result in the lens regeneration suggests that the regeneration process is biphasic, consisting of a period of emergency repair in the whole iris and a period of regeneration only in the dorsal iris. Through such renewed studies, which are also focused on growth factors and tissue proteases, a clearer image of the lens regeneration process will be obtained.

Publication List:

Kodama, R. and Eguchi, G. (1994) The loss of gap junctional cell-to-cell communication is coupled with dedifferentiation of retinal pigmented epithelial cells in the course of transdifferentiation into the lens. *Int. J. Dev. Biol.* **38**, 357–364.

Kodama, R. and Eguchi, G. (1994) Gene regulation and differentiation in vertebrate ocular tissues. *Curr. Opin. Genetics Dev.* **4**, 703–308.

Iio, A., Mochii, M., Agata, K., Kodama, R. and Eguchi, G. (1994) Expression of the retinal pigmented epithelial cell-specific pP344 gene during development of the chicken eye and identification of its product. *Develop. Growth Differ.* **36**, 155–164.

Ono, T., Murakami, T., Mochii, M., Agata, K., Kino, K., Otsuka, K., Ohta, M., Mizutani, M., Yoshida, M. and Eguchi, G. (1994) A complete culture system for avian transgenesis, supporting quail embryos from the single-cell stage to hatching. *Dev. Biol.* **161**, 126–130.

Ono, T., Muto, S., Mizutani, M., Agata, K., Mochii, M., Kino, K., Otsuka, K., Ohta, M., Yoshida, M. and Eguchi, G. (1994) Production of quail chimera by transfer of early blastodermal cells and its use for transgenesis. *Jap. Poultry Sci.* **31**, 119–129.

Orii, H., Hyuga, M., Mochii, M., Kosaka, J., Eguchi, G. and Watanabe, K. (1994) Predominant melanogenesis and lentoidogenesis in vitro from multipotent pineal cells by dimethyl sulfoxide and hexamethylene bisacetamide. *Int. J. Dev. Biol.* **38**, 397–404.

DIVISION OF DEVELOPMENTAL BIOLOGY (ADJUNCT)

Professor: Masaki Iwabuchi
Associate Professor: Masao Tasaka
Research Associates: Norihiro Ohtsubo
Koji Mikami
Visiting Scientist: Michael Filosa[1)]
Graduate Students: Takuya Ito[2)]
Ken-ichiro Taoka [2)]
([1)]from University of Toronto)
([2)]from Kyoto University)

Genes encoding four types of core histones (H2A, H2B, H3, H4) and a linker histone (H1) are coordinately expressed during S phase of the cell cycle. To understand how this coordinate expression is regulated, we have been continuously studying the transcriptional regulation of wheat histone genes.

Within the promoter region of an H3 gene (*TH012*), three types of positive *cis*-acting elements, the hexamer (ACGTCA), the octamer (CGCGGATC) and the nonamer (CATCCAACG) have been found. The hexamer and the octamer constitute a sequence unit designated as type I element. This combined sequence is often found within the promoter region of plant histone H2B, H3 and H4 genes.

We have also found several sequence-specific DNA-binding proteins interacting with the type I element; HBP-1a and HBP-1b specific for the hexamer, and OBRFs for the octamer. Analyses of cloned cDNAs for HBP-1a and HBP-1b revealed that they have the characteristic feature of bZIP (basic region/leucine zipper) type transcription factors.

To analyze the temporal patterns of plant histone gene expression during the cell cycle, we have established the cell cycle synchronization system using suspension culture of rice Oc cells and the inhibitor of DNA synthesis, aphidicolin. S1 analyses of mRNAs transcribed from chimeric genes composed of the H3 promoter and protein coding region of β-glucuronidase gene revealed that the type I element is necessary and sufficient for the S phase-specific activation of the H3 gene expression.

I. Auxin-inducible expression of the wheat histone H3 gene

To define the functions of the type I element *in vivo*, we introduced the H3/GUS chimeric gene (-185WTH3/GUS) into *Arabidopsis* plants *via Agrobacterium* Ti plasmid, then analyzed their expressions in roots of regenerated plants. Histochemical staining experiment showed that the WTH3/GUS gene is expressed at root apical meristem and vascular bundle (Fig. 1A) whereas expression in the latter region was not observed when we examined using regenerated roots of transgenic rice plants. Expression in vascular bundle may occur in the dividing cells that constitute secondary vascular bundle specific for dicots and gymnosperms. We concluded that the H3 gene expression in roots is detected only in dividing cells whose cell cycle is actively progressing.

To determine whether the type I element is also involved in the meristem-specific gene expression, transgenic plants harboring mutant genes, HexM, OctM, and HexM/OctM were tested. Base-substitutions introduced into the hexamer or the octamer abolished the expression in roots (data not shown), suggesting that the type I element is necessary for the H3 gene expression in dividing cells.

Since auxin acts as a mitogen, progression of the cell cycle and expression of cell cycle-dependent genes such as histone genes would be expected to be regulated by auxin. To address this

possibility, we examined the effect of exogenously provided auxin on the meristem-specific expression of the H3/GUS genes. As shown in Fig. 1B, expression of the WTH3/GUS gene was enhanced in both root apical meristem and vascular bundle. This result indicates that the action of auxin is involved in the signal transduction pathway from the passage of G_1/S boundary to the H3 gene expression. Detailed analyses of the H3 promoter sequence that functions in this induction pathway are currently under way.

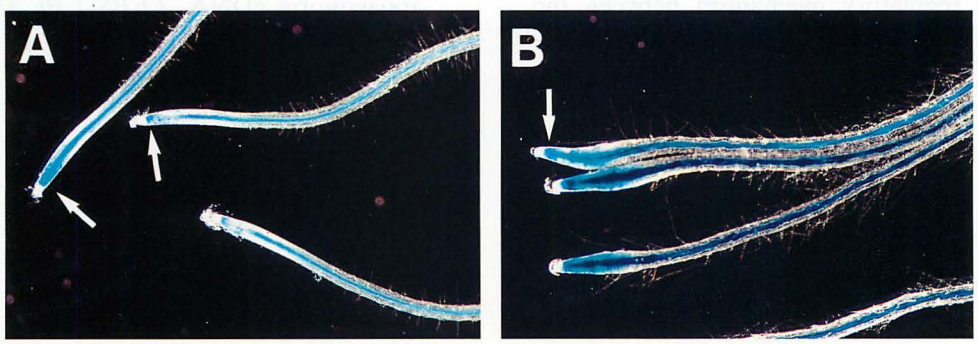
II. Functional analysis of HBP-1b(c38)

When the HBP-1b(c38) expression plasmid was co-transfected into tobacco mesophyll protoplasts with reporter plasmids containing the binding site of HBP-1b(c38), expression of the reporter gene was repressed. However, when the hexamer (a binding site of HBP-1b) was mutated, HBP-1b(c38) acted as an activator. This result represents a dual function of HBP-1b(c38) through direct binding to DNA and protein-protein interactions with the octamer-binding protein(s) or some other transcription factors. In addition, we investigated the functional difference between HBP-1b(c38) and its tobacco homologue, TGA-1a, in this expression system. Swapping experiments aiming at the functional regions of HBP-1b(c38) and TGA-1a showed that the activation of gene expression through the hexamer motif depends on the acidic region of TGA-1a.

To elucidate the functional roles of HBP-1b(c38) in plant development, we made transgenic *Arabidopsis* harboring the plasmids that overexpress HBP-1b(c38) or its antisense RNA and observed morphological effects of transgenic plants. Transformants overexpressing HBP-1b(c38) showed significant morphological changes such as bent stems, leafless plants, and abnormal production of flowers. This result suggests that HBP-1b(c38) is involved in the regulation of cell fate determination, flower development and/or other growth control mechanisms.

III. S phase-specific expression of the wheat histone H1 gene

The genomic clones, *TH315* and *TH325*, for wheat H1 genes, have not the type I element within their promoter region. Instead, they have another type of characteristic sequence unit composed of the octamer and the CAAT-box. This sequence, designated as the type III element, is found not only within the promoter region of wheat histone H1 genes



but also within those of *Arabidopsis* (*AtH1-1*) and tomato H1 genes and wheat H2B genes (*TH123* and *TH153*). Conservation of the order and the spacing (14 bp) between the two components (the octamer and CAAT) represents the possibility that the type III element is involved in the S phase-specific regulation of H1 and H2B gene expression.

Transient expression analyses of 5'-deletion and base-substitution mutants of H1 promoter/GUS chimeric genes revealed that the type III element acts as a positive *cis*-acting element of the H1 (*TH315*) gene.

To determine whether the type III element is involved in the regulation of S phase-specific gene expression, and to understand how the regulation of core histone gene expression during the S phase is different from that of H1 genes, we analyzed the expression of the H1/GUS chimeric genes in transformed rice cells. S1 analyses of the H1/GUS mRNA from synchronized culture of transformed rice cells showed that the peak of H1/GUS mRNA accumulation appeared slightly later (by an hour) than that of H3/GUS mRNA. In addtion, when the type III element of this promoter was deleted by truncation from its 5' end, the S phase-specific change of mRNA level was no longer observed. These results suggest the difference in the regulatory mechanism between the H1 gene and H3 gene, and also represent the contribution of the type III element to the S phase-specific regulation of the H1 gene expression.

Publication List:

Mikami, K., Sakamoto, A. and Iwabuchi, M. (1994) The HBP-1 family of wheat basic/leucine zipper proteins interacts with overlapping cis-acting hexamer motifs of plant histone genes. *J. Biol. Chem.* **269**, 9974–9985.

Morio, T., Takeuchi, I. and Tasaka, M. (1994) Cooperation of positively and negatively acting promoter elements determines prespore-specific transcription of Dp87 gene in Dictyostelium. *Mech. Dev.* **45**, 59–72.

Nakao, H., Yamamoto, A., Takeuchi, I. and Tasaka, M. (1994) Dictyostelium prespore-specific gene Dp87 encodes a sorus matrix protein. *J. Cell Sci.* **107**, 397–403.

Ohtsubo, N. and Iwabuchi, M. (1994) The conserved 3'-flanking sequence, AATGGAAATG, of the wheat histone H3 gene is necessary for the accurate 3'-end formation of mRNA, *Nucl. Acids Res.* **22**, 1052–1058.

Saran, S., Nakao, H., Tasaka, M., Iida, H., Tsuji, F.I., Nanjundiah, V. and Takeuchi, I. (1994) Intracellular free calcium level and its response to cAMP stimulation in developing Dictyostelium cells transformed with jellyfish apoaequorin cDNA. *FEBS Lett.* **337**, 43–47.

