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

1993

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.

In October of 1992, an American journal published an article "Science in Japan" (*Science*, **258**, 562), in which citation indices of papers published in 1981–1991 by different universities and institutes in Japan were shown. The index of the NIBB was highest in Japan and comparable to those of leading universities in the United States. We are justly proud of this and would like to maintain a high level of research activity at the Institute.

A new professor is expected to join the NIBB in early 1994. Dr. Tetsuo Yamamori, Sub-Teamleader of the Riken is to fill 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 14 research associates from other universities including one foreign university, while three research associates were promoted to associate professors and a lecturer 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 "Signal Transduction" and "Transdifferentiation of tissue cells". Based on such programs, the NIBB held the 30th and 31st Conferences in 1993, entitled "Vertebrate germ-line: its development and maturation" (organized by Prof. Nagahama) and "Japan-France collaborative workshops on gene manipulation in aves" (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 1993.

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 (IPS) 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 Facility, Plant Culture Facility, Plant Cell Culture Facility, and Experimental Farm. In addition, seven facilities are operated jointly with the National Institute for Physiological Sciences (IPS); 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:

Ozaki, T., Nakao, H., Orii, H., Morio, T., Takeuchi, I. and Tasaka, M. (1993) Developmental regulation of transcription of a novel prespore-specific gene (Dp87) in *Dictyostelium discoideum*. *Development* **117**, 1299–1308.

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 unicellular eukaryotic organisms, 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
Hitoshi Mori
JSPS-Post-doctoral Fellow: Ryuji Tsugeki
Graduate Students: Kaori Inoue
Akira Kato
Tomoo Shimada
Tetsu Kinoshita
Nagako Hiraiwa[1)]
Masahiro Aoki[2)]
Technical Staff: Maki Kondo
Katsushi Yamaguchi

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

Higher plant cells contain several distinct organelles that play vital roles in cellular physiology. During proliferation and differentiation of the cells, the organelles often undergo dynamic changes. The biogenesis of new organelles may occur; existing organelles may undergo a transformation of function, which other organelles may degenerate. In this division, the regulatory mechanisms underlying organelle transformation are currently studied to examine the dynamic transformation of organellar functions (differentiation of organelles) responsible for differentiation events in higher plant cells.

I. Transformation of leaf peroxisomes to glyoxysomes in senescing pumpkin cotyledons.

Dramatic metabolic changes that underlie the shift from heterotrophic to autotrophic growth occur in greening of seed germination. Accompanying these metabolic changes, many constitutive organelles are functionally transformed. For example, etioplasts differentiate into chloroplasts and mitochondria acquire the ability to oxidize glycine. Glyoxysomes that are microbodies engaged in the degradation of reserve oil via β-oxidation and the glyoxylate cycle, are transformed into leaf peroxisomes that function in several crucial steps of photorespiration. After the functional transition of glyoxysomes to leaf peroxisomes during the greening of pumpkin cotyledons, the reverse microbody transition of leaf peroxisomes to glyoxysomes occurs during senescence. Immunocytochemical labeling with protein A-gold was performed to analyze the reverse microbody transition using antibodies against a leaf-peroxisomal enzyme, glycolate oxidase, and against two glyoxysomal enzymes, namely, malate synthase and isocitrate lyase. The intensity of labeling for glycolate oxidase decreased in the microbodies during senescence whereas in the case of malate synthase and isocitrate lyase intensities increased strikingly. Double labeling experiments with protein A-gold particles of different sizes showed that the leaf-peroxisomal enzymes and the glyoxysomal enzymes coexist in the microbodies of senescing pumpkin cotyledons (Fig. 1), indicating that leaf peroxisomes are directly transformed to glyoxysomes during senescence. These findings indicate that transformation of microbodies is a reversible process and glyoxysomes and that leaf peroxisomes are directly transformed to other microbodies in greening and senescing cotyledons, respectively.