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
Shinji Hirano
Masahito Yamagata
Graduate Students: Hiroki Hamanaka
Junichi Yuasa
Takafumi Shintani
Taeko Nishiwaki
*Visiting Fellow: Haruyuki Matsunaga**
Technical Staff: Tomoko Mori
Shigemi Ohsugi
Masae Mizoguchi

(from Environmental Health Science Laboratory, Sumitomo Chemical Co., Ltd.)*

Our efforts have been devoted to studying molecular and cellular mechanisms which underlie the development of the vertebrate central nervous system. We are seeking for the molecules and structures that regulate various cellular events requisite for 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. Topographical and laminar connection in the chick retinotectal system

Neural connection in the vertebrate brain is selective in two ways, which we refer to as topographical and laminar. Topographical 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 cells/portions of the particular target layer/cell's surface. However, we still have no knowledge about the crucial factors that determine the development of these two classes of neural connection. We have chosen to study a retinotectal system in chickens which have many experimental advantages.

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. For example, retinal axons from the temporal (posterior) retina connect to the anterior part of the tectum. Many works, since the formulation of 'chemoaffinity theory' by Roger Sperry, have supported the involvement of gradient molecules in this topographically organized connection. In positing this hypothesis, we performed subtractive screening to isolate the cDNAs that is graded along the anterio-posterior axis in the retina. Recently, we have found several known and novel molecules, which are selectively expressed in the nasal or temporal half region 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 comparatively 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, and neurochemically-distinguishable subsets of the ganglion cells connect to distinct laminae to establish a functional network (Yamagata et al. submitted). 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 are now preparing new monoclonal antibodies that mark the subsets of laminae or cells, eventually to identify the molecules that guide the lamina-specific connection. To understand the function of these molecules, we will employ a variety of gene transfer techniques, including retroviral and adenoviral vectors.

II. Proteoglycan and brain development

Proteoglycans are a family of proteins bearing sulfated glycosaminoglycans, which bind many extracellular matrix components and growth factors through their core protein and glycosaminoglycan portions. We have been interested in the functional roles of the brain-specific proteoglycans in the development of the nervous tissue because they are the major extracellular matrix components in the tissue.

Previously, we identified a phosphate-buffered saline-soluble chondroitin sulfate proteoglycan with a 300-kDa core protein (6B4 proteoglycan) using a monoclonal antibody (MAb6B4). Immunohistochemical analysis of the adult rat hindbrain showed that 6B4 proteoglycan is expressed in fairly restricted areas such as pontine nuclei and lateral reticular nucleus. Developmental studies of the rat hindbrain indicated that the expression of 6B4 proteoglycan is spatiotemporally correlated with the circuit formation of the mossy fiber system. In the embryonal rat cerebral cortex, in contrast, 6B4 proteoglycan is expressed on the radial glial fiber, a scaffold for neuronal migration.

Recently, we cloned cDNAs encoding this proteoglycan from a λgt11 rat whole brain cDNA library. Nucleotide sequence analysis of the isolated cDNA clones revealed that 6B4 proteoglycan is highly homologous to human receptor-like protein tyrosine phosphatase (PTPase), PTPζ (also known as RPTPβ). In parallel, Maurel et al. reported a cDNA encoding a chondroitin sulfate proteoglycan termed phosphacan, from rat brain, and it turned out that 6B4 proteoglycan is identical to phosphacan. The cDNA analysis including our own revealed that 6B4 proteoglycan/phosphacan is an alternatively spliced extracellular variant of PTPζ. We then attempted to identify proteoglycan-type PTPases in the rat brain, where many types of proteoglycans and PTPases are known to be present.

Membrane-bound proteoglycan fractions were obtained from the postnuclear membrane preparation of the 8-day-old rat brain by DEAE ion exchange chromatography and CsCl density gradient centrifugation. The isolated proteoglycan fraction contained a high PTPase activity with typical PTPase characteristics. Protein renaturation experiments from SDS gels demonstrated that chondroitin

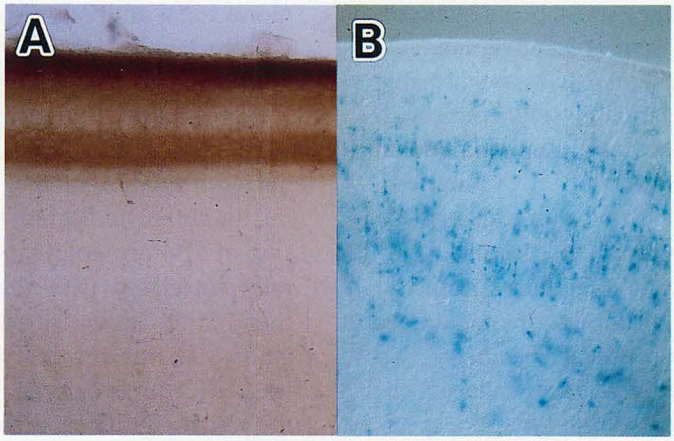


Fig. 1 A) Lamina-specific projection of retinal axons in the chick optic tectum. The anterogradely-labeled retinal terminals (brown) make synapses in some restricted laminae. B) Recombinant adenovirus-mediated gene transfer into the chick optic tectum. Adenovirus carrying lacZ (E. coli β-galactosidase) was injected into the mesencephalic ventricle. After one week, many cells in various laminae express the introduced gene (blue).

sulfate proteoglycans with a 380- and 170-kDa core proteins carried the PTPase activity. The proteoglycan with 380-kDa core protein was identified as PTPζ with the specific antibodies. The PTPase with a 170-kDa core protein did not cross-react with the antibodies against PTPζ, suggesting that these two are not closely related structurally. These findings show that early postnatal rat brain indeed contain multiple proteoglycan-type PTPases.

Proteoglycans may play crucial roles in cell adhesion, motility, growth and differentiation through the process of binding to various extracellular matrix molecules and growth factors. On the other hand, the level of protein tyrosine phosphorylation is regulated by cellular interactions with these molecules through the modulation of protein tyrosine kinase and PTPase activities. Our findings that some membrane-bound PTPases are proteoglycans would explain the functional overlap between the two classes of molecules.

Publication List:

Maeda, N., Hamanaka, H., Shintani, T., Nishiwaki, T. and Noda, M. (1994) Multiple receptor-like protein tyrosine phosphatases in the form of chondroitin sulfate proteoglycan. *FEBS Lett.* **354**, 67–70.

Maeda, N., Hamanaka, H., Oohira, A. and Noda, M. (1995) Purification, characterization and developmental expression of a brain-specific chondroitin sulfate proteoglycan, 6B4 proteoglycan/phosphacan. *Neuroscience.* in press.

Oohira, A., Matsui, F., Watanabe, E., Kushima, Y. and Maeda, N. (1994) Developmentally regulated expression of a brain specific species of chondroitin sulfate proteoglycan, neurocan, identified with a monoclonal antibody 1G2 in the rat cerebrum. *Neuroscience.* **60**, 145–157.

DIVISION OF CELLULAR REGULATION

Professor: Norio Murata
Associate Professor: Hidenori Hayashi
Research Associates: Takao Kondo
Ikuo Nishida
Dmitry A. Los
Atsushi Sakamoto
Monbusho Foreign Scientist: Laszlo Mustardy[1)]
Visiting Scientists: Yasushi Tasaka
Byoung Yong Moon[2)]
Michael P. Malakhov[3)]
Zoltan Gombos[4)]
Carl H. Johnson[5)]
Graduate Students: Katsuzo Noguchi
Patcharaporn Deshnium
Sayamrat Panpoom
Technical staffs: Sho-Ichi Higashi
Miki Ida

([1)] from Biological Research Center, Szeged, Hungary)
([2)] from Inje University, Pusan, Korea)
([3)] from Plant Physiology Institute, Moscow, Russia)
([4)] from Biological Research Center, Szeged, Hungary)
([5)] from Vanderbilt University, TN, USA)

The research effort of this division is directed toward understanding the tolerance and acclimation of plants to temperature extremes, with particular emphasis on the molecular mechanisms by which plants acclimate to or tolerate temperature conditions. In 1994, several significant achievements were made in the following areas with cyanobacteria and higher plants as experimental materials.

I. Molecular cloning and characterization of cyanobacterial desaturases.

Higher plants and most cyanobacteria have high levels of membrane lipids containing polyunsaturated fatty acids, which are important in their response to ambient temperature. We have isolated the *desA*, *desB*, and *desC* genes of *Synechocystis* sp. PCC 6803 that encode the Δ12, ω3, and Δ9 desaturases, respectively, of the acyl-lipid type. We overexpressed the *desA* and *desC* genes in *Escherichia coli* using the bacteriophage T7 RNA polymerase system. The desaturases, thus overexpressed in *E. coli*, were active *in vitro* when reduced ferredoxin was supplemented as an electron donor. The mode of fatty acid desaturation in the transformed *E. coli* cells demonstrates that the Δ9 and Δ12 desaturases are specific to the *sn*-1 position of the glycerol moiety and the C18 fatty acids, and are nonspecific with regard to the polar head group of the lipid.

II. Regulation of the expression of the desaturase genes.

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

We have shown that the levels of the transcripts of the *desA*, *desB*, and *desD* (for Δ6 desaturase) genes increase about 10-fold during a downward shift of temperature from 34°C to 22°C. However, the level of the transcript of the *desC* gene remains constant after the temperature shift, suggesting that the expression of the gene is constitutive. Determination of the 5′-termini of the desaturases indicated that the sites of initiation of

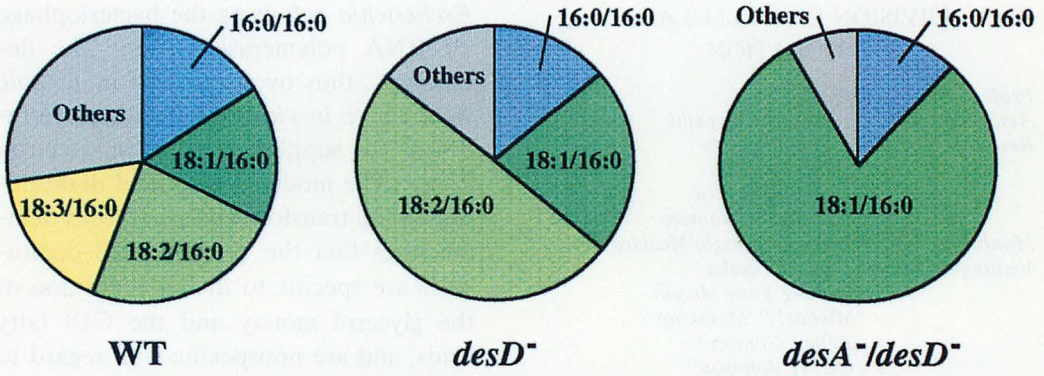


Figure 1. Changes in composition of lipid molecular species by step-wise depletion of desaturases in *Synechocystis* sp. PCC 6803. WT, wild-type strain; *desD*⁻, a mutant of Δ6 desaturase in which the *desD* gene was disrupted by insertion of a chloramphenicol resistance gene cartridge; *desA*⁻/*desD*⁻, double mutant of Δ6 and Δ12 desaturases in which the *desA* gene in the *desD*⁻ mutant was mutated by insertion of a kanamycin resistance gene cartridge.

transcription are significantly similar among all four desaturase genes. Identification of the regulatory elements of the desaturase genes and cloning of their transacting factors are under way.

III. Importance of membrane-lipid unsaturation in tolerance to low-temperature photoinhibition.

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 mutation greatly modified the extent of unsaturation of the fatty acids of the membrane lipids (Figure 1). In the wild-type strain, each of the saturated, monounsaturated, diunsaturated, and triunsaturated lipid molecules amounted to 15% of the total membrane lipids; in the *desD*⁻ mutant, the triunsaturated lipid molecule was not present; in the *desA*⁻/*desD*⁻ mutant, 80% of the total membrane lipids were monounsaturated lipid molecules and only 15% were fully saturated molecules. These decreases in the unsaturation of membrane lipids greatly reduced the tolerance of the cyanobacterium to low temperature in the presence of light.

These results together with other observations suggest that unsaturated fatty acids play an important role in protection of the photosynthetic machinery meant to protect against low-temperature photoinhibition. Because 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, we separated the two processes using protein synthesis inhibitors. The results demonstrated that the unsaturation of the membrane lipids has no effect on the light-induced inactivation process, but accelerates the recovery of the photosystem II complex from the photoinactivated state. Essentially, the same results were obtained from studies of a higher plant, namely, tobacco, in which the level of unsaturation of the thylakoid membrane lipids was genetically modulated by overexpression of glycerol-3-phosphate acyltransferase from squash.

IV. Heat stability of photosynthesis.

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

We have studied an effect for the heat stability of photosynthesis in the cyanobacterium, *Synechococcus* sp. PCC 7002. When the thylakoid membranes isolated from the cyanobacterial cells were treated with a low concentration of Triton X-100, the heat stability of oxygen evolution was decreased by 4°C. From the extracts obtained with Triton X-100, we purified a protein that re-established the heat stability of oxygen evolution. The protein was identified as cytochrome *c*-550 which has a low redox potential and a molecular mass of 16 kDa. These results indicate that cytochrome *c*-550 is involved in the mechanism of heat stability of oxygen evolution and, therefore, in the heat stability of photosynthesis. We isolated the gene encoding this cytochrome of *Synechococcus* sp. PCC 7002. Disruption of this gene in *Synechococcus* sp. PCC 7002 is in progress in this laboratory. We also studied the heat tolerance of photosynthesis in cultured soybean cells. The heat stability of oxygen evolution in these cells was enhanced by 3°C upon an increase of growth temperature from 25°C to 35°C.

V. Two genes homologous to *groEL* in cyanobacteria.

GroEL is a highly conserved heat shock protein in prokaryotes and a homologue of the eukaryotic heat shock protein with a molecular mass of about 60 kDa (HSP60). These heat shock proteins act as molecular chaperones that assist proper folding and assembly of other proteins. We discovered two kinds of *groEL* homologue in *Synechococcus* sp. PCC 7002 and designated them as *groEL*-α and *groEL*-β. The *groEL*-α gene forms an operon together with the upstream component of the *groES* gene, as is the case with the *groEL* of *E. coli*. The *groEL*-β gene is not accompanied by the *groES* gene, but *groEL*-β retains the carboxyl terminal repeat of Gly-Gly-Met as in the *E. coli* GroEL.

We mutated the *groEL*-β gene in *Synechococcus* sp. PCC 7002 by inserting an antibiotic resistance gene cartridge and examined the heat shock response of the mutant cells. The heat shock response was greatly reduced in the *groEL*-β mutant cells. This was evaluated from examining the viability at high temperatures after heat shock treatment, for example, viability at 48°C after exposure to 45°C. These results demonstrate that not only the *groEL*-α but also the *groEL*-β gene products contribute to the heat shock response in the cyanobacterium. However, the thermal tolerance of photosynthetic oxygen evolution was not modified by the *groEL*-β mutation, indicating that *GroEL*-β does not contribute to the thermal tolerance of photosynthesis.

VI. Genetic modification of salinity tolerance of a cyanobacterium.

Under saline conditions, some plants produce compatible solutes to avoid deleterious effects caused by salt intolerance. Glycine betaine, a quaternary ammonium compound, is one of such solute found in halotolerant plants and bacteria. We discovered that this compound protects the photosystem II protein

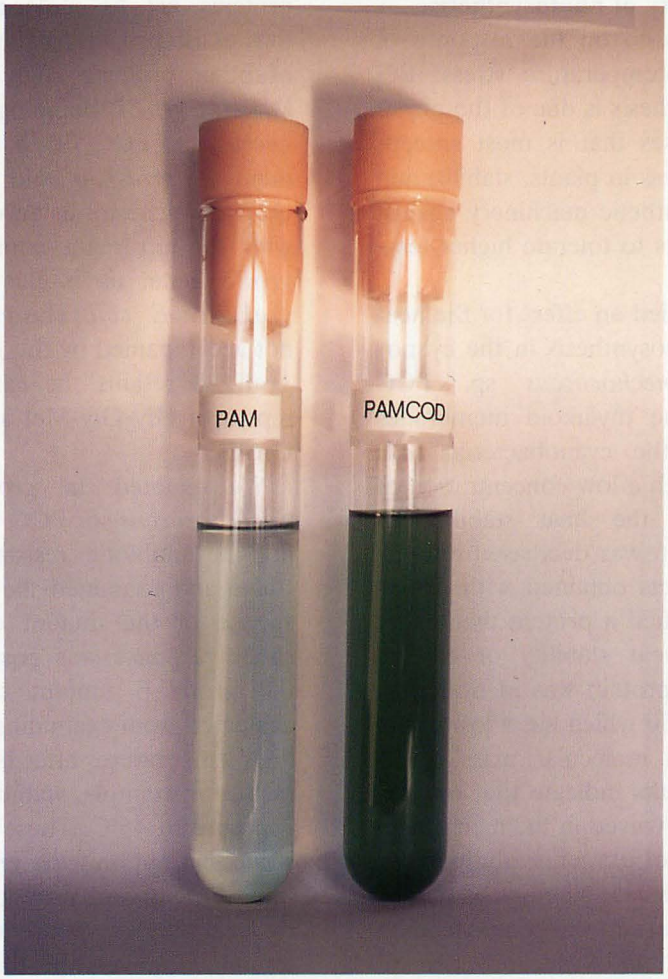


Figure 2. Growth under saline conditions in control cells (PAM) and transformed cells (PAMCOD) in which the *codA* gene (for choline oxidase) was introduced in the chromosome of *Synechococcus* sp. PCC 7942 and was overexpressed under control of the ConII promoter. Cells were grown at 30°C for 6 days in a medium containing 0.4 M NaCl.

complex from inactivation of oxygen evolution in saline conditions. To examine the effect of glycine betaine on photosynthesis *in vivo*, we transformed the salinity-sensitive cyanobacterium, *Synechococcus* sp. PCC 7942, with the *codA* gene for choline oxidase of *Arthrobacter globiformis*, which can oxidize choline into glycine betaine. The transformed cells accumulated glycine betaine to a concentration of about 85 mM and grew in the medium containing 0.4 M NaCl (lethal conditions for the wild-type cells) (Figure 2). The salinity tolerance of the oxygen-evolving activity was enhanced by the transformation. These observations demonstrate the *in vivo* action of glycine betaine in protecting the cyanobacterial cells from salinity-induced stresses.

Publication List:

(1) Original papers

Gombos, Z., Wada, H. and Murata, N. (1994) The recovery of photosynthesis from low-temperature photoinhibition is accelerated by the unsaturation of membrane lipids: a mechanism of chilling tolerance. *Proc. Natl. Acad. Sci. U.S.A.* **91**, 8787–8791.

Gombos, Z., Wada, H., Hideg, E. and Murata, N. (1994) The unsaturation of membrane lipids stabilizes photosynthesis against heat stress. *Plant Physiol.* **104**, 563–567.

Kondo, T. and Ishiura, M. (1994) Circadian rhythms of cyanobacteria: Monitoring the biological clock of individual colonies by bioluminescence. *J. Bacteriol.* **176**, 1881–1885.

Kondo, T., Tsinoremas, N.F., Golden, S.S., Johnson, C.H., Kutsuna, S. and Ishiura, M. (1994) Circadian clock mutants of cyanobacteria. *Science* **266**, 1233–1236.

Los, D.A. and Murata, N. (1994) Low-temperature induced accumulation of the desaturase gene transcript in *Synechocystis* PCC 6803 results from both acceleration of transcription and increase in mRNA stability. *Russian J. Plant Physiol.* **41**, 147–151.

Malakhov, M.P., Wada, H., Los, D.A., Semenenko, V.E. and Murata, N. (1994) A new type cytochrome *c* from *Synechocystis* PCC 6803. *J. Plant Physiol.* **144**, 259–264.

Nishiyama, Y., Hayashi, H., Watanabe, T. and Murata, N. (1994) Photosynthetic oxygen evolution is stabilized by cytochrome *c*-550 against heat inactivation in *Synechococcus* sp. PCC 7002. *Plant Physiol.* **105**, 1313–1319.

Sakamoto, T., Los, D.A., Higashi, S., Wada, H., Nishida, I., Ohmori, M. and Murata, N. (1994) Cloning of ω3 desaturase from cyanobacteria and its use in altering the degree of membrane-lipid unsaturation. *Plant Mol. Biol.* **26**, 249–264.

Sakamoto, T., Wada, H., Nishida, I., Ohmori, M. and Murata, N. (1994) Identification of conserved domains in the Δ12 desaturase of cyanobacteria. *Plant Mol. Biol.* **24**, 643–650.

Sakamoto, T., Wada, H., Nishida, I., Ohmori, M. and Murata, N. (1994) Δ9 acyl-lipid desaturase of cyanobacteria: Molecular cloning and substrate specificities in terms of fatty acids, *sn*-positions, and polar head groups. *J. Biol. Chem.* **269**, 25576–25580.