II. Characterization of aconitase responsible for glyoxylate cycle.

As a step to understand the regulatory mechanisms that operate during microbody transition in pumpkin cotyledons (*Cucurbita* sp., Amakuri Nankin), nine microbody enzymes, i.e., three glyoxysomal enzymes, three leaf peroxisomal enzymes and three enzymes present in both microbodies have been purified

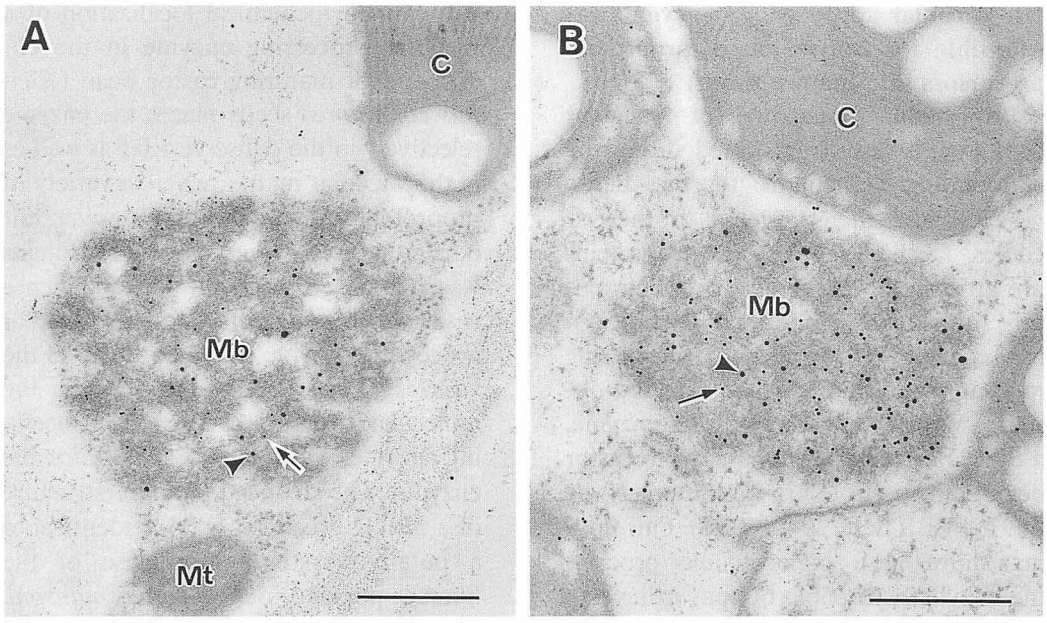

Fig. 1. Double immunogold-labeling of senescing pumpkin cotyledons using antibodies against a leaf-peroxisomal enzyme, glycolate oxidase, and two glyoxysomal enzymes, malate synthase and isocitrate lyase. The leaf-peroxisomal enzyme and the glyoxysomal enzymes coexist in the same microbody in senescing pumpkin cotyledons. Bars represent 0.5 μm. Mb microbody, Mt mitochondrion, C chloroplast.
(A) Large gold particles (size 15 nm, arrowhead) glycolate oxidase, small gold particles (size 10 nm, arrow) malate synthase.
(B) Large gold particles (size 15 nm, arrowhead) glycolate oxidase, small gold particles (size 10 nm, arrow) isocitrate lyase.

and characterized. The developmental changes in the level of mRNA and protein have been analyzed. This year, we will start analyzing aconitase which catalyzes the reversible interconversion of citrate, isocitrate, and cis-aconitate. Aconitase is involved in the Krebs cycle, localized in mitochondria, and in the glyoxylate cycle in glyoxysomes, but the enzyme has also been reported to be localized in the cytosol. Aconitase has been detected in glyoxysomes, but only less than 0.5% of the total aconitase activity was recovered in glyoxysomal fractions after sucrose gradient centrifugation. Whether mitochondria and glyoxysomes contain specific isoenzymes of aconitase remains to be confirmed. Three isoforms of aconitase (AcoI, AcoII, AcoIII) were found in etiolated cotyledons of pumpkin and two of them (AcoI, AcoII) were purified. The specific antibody raised against AcoI crossreacted with all isoforms of aconitase. Subcellular fractionation and immunoblot analyses showed that the activity of aconitase and the immunopositive polypeptide were not detected in glyoxysomes. These findings indicate that aconitase is not localized in glyoxysomes although the enzyme is a member of the glyoxylate cycle. cDNA cloning and immunocytochemical analysis are in progress.