Wada, H., Gombos, Z. and Murata, N. (1994) Contribution of membrane lipids to the ability of the photosynthetic machinery to tolerate temperature stress. *Proc. Natl. Acad. Sci. U.S.A.* **91**, 4273–4277.

(2) Reviews

Murata, N. (1994) Genetic and temperature-induced modulation of cyanobacterial membrane lipids. *In* Temperature Adaptation of Biological Membranes (A.R. Cossins, ed.), Portland Press, London, pp. 155–162.

Murata, N. (1994) Genetic engineering of phosphatidylglycerol and chilling sensitivity in higher plants. *In* Temperature Adaptation of Biological Membranes (A.R. Cossins, ed.), Portland Press, London, pp. 163–167.

(3) Proceeding

Hayashi, H., Nishida, I., Ishizaki-Nishizawa, O., Nishiyama, Y. and Murata, N. (1994) Genetically engineered modification of plant chilling sensitivity and characterization of cyanobacterial heat shock proteins. *In*

Biochemical and Cellular Mechanisms of Stress Tolerance in Plants (J.H. Cherry, ed.), Springer-Verlag, Berlin, pp. 543–555.

DIVISION OF BIOLOGICAL REGULATION AND PHOTOBIOLOGY (ADJUNCT)

Professor (adjunct): Kimiyuki Satoh
Associate Professor (adjunct): Hirokazu Kobayashi
Research Associates: Satoru Tokutomi, Noritoshi Inagaki
Graduate Students: Kyoichi Isono, Yoshihiro Narusaka*, Tatsuya Tomo*, Akira Matsumoto**

*(*from Okayama University)*

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 reinhardtii*. 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 function of 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 multi-component pigment-protein complexes.

I. Structural organization of photosystem II reaction center

The structure and molecular interactions of the primary donor in the photosystem II reaction center have been investigated by detecting light-induced FT-IR difference spectra upon the formation of its triplet state. From the band positions of the keto and carbomethoxy C=O stretches, the hydrogen-bonding properties of the two chlorophylls of P-680 were found to be asymmetrical; in one chlorophyll (P1) both the keto and carbomethoxy C=O groups form hydrogen bonds, while in the other chlorophyll (P2) the keto C=O is not hydrogen-bonded whereas the carbomethoxy C=O probably is hydrogen-bonded. Considering the orientation of P-680 analyzed by EPR and the structure of bacterial reaction center determined by X-ray crystallography, together with the sequence homology between the D1 and D2 subunits of photosystem II and the L and M subunits of purple bacteria, a model of the structure of P-680 and its interactions with apoproteins has been proposed (Noguchi, T., Inoue, Y. and Satoh, K., 1993, *Biochemistry*, **32**, 7186–7195). Site-directed modification

using *Chlamydomonas reinhardtii* of the amino acid side chains on D1 protein presumably involved in the hydrogen-bonding interaction (Ser-191 and Thr-192) has been succeeded and the analysis of these mutants is now in progress in order to prove this hypothesis.

Chemical cross-linking analysis has also been conducted for the isolated photosystem II reaction center to analyze the gross structure; *i. e.*, the nearest neighbors of the D1 and D2 subunits and amino acid residues in cross-contact in the reaction center complex.

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

(1) The D1 subunit of 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 processing has been identified and the partial amino acid sequences have been determined for spinach. Based on these data, a gene coding for the enzyme has been identified and sequenced (paper in preparation).

(2) The recognition signal on substrate was analyzed for the C-terminal processing protease using substituted synthetic oligopeptides corresponding to the C-terminal sequence of D1 precursor protein. The efficiency of these synthetic oligopeptides, both as substrate and as inhibitor for the C-terminal cleavage, were examined. The result suggests that the presence of a hydrophobic amino acid side chain at the -2 position, in addition to the secondary structure around the cleavage site, is crucial in the recognition (Taguchi, F., Yamamoto, Y. and Satoh, K., *J. Biol. Chem.*, in press).

(3) 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. The isolated chloroplasts was shown to accumulate translation intermediate(s) of D1 protein in the presence of externally added ATP. The result suggested that the translation of D1 protein at the specific stage of polypeptide elongation requires factor(s) caused by illumination, in addition to energy supply.

(4) Photo-tolerant mutants of an unicellular cyanobacterium *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, in order to analyze the damage-repair cycle of D1 protein in photoinhibition of photosystem II reaction center. The mechanistic analysis of photo-tolerance caused by these amino acid displacements on D1 protein is now in progress. **Figure** shows a photo-tolerant mutant (lower), together with the control strain (upper), grown at the photon flux density of 50μE (left) and those after exposure to 320μE (right).

(5) Synthesis of some photosystem proteins are regulated by light signals received by phytochrome. Molecular mechanism of the photoreception of phytochrome were studied by circular dichroism and resonance Raman spectroscopies. The former shows that the phototransformation of pea phytochrome from Pr to Pfr induces unfolding of α-helix in the 6-kDa N-terminal domain which is requisite for its function. The latter proposes that a proton associated to the chromophore in Pr migrates to the apoprotein during a phototransformation process(s) to the bleached intermediate,

which presumably triggers conformational changes in the molecule.

Publication List:

Deforce, L., Tokutomi, S. and Song, P.-S. (1994) Phototransformation of pea phytochrome A induces an increase in α-helical folding of the apoprotein: Comparison with a monocot phytochrome A and CD analysis by different methods. *Biochemistry* **33**, 4918–4922.

Mizutani, Y., Tokutomi, S. and Kitagawa, T. (1994) Resonance Raman spectra of the intermediates in phototransformation of large phytochrome: Deprotonation of the chromophore in the bleached intermediate. *Biochemistry* **33**, 153–158.

Nozawa, T., Kobayashi, M., Wang, Z.-Y., Itoh, S., Iwaki, M., Mimuro, M. and Satoh, K. (1994) Magnetic circular dichroism investigation on chromophores in reaction centers of photosystem I and II of green plant photosynthesis. *Spectrochimica Acta*, in press.

Tomo, T. and Satoh, K. (1994) Nearest neighbor analysis of D1 and D2 subunits in the photosystem II reaction center using bifunctional cross-linker, hexamethylene diisocyanate. *FEBS Lett.* **351**, 27–30.

DIVISION OF BEHAVIOR AND NEUROBIOLOGY (ADJUNCT)

Professor: Masatoshi Takeichi
Research Associate: Akinao Nose
Kazuaki Tatei
Institute Research Fellow: Tomoko Tominaga
Graduate Student: Tatsuo Umeda
Visiting Scientist: 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.

By using an enhancer trap method, several genes have previously been identified that are expressed in small subsets of muscle fibers prior to innervation, and are thus good candidates for such recognition molecules. Two of them, *connectin* and *Toll*, were shown to encode cell recognition molecules which belong to the leucine-rich repeat (LRR) family. In paticular, Connectin is expressed not only on a subset of muscle fibers (primarily lateral muscles) but also on the axons and growth cones of the very motoneurons which innervate these muscles (primarily SNa motoneurons, see Figure 1) 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. We are currently studying the function of Connectin by molecular genetic methods and also trying to clone novel genes implicated in the neuromuscular connectivity.

I. Molecular genetic analysis of the function of Connectin

To study the role of Connectin in vivo, we first ectopically expressed Connectin on muscle fibers that normally do not express the molecule by using Toll enhancer. Toll is expressed on ventral muscles 6, 7, 14–17 and 28. These muscle fibers do not normally express Connectin, and are innervated by SNb motoneurons. We used a 7 kb Toll upstream sequence sufficient for the muscle expression to express Connectin on these muscle fibers in P-element mediated transgenic flies (Figure 1).

The analysis of the transformants (*Toll-connectin*) showed that the development of SNb is abnormal. The SNb growth cones change their morphology and their trajectory as they encounter ectopic Connectin-positive ventral muscles, displaying "bypass", "detour" and "stall" phenotypes. Moreover, SNb synapse formation is prevented by Connectin expression on ventral muscles. These results revealed a repulsive function for Connectin during motoneuron growth cone guidance and synapse formation.

In *Toll-connectin*, we did not observe any abnormality in the SNa motoneurons. To further study the possible role of Connectin as an attractive guidance molecule for SNa, we then ectopically expressed Connectin on all muscles by

using MHC (myosin heavy chain) promoter (*MHC-connectin*, see Figure 1). In *MHC-connectin*, SNa nerves were observed to send an extra axon branch that

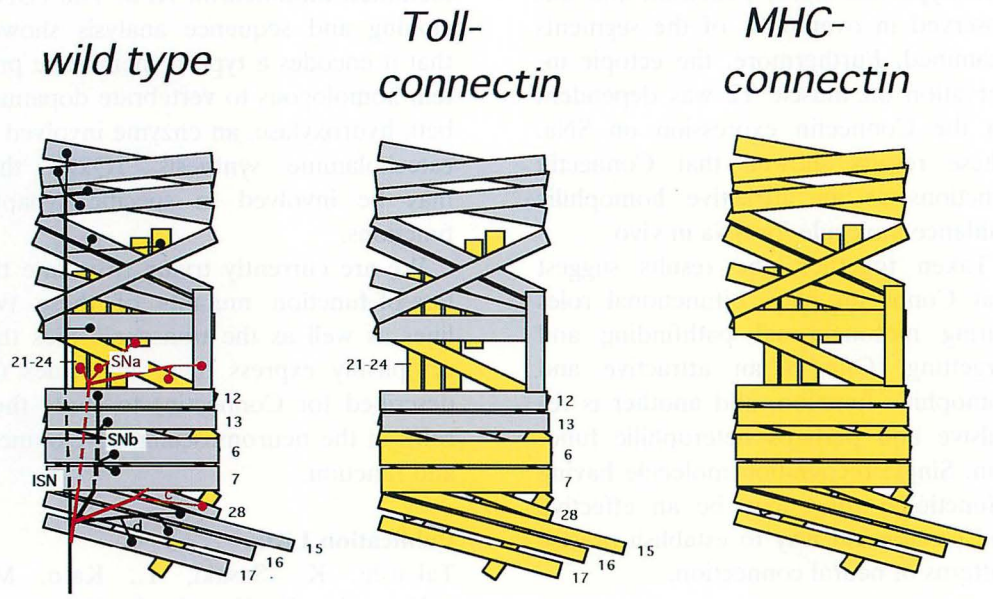


Fig. 1. Ectopic expression of Connectin.
Schematic diagram showing wild type and ectopic Connectin expression on embryonic muscles and motor nerves. In wild type, Connectin is expressed on a subset of motoneurons (SNa and SNc, shown in red) and muscles (primarily lateral muscles, shown in yellow). In *Toll-connectin*, Connectin is ectopically expressed on a subset of ventral muscles (also shown in yellow). In *MHC-connectin*, Connectin is ectopically expressed on all muscles.

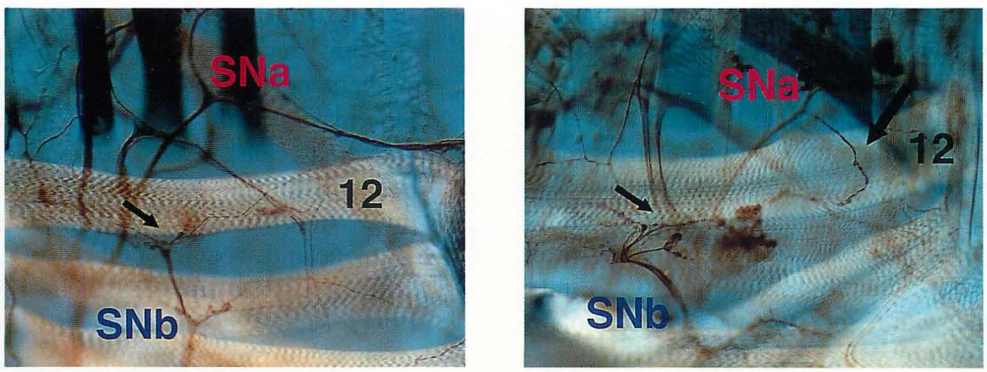


Fig. 2. Connectin functions as an attractive guidance molecule.
In wild type, muscle 12 is innervated by SNb motoneurons (short arrow). Connectin-positive SNa motoneurons innervate more distal muscles. Ectopic Connectin expression on muscle 12 in *MHC-connectin* changed SNa trajectories: SNa sends an extra axon branch that forms an ectopic nerve ending on muscle 12 (large arrow).

forms an ectopic nerve endings on muscle 12, a muscle they would never innervate in wild type (Figure 2). 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 homophilic guidance molecule for SNa in vivo.

Taken together, the results suggest that Connectin plays bifunctional roles during motoneuronal pathfinding and targetting. One is an attractive and homophilic function, and another is repulsive and perhaps heterophilic function. Single recognition molecule having bifunctional roles may be an effective and economical way to establish precise patterns of neural connection.

II. Cloning of novel genes implicated in the neuromuscular connectivity in Drosophila

We are conducting molecular and genetic analysis of two other enhancer trap lines that are expressed in specific subsets of muscles and/or motoneurons. One of the line, AN34 expresses the reporter gene (β-gal) in a single muscle fiber18 per hemisegment. The remarkable specificity in its expression pattern (one out of 30 muscle fibers) makes it a good candidate for the muscle target recognition molecule. The cDNA cloning and sequencing revealed that AN34 encodes a putative 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.

Another line, rQ224 expresses β-gal in a small subset of neurons including an identified motoneuron RP3. The cDNA cloning and sequence analysis showed that it encodes a type II membrane protein homologous to vertebrate dopamine beta-hydroxylase, an enzyme involved in catecholamine synthesis. rQ224 thus may be involved in specific synaptic functions.

We are currently trying to isolate the loss-of-function mutants of these two lines as well as the transgenic flies that ectopically express these molecules (as described for Connectin) to study their roles in the neuromuscular development and function.

Publication List:

Takaishi, K., Sasaki, T., Kato, M., Yamochi, W., Kuroda, S., Nakamura, T., Takeichi, M. and Takai, Y. (1994) Involvement of Rho p21 small GTP-binding protein and its regulator in the HGF-induced cell motility. *Oncogene* **9**, 273–279.

Shibamoto, S., Hayakawa, M., Takeuchi, K., Hori, T., Oku, N., Miyazawa, K., Kitamura, N., Takeichi, M. and Ito, F. (1994) Tyrosine phosphorylation of b-catenin and plakoglobin enhanced by hepatocyte growth factor and epidermal growth factor in human carcinoma cells. *Cell Adhesion & Commun.* **1**, 295–305.

Uchida, N., Shimamura, K., Miyatani, S., Copeland, N.G., Gilbert, D.J., Jenkins, N.A. and Takeichi, M. (1994) Mouse aN-catenin, two isoforms, specific expression in the nervous sytem and chromosomal localization of the gene. *Develop. Biol.* **163**, 75–85.

Shiomi, K., Takeichi, M., Nishida, Y., Nishi, Y. and Uemura, T. (1994) Alternative cell fate choice induced by low-level expression of a regulator of protein phosphatase 2A in the Drosophila peripheral nervous system. *Development* **120**, 1591–1599.

Uchiyama, N., Hasegawa, M., Yamashina, T., Yamashita, J., Shimamura, K. and Takeichi, M. (1994) Immunoelectron microscopic localization of E-cadherin in dorsal root ganglia, dorsal root and dorsal horn of postnatal mice. *J. Neurocytol.* **23**, 460–468.

Shimamura, K., Hirano, S., McMahon, A.P. and M. Takeichi. (1994) Wnt-1-dependent regulation of local E-cadherin and aN-catenin expression in the embryonic mouse brain. *Development* **120**, 2225–2234.

Watabe, M., Nagafuchi, A., Tsukita, S. and Takeichi, M. (1994) Induction of polarized cell-cell association and retardation of growth by activation of the E-cadherin-catenin adhesion system in a dispersed carcinoma line. *J. Cell Biol.* **127**, 247–256.

Tong, K.I., Yau, P., Overduin, M., Bagby, S., Porumb, T., Takeichi, M. and Ikura, M. (1994) Purification and spectroscopic characterization of a recombinant amino-terminal polypeptide fragment of mouse epithelial cadherin. *FEBS letters* **352**, 318–322.

Nose, A., Takeichi, M. and Goodman, C.S. (1994) Ectopic expression of connection reveals a repulsive function during growth cone guidance and synapse formation. *Neuron* **13**, 525–539.

Oda, H., Uemura, T., Harada, Y., Iwai, Y. and Takeichi, M. (1994) A Drosophila homolog of cadherin associated with Armadillo and essential for embryonic cell-cell adhesion. *Develop. Biol.* **165**, 716–726.

Hatta, M. and Takeichi, M. (1994) Complex cell type-specific transcriptional regulation by the promoter and an intron of the mouse P-cadherin gene. *Develop. Growth & Differ.* **36**, 509–519.

LABOLATORY OF GENE EXPRESSION AND REGULATION

Head: Goro Eguchi

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

DIVISION OF GENE EXPRESSION AND REGULATION I

Associate Professor: Kiyotaka Okada
Research Associate: Sumie Ishiguro
Graduate Students: Azusa Yano
Yoichi Ono
Toshiro Ito[1)]
Takuji Wada
Tokitaka Oyama[1)]
Sinichiro Sawa[2)]
Technical Staff: Hideko Nonaka
Akiko Kawai

([1)] from Kyoto University)
([2)] from Nagoya University)

The principal interest of this laboratory is molecular genetic studies on the regulatory systems of organ development and on growth control by several environmental stimuli in higher plants. For these studies, we have mainly used a small crusifer, *Arabidopsis thaliana.* This plant is called "botanical *Drosophila*", because it has some remarkable features, such as a small genome size (1×10^8 base pairs per haploid), short life-cycle (5—6 weeks), small size (20—30 cm in height), and ease of propagation. These features make the plant ideally suited for genetic and molecular biological studies. In addition, more than 360 loci and more than 650 RFLP and RAPD markers are mapped on 5 chromosomes. Experimental techniques such as transformation, regeneration of transgenic plants and gene tagging have been improved. Using this plant, we have isolated and characterized many mutants defective in flower development and morphogenesis or in root formation and responses toward physical stimuli such as gravity, light or touching.

I. Development and morphogenesis of flowers

The process of flower development can be divided into four major steps: phase transition from vegetative to reproductive growth, formation of inflorescence meristem, formation and identity determination of floral organs, and growth and maturation of floral organs. Several different types of signaling mechanism, between or within cells, must have important roles in each step of flower development, because each step requires cell division, cell growth and cell differentiation in a concerted fashion. In order to unveil the signaling mechanism and its genetic regulatory systems, we are carrying out genetic, biochemical, anatomical and physiological analyses on the processes of flower development using *A. thaliana.*

The *filamentous flower* (*fil*) mutant shows several structural defects in flowers and inflorescences. After flower initiation, *fil* mutant elongates an inflorescence axis normally, and forms about 10 flowers that show some structural abnormalities (phase 1). Then formation of floral buds is stopped at the inflorescence meristem, and instead, more than 10 filaments and more than 10 sepal-like structures are formed (phase 2). After forming the phase 2 cluster, a cluster of flowers is formed again (phase 3). One possible explanation of the phase shift is that the amount of the factor(s) or the amount of receptor molecules of the factor(s) changes to high-low-high as plants grow. In order to examine the genetic relationships of *FIL* gene with other known flower genes, we have constructed a series of double mutants. The results indicate that *FIL* gene is required for formation and maintenance of floral meristem in combination with *APETALA 1, LEAFY* and *CAULIFLOWER.*

The *AGAMOUS* (*AG*) gene of *A. thaliana* is a homeotic gene involved in the development of stamens and carpels.

This gene encodes a putative DNA-binding protein sharing a homologous region with the DNA-binding domains, MADS boxes, of yeast MCM1 and mammalian SRF. We confirmed that the AG protein is localized in the nuclei of the cell in stamen- and carpel-primodia. In order to identify AG target genes, we isolated the DNA fragments bound by AG protein in native chromatin by immunopurification, and then identified genes within or near the isolated fragments. Characterization of these genes is in progress.

II. Development and stimulus-response reactions in root

The root of *A. thaliana* has many advantages of simplicity, transparency and strict organization of cells to analyze the development of roots. One of the thick root mutant, RH32, has 10 cortical cells in the root, whereas wild type has 8 cortical cells. The epidermal cells of RH32 mutant in different tissues, such as root, hypocotyl, stem and sepal, are rounder than those of wild type. RFLP mapping has shown that RH32 gene is located on the lower portion of chromosome 1.

A T-DNA-tagged mutant K293 has a few root hairs of the primary root. Map position of K293 gene is on the lower portion of chromosome 2. We are now in progress of cloning of this gene.