III. Vacuolar processing enzyme responsible for conversion of proprotein precursors into mature forms.

Proprotein precursors of vacuolar components are transported from the endoplasmic reticulum to the dense vesicles, and then targeted to the vacuoles. In the vacuoles, the proproteins are processed proteolytically to their mature forms by a unique vacuolar processing enzyme. However, the processing mechanism in plant vacuoles is very obscure. We isolated a processing enzyme, a 37-kD cysteine proteinase, from castor bean endosperm (Hara-Nishimura *et al.*, 1991, FEBS Lett. 294, 89-93). Our findings show that a single vacuolar processing enzyme is capable of converting several proprotein precursors with a large variability of molecular structure into their mature forms.

i) Molecular characterization and localization of a vacuolar processing enzyme.

Immunocytochemical localization of a vacuolar processing enzyme in the endosperm of maturing castor bean (*Ricinus communis*) seeds places the enzyme selectively in the dense vesicles as well as in the vacuolar matrix, where a variety of proproteins is also present. However, endogenous processing of the proproteins was not observed in the isolated dense vesicles. This suggests that the vacuolar processing enzyme is a latent form in the vesicles (Fig. 2). To characterize the molecular structure of vacuolar processing enzyme, we isolated a cDNA for the enzyme. The deduced primary structure of a 55-kD precursor is 33% identical to a putative cysteine proteinase of the human parasite *Schistosoma mansoni.* The precursor is composed of a signal peptide, a 37-kD active processing enzyme domain, and a propeptide fragment. Although the precursor expressed in *Escherichia coli* has no vacuolar processing activity, a 36-kD immunopositive protein expressed in *E. coli.* is active.

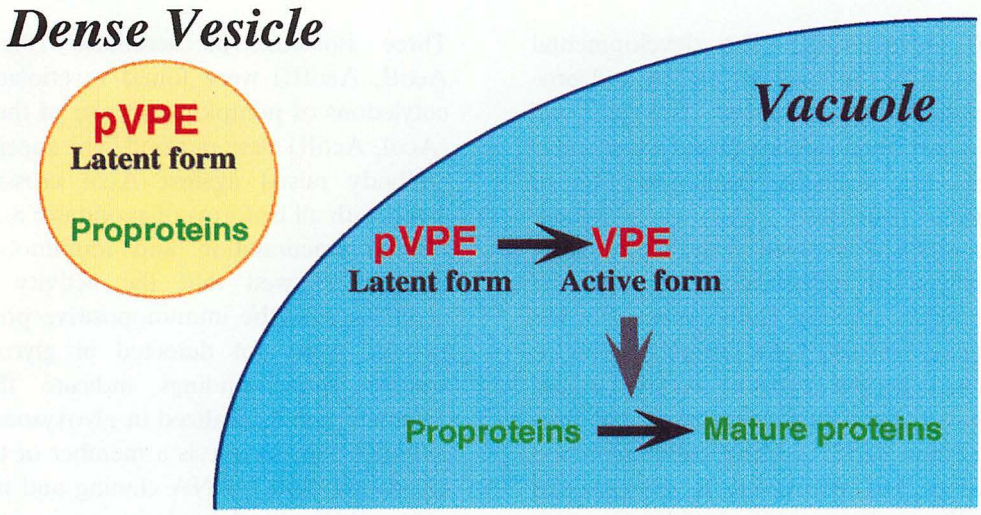

Fig. 2. Maturation system for proprotein precursors of various vacuolar proteins. A latent form of the vacuolar processing enzyme is transported into vacuole via dense vesicle along with proprotein precursors. The precursors are converted into the respective mature forms by the action of the vacuolar processing enzyme that is activated in the vacuole.

active. These findings suggest that the activation of the vacuolar processing enzyme requires proteolytic cleavage of the 14-kD C-terminal propeptide fragment of the precursor.

ii) Developmental changes of a vacuolar processing enzyme in maturing and germinating seeds.