Normal root hairs of *A. thaliana* are short and formed perpendicular to the root surface, when roots grow on agar plates stood vertical ("agar" form). The long and tilted root hairs are formed when roots grow in the air ("air" form). We showed that the "agar" form hairs are formed by the cells that have passed through the elongation zone, whereas the "air" form hairs are generated within the elongation zone. Mutants defective in these morphological alteration of root hairs are isolated and classified into three categories: namely, mutants generating root hairs of either the "agar" or the "air" form in both condition (class I); mutants defective in the "agar" form hair formation but normal in the "air" form hair formation (class II); and mutants defective in both hair formation (class III).

Roots alter their growth direction when their relative orientation against gravity is changed (gravitropic response), when they are illuminated from aside (phototropic response), or when they encounter obstacles (obstacle-escaping response). These responses were analyzed using agar plates. Young seedlings grown on vertical agar plates have roots which grow straight downward on the agar surface. When the plates are put aside, roots bend 90° and grow to the altered direction of gravity. If the plates are covered with black cloth and illuminated from a side, roots grow to the opposite direction of the incoming light. On the surface of agar plates which are set at an angle of 45° to the vertical, the roots exhibit a wavy growth pattern that is caused by periodic reversion of rotation of the root tip. Using these systems, mutants which show abnormal graviresponse, photoresponse, or obstacle-escaping responses were isolated. Several mutants with abnormal obstacle-escaping response also show abnormal gravitropism and/or phototropism. These results indicate that root gravitropic, phototropic and obstacle-escaping responses share at least in part a common genetic regulatory mechanism. Physiological characterization and molecular cloning of these mutants are in progress.

A mutant *hy5* of *A. thaliana*, which was isolated as a long hypocotyl mutant,

Filamentous flower mutant forms clusters of deformed flowers and of filaments with a sepal-like structure at its top (left). In *leafy* mutant, flowers are homeotically transformed to shoots (middle). A double mutant carrying mutations in both *FILAMENTOUS FLOWER* and *LEAFY* genes fails to generate either flowers or shoots, and instead continues to form filaments and sepal-like structures (right).

shows a variety of abnormalities in development and stimulus-response of roots. *HY5* gene therefore plays a key function in signaling pathways of root morphogenesis as well as photomorphogenesis of hypocotyl. We have isolated a *hy5* mutant allele from T-DNA inserted lines and cloned the putative *HY5* gene. This gene encodes an open reading frame of 168 amino acid residues which include a bZIP motif at the C-terminus. Therefore, *HY5* gene may function as a transcriptional regulator in the signaling pathways.

III. Technical improvement for molecular cloning

In order to isolate the genes responsible for the mutants, some technical improvements for gene tagging systems in *A. thaliana* were attempted. For such experiments, it is absolutely necessary to develop a good, efficient system of transformation mediated by *Agrobacterium.* We carried out so called *in planta* transformation, in which adult *A. thaliana* plants are directly infected with *Agrobacterium* and gene transfer occurs during floral development. In this system, it is expected that problems caused by somaclonal variation are avoided. About 1,100 lines of transformants were generated. Several interesting mutants have been identified and characterized.

Publication List:

Okamoto, H., Yano, A., Shiraishi, H., Okada, K. and Shimura, Y. (1994) Genetic complementation of a floral homeotic mutation, *apetala 3*, with an *Arabidopsis thaliana* gene homologous to *DEFICIENS* of *Antirrhinum majus. Plant Mol. Biol.* **26**, 465–472.

Ito, T., Shiraishi, H., Okada, K. and Shimura, Y. (1994) Two amidophosphoribosyltransferase genes of *Arabidopsis thaliana* expressed in different organs. *Plant Mol. Biol.* **26**, 529–533.

Okada, K. and Shimura, Y. (1994) Genetic analyses of signaling in flower development using *Arabidopsis*. *Plant Mol. Biol.* **26**, 1357–1377.

Okada, K. and Shimura, Y. (1994) Modulation of root growth by physical stimuli. *In Arabidopsis* (E.M. Meyerowitz and C. Somerville, eds.). Cold Spring Harbor Lab. Press, pp.665–684.

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: 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 *E. coli* recombinational hot spots, Hot.

We identified hotspots of recombination (termed Hot) on the *E. coli* chromosome. These sites are specifically activated under *rnh*$^-$ (RNase H-defective) conditions (Nishitani et al., MGG **240**, 307, 1993). 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 (called *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.

If the ds-break is induced by replication fork arrest, physiological effects on the cell would be evident. To elucidate effects of fork arrest at *Ter* sites, we constructed an *E. coli* strain, termed a blocked strain, in which oligonucleotides with the *TerA* sequence were inserted into the *lacZ* gene in an orientation so as to block the fork of clockwise (normal direction) replication. In this strain, replication forks were expected to be blocked at both the *TerL* in the *lacZ* and at *TerA, D* and *E* sites. While the blocked strain grew somewhat more slowly than a control strain, it had abnormal phenotypes similar of *E. coli dam*$^-$ mutants, i.e., hyper-Rec phenotype, *recA*$^+$- and *recB*$^+$ (*C*$^+$)-dependent growth, and constitutive SOS expression. We propose that the following sequential events may occur in both strains (see Figure 1). A ds-break occurs at the blocked replication fork in the blocked strain and at the ongoing fork in the *dam*$^-$ mutant, through which RecBCD enzyme enters and degrades the ds-DNA molecule, and the degradation product serves as the signal molecule for SOS induction. When RecBCD enzyme meets an appropriately oriented Chi sequence, its DNase activity is converted to recombinase enzyme, which is able to form a new replication fork, recombinationally with RecA and SSB proteins. This model (i) explains the puzzling phenotype of *recA*$^-$ and *recB*$^-$ (*C*$^-$) mutants and SOS-inducing phenotype of *polA, lig* and *dna* mutants under restrictive conditions, (ii) provides an interpretation for the role of the Chi sequence, and (iii) suggests a possible key role for homologous recombination with regard to cell survival following the arrest of DNA replication (Horiuchi and Fujimura; *J. Bacteriol.* in press).

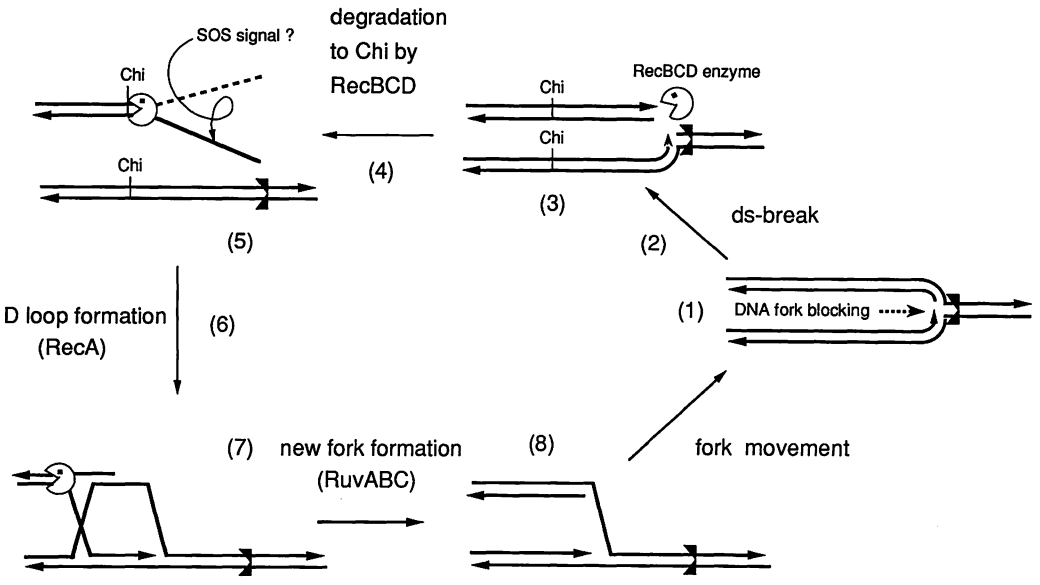


Fig. 1. Model of rescue from blocked DNA replication in *E. coli*, as applicable to a spontaneously stalled replication fork. Essentially the same sequential reactions would probably occur in *S. cerevisiae*.

II. 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. By assaying Sog 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 SOG 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 SOG and the hotspot activity in HOT1, we first isolated mutants defective in Hot1 activity and examined whether these mutations would also affect Sog 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. Genetic analysis of these mutants revealed that all four were recessive for the Sog 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 *FOB* gene by selecting a DNA fragment which had suppressive activity for Hot deficiency of the mutant. The minimal *FOG* plasmid was shown to complement both the Hot^- and Sog^- phenotype of the *fob1-4* mutant, suggesting that both phenotypes are caused by a mutation in the *FOB1* gene. 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. This gene has no homology with any DNA sequence registered in Genbank. 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.

Because there is an extraordinary functional similarity between factors required for the hotspot activity in *E. coli* and *S. cerevisiae*, in both organisms ds-break and repair would occur when the DNA replication fork is arrested at fork blocking sites and probably was spontaneously blocked (Figure 1).

Publication List:

Horiuchi, T., Fujimura, Y., Nishitani, T., Kobayashi, T. and Hidaka, M. (1994) The DNA replication fork blocked at the *Ter* site may be entrance for the RecBCD enzyme into duplex DNA. *J. Bacteriol.* **176**, 4656–4663.

DIVISION OF SPECIATION MECHANISMS

Professor: Tetsuo Yamamori

Our research goal is to understand mechanisms underlying evolution of the nervous system. To approach this question, we are currently focusing on two different subsystems which may respectively represent some molecular and cellular aspects of the system.

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

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

Bazen, first pointed out that the cytokine receptor family may be evolved from an ancestral module of immunoglobulin (1990). Based on Bazen's model 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. 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. It has been demonstrated by Ito and his colleagues (1982) that in the cerebellum the conjunctive stimuli of a parallel fiber 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).

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

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

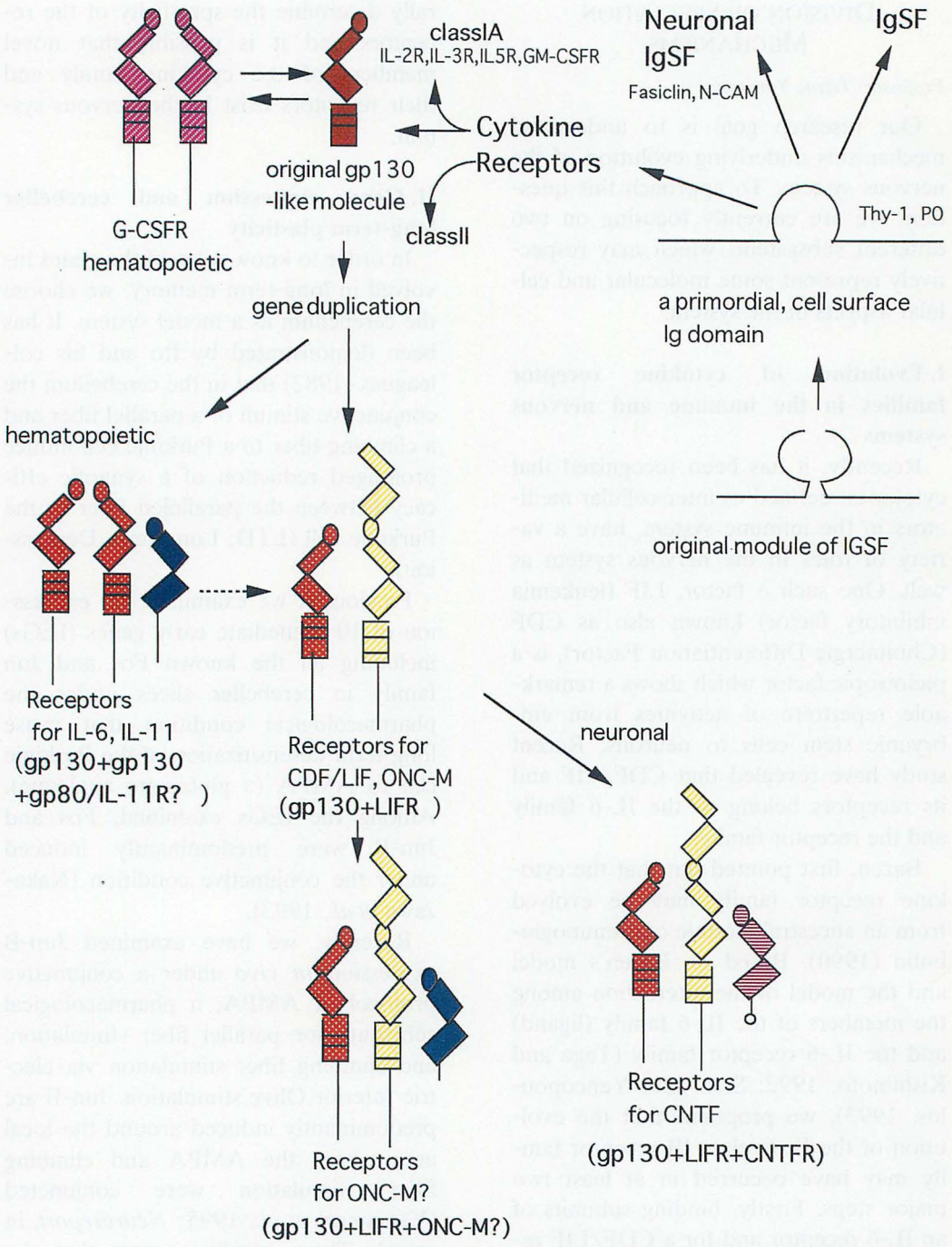


Fig. 1. Evolution of the IL-6 receptor family in the immune and nervous system (a Model). Modified from the figure 4 of Yamamori and Sarai (1994) including recent results from Hilton et al. (EMBO J., 13, 4765–4775, 1994.)

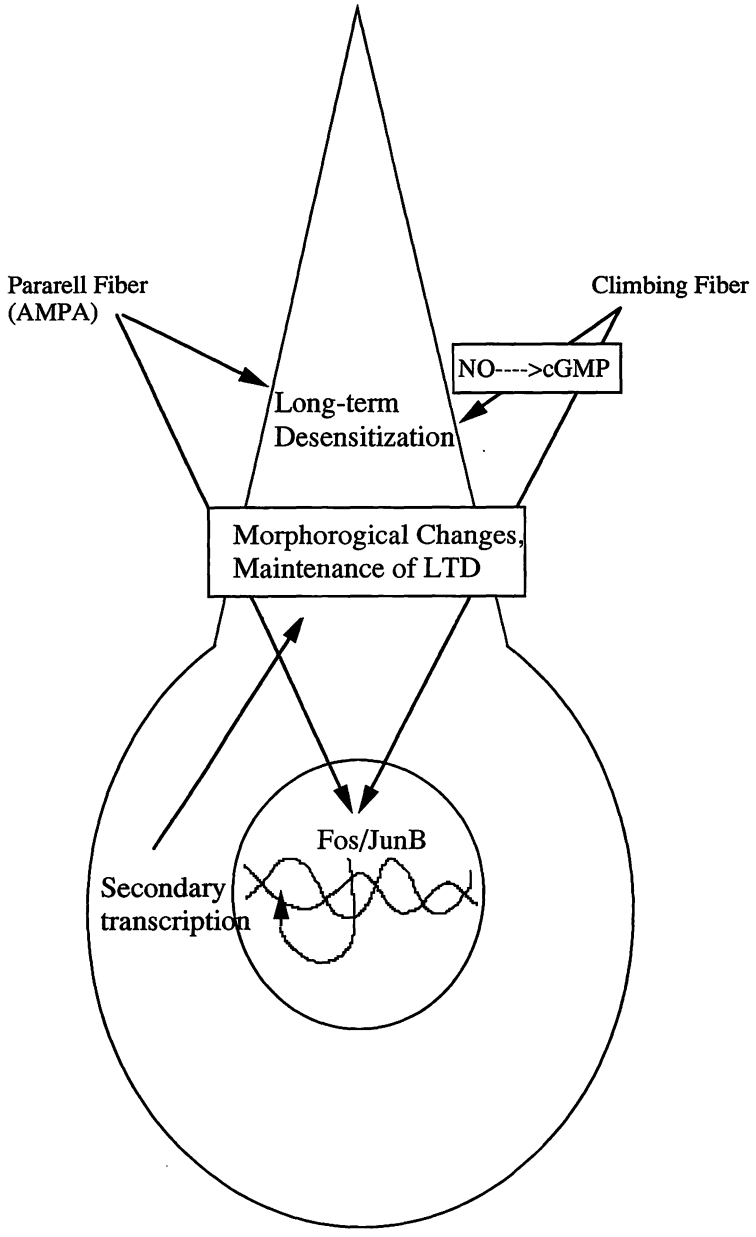


Fig. 2. Gene Expression and Long-term Memory (a Model)

Publication List:

Yamamori, T. and Sarai, A. (1994) Evolution of the IL-6/class IB cytokine receptor family in the immune and nervous systems. *J. Physiology (Paris).* **88**, 165–171.

TECHNOLOGY DEPARTMENT

Head: Hiroyuki Hattori

Common Facility Group
Chief: Kazuhiko Furukawa

Research Support Facilities
Mamoru Kubota
Chieko Nanba
Toshiki Ohkawa
Tomoki Miwa
Kaoru Sawada
Kimiko Yamamiya

Radioisotope Facility
Yousuke Kato
Yosimi Matsuda

Center for Analytical Instruments
Yukiko Kabeya
Yumiko Makino
Sonoko Ohsawa
Takeshi Mizutani

Glassware Washing Facility
(Kazuhiko Furukawa)
(Toshiki Ohkawa)

Research Support Group

Cell Biology Group
Akio Murakami (Unit Chief)
Masayo Iwaki
Maki Kondo
Katsushi Yamaguchi

Developmental Biology Group
Hiroko Kobayashi (Unit Chief)
Miyuki Ohkubo
Sachiko Fukada
Chikako Inoue
Chiyo Takagi
Hisae Urai

Regulation Biology Group
Shoichi Higashi
Miki Ida
Tomoko Mori
Shigemi Ohsugi

Gene Expression and Regulation Group
Hideko Nonaka
Koji Hayashi
Akiko Kawai
Yasushi Takeuchi

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

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

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

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

RESEARCH SUPPORT FACILITY

Head of Facility: Yoshiaki Suzuki
Associate Professor: Masakatsu Watanabe
Research Associates:
Yoshio Hamada (Tissue and Cell Culture)
Kenta Nakai (Computer)
Technical Staff: Mamoru Kubota
Chieko Nanba
Toshiki Ohkawa
Kaoru Sawada
Tomoki Miwa
Kimiko Yamamiya

The Facility provides large- and medium-scale equipments 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-surface 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, CR-599-01) (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 2 μm in beam diameter) with an infrared phase-contrast observation system.

2. *Tissue and Cell Culture Laboratory*

Various equipments for tissue and cell culture are provided. This laboratory is equipped with safety 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 needes in this Institute, various computers are equipped: VAX/VMS machines, UNIX workstations (SPARCstation 10 and others), and personal computers of various vendors. Through the Ethernet or the CDDI, all of them are linked to the backbone FDDI of this institute, which is further linked to the hyper-multimedia network of Okazaki National Research Institutes (the ORION network). Each laboratory has several computers connected to the network and full resources of the Internet are accessible from there. In addition, NetWare server machines

work as file servers and printer servers. Latest databases and various useful softwares are also maintained. Information of this institute, including this document is open to the world through the World Wide Web (WWW; URL ishttp://www.nibb.ac.jp).

4. *Plant Culture Laboratory*

There are a large number of culture boxes, cubicles, and a limited number of rooms with environmental control for plant culture.

5. *Experimental Farm*

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

6. *Plant Cell Culture Laboratory*

Autotrophic and heterotrophic culture devices and equipped for experimental cultures of plant and microbial cells.

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, biochemical, and physiological evaluations of transgenic plants. The laboratory is also a base of domestic and international collaborations devoted to the topic of Stress-Resistant Transgenic Plants.

II. Research Activities

1. *Faculty*

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

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

(2) Developmental Biology: Myosin is a ubiquitous protein in eukaryotes. It is presumed that this molecule plays a key role in cell motility as well as other functions. The role of myosis in non-muscle cells might be better understood if wild-type cell could be compared with mutant cells with altered myosin molecules, though such mutants have not been isolated. The faculty intends to examine the function of the myosin heavy chain in vertebrate non-muscle cells by interrupting its synthesis with the introduction of antisense RNA molecules.

(3) Computational Biology: Efforts to develop new methodology for sequence analysis have been continued. One of the projects is to develop an expert system to predict various protein localization-sites from amino acid sequence data. This work is done in collaboration with Drs. S. Miyano (Kyushu Univ.), T. Shimizu (Hirosaki Univ.) and A. Bairoch (Geneva Univ.). Some results have been Published in Shimizu and ZK. Nakai

(Proc. Genome Informatics Workshop 1994, 148–149) and in K. Nakai, A. Shinohara, and S. Miyano (Proc. GIW 1994, 170–171). Another is to construct a prediction system of mature mRNA sequence from their precursors. It is in collaboration with Drs. H. Sakamoto (Kobe Univ.), Y. Akiyama (Kyoto Univ.) and S. Stamm (Cold Spring Harbor Lab.). The activities of computer assistance for other experimental researchers can be exemplified in M. Tomita, J. Kobayashi, AA. Mori, K. Hagiwara, K. Nakai, Y. Suzuki, and S. Miyajima, Protein Eng., 7, in press.