During seed maturation of castor bean, an increase in the activity of the vacuolar processing enzyme in the endosperm precedes the increase in the amount of total protein. The enzymatic activity reaches a maximum at the late stage of seed maturation and then decreases during seed germination concomitantly with the degradation of seed storage proteins. We examined the distribution of the enzyme in different tissues of various plants. The processing enzyme was found in cotyledons of castor bean, pumpkin and soybean, as well as in endosperm, and a slight processing activity was also detected in roots, hypocotyls and leaves of castor bean, pumpkin, soybean, mung bean and spinach. These findings suggest that the proprotein-processing machinery is widely distributed in vacuoles of various plant tissues.

IV. Mechanism for biosynthesis and vacuolar processing of 2S albumin, a major seed protein of pumpkin.

Cell fractionation of pulse-chase-labeled developing pumpkin cotyledons demonstrated that the proprotein precursor to 2S albumin is transported from the endoplasmic reticulum to dense vesicles and then to the vacuoles, in which pro2S albumin is processed to the mature 2S albumin. Immunocytochemical analysis showed that dense vesicles of about 300 nm in diameter mediate the transport of pro2S albumin to the vacuoles. The primary structure of the precursor to 2S albumin has been deduced from the nucleotide sequence of an isolated cDNA insert. The presence of a hydrophobic signal peptide at the N-terminus indicates that the precursor is a preproprotein that is converted into pro2S albumin after cleavage of the signal peptide. N-terminal sequencing of the pro2S albumin in the isolated vesicles revealed that the signal peptide is cleaved off co-translationally on the C-terminal side of alanine residue 22 of prepro2S albumin. By contrast, post-translational cleavage occurs on the C-terminal side of both asparagine residues 35 and 74, which are conserved among precursors to 2S albumin from different plants. Hydropathy analysis revealed that the two asparagine residues are located in the hydrophilic regions of pro2S albumin. These findings suggest that a vacuolar processing enzyme can recognize exposed asparagine residues on the molecular surface of pro2S albumin and cleave the peptide bond on the C-terminal side of each asparagine residue to produce mature 2S albumin in the vacuoles.

V. Role of molecular chaperones on translocation of proteins into chloroplasts.

Molecular chaperones are a class of cellular proteins that function in the folding and assembly into oligomeric structures of certain other polypeptides but that are not components of the final oligomeric structure. To investigate the role(s) of molecular chaperones in the chloroplasts, we examined whether the homologues of the 70-kDa heat-shock protein (Hsp70) and chaperonin 60 (Cpn60) interact with newly imported

proteins to assist in their maturation. Ferredoxin $NADP^+$ reductase (FNR) imported into chloroplasts in vitro could be immunoprecipitated with antisera raised against the homologue of Hsp70 from pumpkin chloroplasts and against GroEL from *Escherichia coli*, which is a bacterial homologue of chaperonin 60 (Cpn60), in an ATP-dependent manner, an indication that newly imported FNR interacts physically with homologues of Hsp70 and Cpn60 in chloroplasts. Time-course analysis of the import of FNR showed that imported FNR interacts transiently with the homologue of Hsp70 and that the association of FNR with the homologue of Hsp70 precedes that with the homologue of Cpn60. These findings suggest that homologues of Hsp70 and Cpn60 in chloroplasts sequentially assist in the maturation of newly imported FNR in an ATP-dependent manner. cDNA for chloroplast Cpn10 has been isolated and characterized. Further investigation on the function of Cpn10 in the transport of protein into chloroplasts is in progress.

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Hiraiwa, N., Takeuchi, Y., Nishimura, M. and Hara-Nishimura, I. (1993) A vacuolar processing enzyme in maturing and germinating seeds: Its distribution and associated changes during development. *Plant Cell Physiol.* **34**, 1197–1204.

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DIVISION OF BIOENERGETICS

Professor: Yoshihiko Fujita
Associate Professor: Sigeru Itoh
Research Associates: Mamoru Mimuro
Katsunori Aizawa
*Visiting Scientist: Sung-Jun Kim**
JSPS-Post-doctral Fellow: Tohru Ikeya
*Visiting Fellow: Yuji Nakajima***
Technical Staff: Akio Murakami

*(*from Kyngpook National University)*
*(**from Advanced Technology Research Center, Mitsubishi Heavy Industries, LTD)*

Oxygenic photosynthesis is an energy yielding metabolism that supports autotrophic life of plants. Primary process of photosynthesis, light-energy conversion, in thylakoids is the target for the study of this research division. The research has been conducted for regulatory aspects of thylakoid system in response to photosynthetic environments and also for molecular mechanism of light-capturing and conversion in functional molecules.

I. Regulation of PSI/PSII stoichiometry

Light-energy conversion in oxygenic photosynthesis is driven by the two light reactions of the two photosystems, PSI and PSII. Imbalance between the two light reactions causes lowering the efficiency of energy conversion. Our previous study with cyanophytes has revealed that plants have an ability for regulating the stoichiometry of the two photosystems so as to compensate imbalance between the two light reactions. Further, at least in the cyanophyte *Synechocystis* PCC 6714, the stoichiometry is regulated by the control of PSI formation, which occurs in response to the signal comming from the state of the electron transport between the two photosystems.

Controlling mechanism for PSI formation has been conducted in two ways; determining the target sites for regulation (1)in Chl *a* synthesis and (2)in apoprotein synthesis. Analysis for dynamics of intermediates of Chl *a* synthesis yielded following results (Fujita et al., in press): (1) Pchlide reduction is regulated in parallel with regulation of PSI formation. The activity is suppressed under conditions for suppression of PSI formation, such as light regime preferentially exciting PSI (PSI light). The suppression is released by the conditions for stimulation of PSI formation, such as light regime exciting preferentially PSII (PSII light). (2) Changes in the activity of Pchlide reduction are neither due to acceleration-deceleration of PSI synthesis nor due to differential occurrence of light-dependent Pchlide reduction. The enzyme activity itself is regulated. Analysis for synthesis of PsaA/B peptides, core peptides of PSI complex, with pulse-labeling method revealed that (1) inhibitors for peptide elongation suppresses PsaA/B synthesis differently in accelerated and decelerated PSI formations. Inhibition at low inhibitor concentrations was stronger in the former than that in the latter, suggesting that pausing of pepetide elongation becomes longer under conditions for suppression of PSI formation. (2) The inhibitor for initiation of translation showed similar effect. (3) Effect of chrolamphenicol suggested that initiation of translation is also regulated; it is suppressed by PSI light, and PSII light releases from suppression. Together with previous results, evidences obtained show that regulation of PSI formation in PSI/PSII adjustment occurs at the translation level of PsaA/B synthesis. Since Chl *a* stabilizes PsaA/B peptides, we assume that regulation of *psaA/B* expression at translation level is achieved at least by Chl *a* supply.

Changes in thylakoid composition in

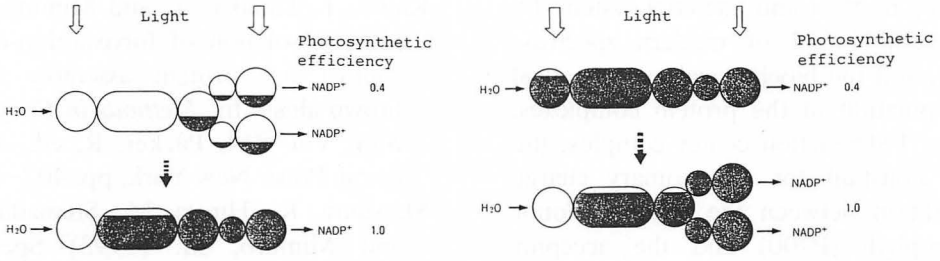

Fig. 1. Schematic presentation of regulation of PSI/PSII stoichiometry and electron transport state. Shaded area in each circle indicates level of reduced state of each component. Reduced PSII and oxidized PSI are photochemically inactive, respectively, and large occurrence of such states causes low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