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, in press).

Publication List:

I. Faculty

Nakai, K. and Sakamoto, H. (1994) Construction of a novel database containing aberrant splicing mutations of mammalian genes, *Gene*, **141**, 171–177.

Nakai, K., Tokimori, T., Ogiwara, A., Uchiyama, I., and Niiyama, T. (1994) Gnome – an Internet-based sequence analysis tool, *Comp. Appl. Biosci.*, **10**, 547–550.

II. Cooperative Research Program for the Okazaki Large Spectrograph

Andrady, A.L., Amin, M.B., Hamid, S.H., Hu, X. and Torikai, A. (1994) Effects of increased solar ultraviolet radiation on materials. *In* Environmental Effects of Ozone Depletion: 1994 Assessment (J.C. van der Leun, X. Tang and M. Tevini, eds.), United Nations Environment Programme (UNEP), Nairobi. pp. 101–110.

Hashimoto, T. (1994) Requirements of blue, UV-A, and UV-B light for normal growth of higher plants, as assessed by action spectra for growth and related phenomena. *In* Proceedings of International Lighting in Controlled Environments Workshop (T.W. Tibbitts, ed.), NASA Ames Research Center, Moffett Field, CA., USA, pp. 143–157.

Hashimoto, T. (1994) UV-B effects on plant: The present knowledge and prospect for future studies. *In* Proceedings of the Tsukuba Ozone Workshop – Grobal Environment Tsukuba '94, (K. Takemoto and S. Nishioka, eds.), Center for Grobal Environmental Research, Japan, pp. 134–145.

Nikaido, O., Ohta, M. and Matsunaga, T. (1994) Detection of the Dewar photoproducts in solar light-exposed DNA by a damage specific monoclonal antibody and the repair of the photoproducts in human cells. *In* The 13th UOEH International Symposium & The 2nd Pan Pacific Cooperative Symposium on Inpact of Increased UV-Exposure on Human Health and Ecosystem (Y. Kodama and Si Duk Lee, eds.), University of Occupational and Environmental Health, pp. 190–193.

Sasaki, M., Takeshita, S., Sugiura, M. and Sakata, T. (1994) An increase in

the global solar ultraviolet-B irradiance at 35oN in Japan since 1990. *J. Geomag. Geoelectr.* **46**, 827–834.

Todo, T., Ryo, H., Takemori, H., Toh, H., Nomura, T. and Kondo, S. (1994) High-level expression of the photorepair gene in *Drosophila* ovary and its evolutionary implications. *Mutation Research, DNA Repair* **315**, 213–228.

RADIOISOTOPE FACILITY (managed by NIBB)

Head of Facility: Takashi Horiuchi
Techinical Staffs: Kazuhiko Furukawa (Radiation Protection Supervisor)
Yosuke Kato
Yoshimi Matsuda (Radiation Protection Supervisor)

The facility is composed of a main center and two subcenters, one in the NIBB and the other in the NIPS. The facility is being used for molecular analyses of organisms. A laboratory facility for recombinant DNA research is installed in the center. The subcenter in the NIBB is also equipped with a recombinant DNA research laboratory. The members of the Radioisotope Facility maintain and control the center and subcenter, and provide users appropriate guidance for radioisotope handling.

Appendix: Maximum Permissible Quantities and Nuclides

Place	Nuclide	State	Maximum Permissible Quantity			
			Year	3 Months	Week	Day
Main center	^{22}Na	liquid	150 MBq	150 MBq	150 MBq	50 MBq
	^{36}Cl	liquid	2 GBq	2 GBq	1400 MBq	200 MBq
	^{45}Ca	liquid	2 GBq	2 GBq	1400 MBq	200 MBq
	^{89}Sr	liquid	500 MBq	500 MBq	350 MBq	50 MBq
	^{125}I	liquid	15 GBq	7.215 GBq	555 MBq	185 MBq
	^{28}Mg	liquid	500 MBq	500 MBq	100 MBq	50 MBq
	^{32}P	liquid	30 GBq	30 GBq	3500 MBq	500 MBq
	^{35}S	liquid	30 GBq	30 GBq	3500 MBq	500 MBq
	^{42}K	liquid	500 MBq	500 MBq	500 MBq	100 MBq
	^{3}H	liquid	20 GBq	20 GBq	3500 MBq	500 MBq
	^{14}C	liquid	5 GBq	5 GBq	3500 MBq	500 MBq
NIBB subcenter	^{32}P	liquid	7.4 GBq	7.4 GBq	1295 MBq	185 MBq
	^{35}S	liquid	7.4 GBq	7.4 GBq	1295 MBq	185 MBq
	^{3}H	liquid	7.4 GBq	6.73 GBq	518 MBq	74 MBq
	^{14}C	liquid	740 MBq	740 MBq	518 MBq	74 MBq
NIP subcenter	^{45}Ca	liquid	3.7 GBq	3.36 GBq	259 MBq	37 MBq
	^{125}I	liquid	1.85 GBq	1.68 GBq	129.5 MBq	18.5 MBq
	^{32}P	liquid	3.7 GBq	3.7 GBq	777 MBq	111 MBq
	^{35}S	liquid	3.7 GBq	3.7 GBq	518 MBq	74 MBq
	^{3}H	liquid	7.4 GBq	6.73 GBq	518 MBq	74 MBq
	^{14}C	liquid	740 MBq	740 MBq	518 MBq	74 MBq

CENTER OF FACILITY FOR ANALYTICAL INSTRUMENTS (managed by NIBB)

Head of Facility: Mikio Nishimura
Technical Staffs: Yukiko Kabeya
Yumiko Makino
Sonoko Ohsawa
Takeshi Mizutani

This center provides analytical instruments for biological and biophysical studies. These instruments consists of the following four groups and are supported by experienced technical staff. 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 Sequencer
  ABI 370A, 373A-36
- DNA/RNA Synthesizer
  ABI 381A, 392
- Nucleic Acid Extractor
  ABI 340A
- Peptide Synthesizer
  ABI 430A, 431A
- Portein Sequencer
  ABI 470A, 473A

Instruments for Chemical Analysis

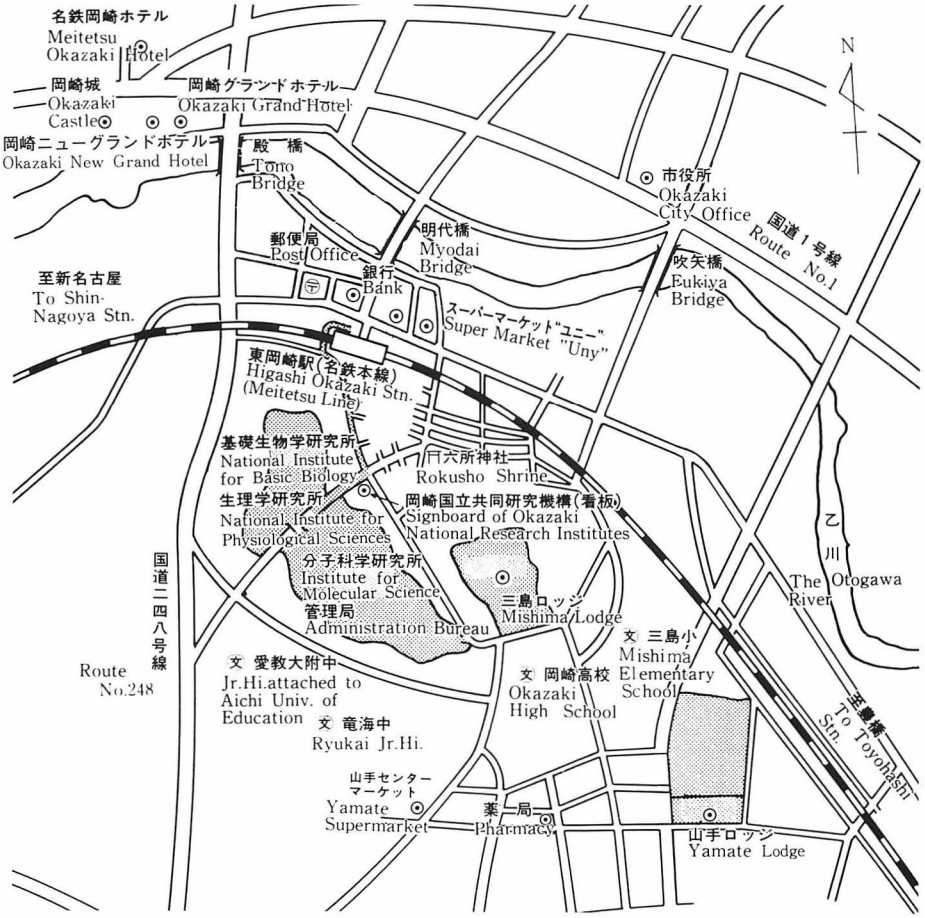
- Capillary Electrophoresis System
  ABI 270A
- Gas Chromatograph
  SHIMADZU GC-7APTF, GC-14APFSC
- Glycoprotein Analysis System
  TAKARA Glyco-Tag
- High Performance Liquid Chromatograph
  SHIMADZU LC-10AD, 6AD
- Micro Preparative Electrophoresis System
  ABI 230A
- Preparative Ultracentrifuge
  BECKMAN L8-80
- 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/MS Spectrometer
  HITACHI M-80, 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
- Spectrofluorometer
  HITACHI 850
  SHIMADZU RF-5000
- Spectrophotometer
  HITACHI 330
- Spectropolarimeter
  JASCO J-40S

Instruments for Microscopic and Image Analysis

- Bio Imaging Analyzer
  FUJIFILM BAS 2000
- Confocal Laser Scanning Microscope
  BIO-RAD MRC-500
- Imaging Analyzer
  KONTRON IBAS-I & II
- Electrophoresis Imaging System
  PDI The Discovery Series
- Microdensitometer
  JOYCE LOEBL 3CS
- Microscope
  CARL ZEISS Axiophot, ICM405s
- Microscope Photometer
  CARL ZEISS MPM 03-FL



National Institute for Basic Biology
Okazaki 444, Japan