the green alga *Chlamydomonas reinhardtii* in response to light quality were determined under collaboration with Dr. A. Melis (Univ. Cal., Berkeley). Results indicated that the pattern of change was similar to that in the cyanophyte *Synechocystis* PCC 6714. Abundance of PSI was markedly changed while that of PSII and Cyt b_6-f remained fairly constant, resulting higher PSI/PSII ratio under PSI light and lower ratio under PSI light. Changes in PSI/PSII improved photosynthetic efficiency under respective light regimes. Differently from cyanophyte system, a marked decrease in LHC, in parallel with PSI decrease, occurred under PSI light. Results suggest that Chl supply to assembling of Chl-protein complexes is a primary determinant for adjustment of PSI/PSII stoichiometry in this organism also.

II. Mechanism of energy transfer in light-capturing pigment system

Examination was made for function of carotenoids in photosynthesis. Relationship between molecular structure and optical properties in the excited state was mainly analyzed. (1) The forbidden S_1 state of neurosporene and spheroidene, which is responsible for the energy transfer to bacteriochlorophylls *a*, was directly measured by the one-photon absorption spectroscopy; the energy level was higher than and close to that of acceptors. (2) It was proved that the relaxation processes from the excited state of carotenoids follow the energy gap law of internal conversion. Presence of a keto carbonyl group induced a rapid internal conversion to the S_1 state, which was favorable for energy transfer to chlorophylls *a*. This corresponds to the case for fucoxanthin in brown algae and diatom. (3) The S_2 lifetime of β-carotene was directly measured by the up-conversion method to be 195 ± 10 fs with an isotropy ratio of 0.39 ± 0.02. (4) Theoretical analysis of the energy transfer processes was carried out by calculation of the energy transfer matrix elements between neurosporene and bacteriochlorophyll *a*. The electron exchange mechnism is concluded to be less probable, even if the forbidden singlet state is involved. (5) Based on these results, the energy transfer through the dipole-multipole interaction from the S_1 state to (bacterio)chlorophyll *a* is proposed.

III. Mechnism of electron transfer in reaction center complex

Mechanism of the primary photoenergy conversion in photosynthesis was

studied in plant and bacterial systems by the combination of modern spectroscopy, and the biochemical and genetical manipulation of the protein complexes. In the PSI reaction center complex, the time constant for the primary charge separation between the electron donor chlorophyll (P700) and the acceptor chlorophyll was measured to be 7×10^{-12} s. The following reaction with phylloquinone was shown to take place with a time constant of 38×10^{-12} s. The rate varied when various artificial quinones were introduced into the reaction center complex in place of native phylloquinone. Functions of quinones in various reaction center preparations were also studied. It is shown that the fine adjustment of the energy levels of native electron transfer components enable the highly efficient, ultra-fast electron transfer reactions. We isolated and characterized the reaction center complex of green bacteria, which seems to be ancestry to PSI reaction center. The complex was shown to have a polypeptide composition simpler than that of plant PSI but to undergo almost similar activities. Unique nature of the iron-sulfur centers in this complex was shown by cryogenic EPR studies. The structure-function relationship of the iron-sulfur centers of the photosynthetic bacterium *Rhodobacter capsulatus* was also investigated by the site-directed mutagenesis.

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DIVISION OF CELL PROLIFERATION (ADJUNCT)

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Research Associate: Yoshiyuki Imai
NIBB Post-doctoral Fellow: Masuo Goto
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Visiting Scientist: Makiko S. Okumura

Meiosis is a crucial step in gamete formation and is essential for sexual reproduction. Meiosis is highly conserved among eukaryotic genera, from yeast to mammals. This Division aims to explore the regulation of meiosis in higher organisms. The major strategy currently taken is as follows. In the fission yeast *Schizosaccharomyces pombe*, which is a unicellular eukaryotic microorganism, many genes involved in control of meiosis have been cloned and characterized. Mutant cells that have a defect in these genes have been obtained either by classical genetics or by chromosome manipulation. We thus rationalize that it is possible to isolate homologs of these genes from animals and plants, by using either similarity in nucleotide sequences or functional complementation of the mutants. The main project of this Division has been performed along this line. We have also carried out two additional projects. One of them was to characterize the mating pheromone of the fission yeast and examine its potential to affect cell growth and sexual development of this yeast. The other was to study the effect of calcium on cell proliferation in the budding yeast. The results obtained in these three projects are described below.

I. Screening for mammalian and plant genes that can replace genes essential for meiosis in the fission yeast.

We prepared cDNA libraries from mouse testis and *Arabidopsis thaliana*. The vector carries a promoter functional in the fission yeast and thus the cloned genes can be expressed in fission yeast cells. We transformed two meiotic mutants of *S. pombe* with these libraries. One is the *sme2* mutant that cannot perform the first meiotic division. The *sme2* gene product has been shown to function as an RNA molecule. The other mutant is *mes1*, which cannot perform the second meiotic division. We obtained five mouse genes and four *Arabidopsis* genes that can rescue *sme2*. The *Arabidopsis* genes include those encoding a novel protein kinase family. We isolated a fission yeast homolog of this kinase family and have shown that it also has the ability to rescue *sme2*. So far we obtained only one mouse gene that can rescue *mes1*. This mouse gene generates a specific transcript in testis that is not detectable in other tissues tested. Further characterization of the isolated genes is in progress.

II. Characterization of the fission yeast mating pheromone P-factor.

S. pombe h^+ cells secrete a diffusible mating pheromone called P-factor. Although M-factor secreted by h^- cells was previously characterized, the identity of P-factor was not known. We now have shown that the *map2* gene, a defect of which confers mating deficiency only in h^+ cells, encodes the precursor of P-factor. We purified P-factor from cells overexpressing *map2* and determined its amino acid sequence. P-factor is a peptide of 23 residues, with the sequence Thr-Tyr-Ala-Asp-Phe-Leu-Arg-Ala-Tyr-Gln-Ser-Trp-Asn-Thr-Phe-Val-Asn-Pro-Asp-Arg-Pro-Asn-Leu. We synthesized a peptide of this sequence. It has the same specific activity and chromato-

graphic profile as the purified P-factor, suggesting that P-factor is unmodified. By using mutant cells that are derepressed for sexual development in rich medium, we could show that P-factor can induce not only responses towards mating but also arrest of the cell cycle at the G1 phase in h^- cells (Figure 1). This proves that the *S. pombe* mating pheromone has the ability to arrest cell cycle progression, which has previously been obscured by the usual requirement for mating of nutritional starvation and subsequent growth arrest (Y. Imai and M. Yamamoto, *Genes Dev.*, **8**, 328–338, 1994).

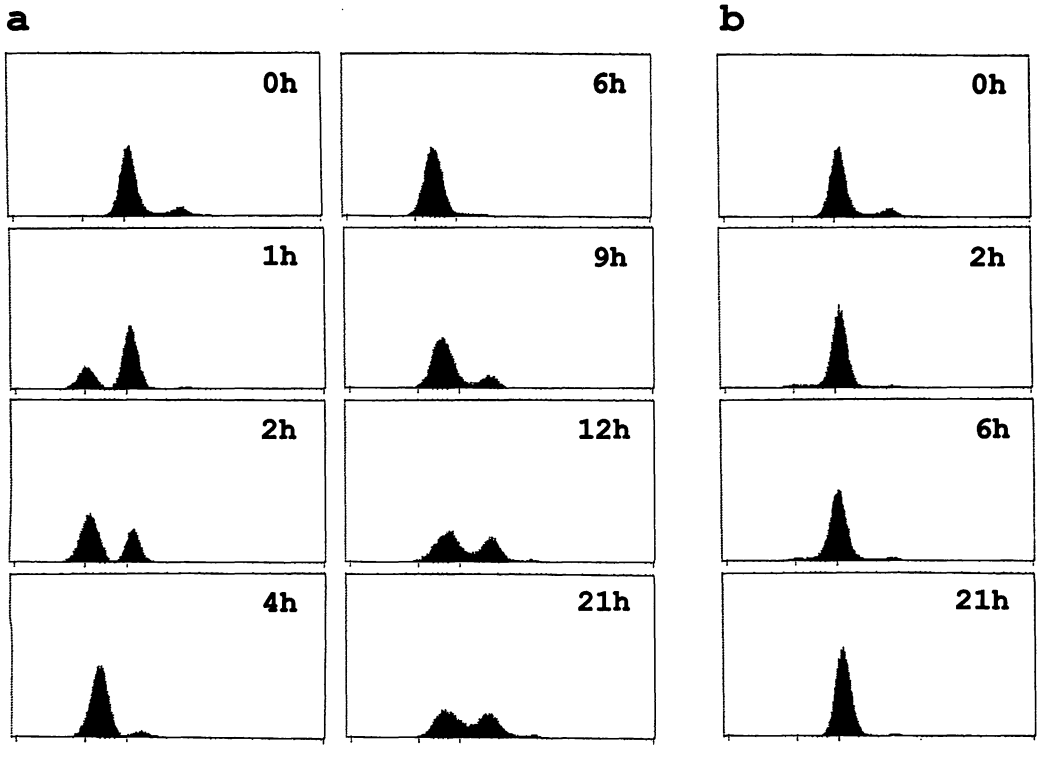

Figure 1. The ability of P-factor to arrest the cell cycle of h^- cells at the G1 phase. Flow cytometric analysis of the DNA content was done in *S. pombe* h^- cells (sexually-derepressed mutant) exposed to P-factor. (a) Cells grown in the presence of 2 μg/ml P-factor; and (b) with no P-factor. The main peaks at 0h in (a) and (b) correspond to 2C (G2-equivalent) DNA content, whereas the main peak at 6h in (a) corresponds to 1C (G1-equivalent) DNA content. The G1 arrest is transient and cells gradually resume growth (a, 9-21h). Without P-factor (b), cells show 2C DNA content because rapidly growing *S. pombe* cells have only short G1 and S phases and they undergo these phases while the daughter cells are not completely separated.

III. Genetic analysis of the role of calcium during sexual differentiation in the budding yeast.

We isolated *Saccharomyces cerevisiae* mutants apparently defective in the Ca^{2+} signalling, on the basis of our discovery that Ca^{2+} is essential for maintaining viability of the cells destined for mating. *S. cerevisiae* cells do not survive if they cannot take up enough Ca^{2+} when they are exposed to the mating pheromone. The mutants, named *mid* (mating

pheromone-induced death), die even in the presence of a sufficient amount of Ca^{2+} if they are exposed to the mating pheromone. The *MID1* and *MID2* genes have been cloned. Both genes apparently encode membrane-associated proteins. Characterization of these and other *MID* genes and the functional relationship among them are currently under study.

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DIVISION OF CELLULAR COMMUNICATION (ADJUNCT)

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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 acquire their own identities and how their axons find their own pathways and finally recognize their proper targets. Our current interest is mainly focused on the motoneurons because of their accessibility to various cellular manipulations. 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 to these questions both at the molecular and cellular levels.

I. Molecular Developmental Neurobiology of Zebrafish

Embryos of zebrafish stay transparent throughout most period of their development. Most neurons in the central nervous system of early embryos are identifiable. In the embryonic brain, a primitive neuronal network is formed by the early born neurons (primary neurons) and presents the initial scaffold for the later extending axons from the secondary neurons. Our current goal is to identify the molecules which are involved in the final determination of the identities or the axonal pathways by individual primary neurons.

1. ZISH (zebrafish Isl-1 homologues) genes in the specification of primary motoneurons

Zebrafish embryos have three subtypes of primary motoneurons (RoP, MiP and CaP) per hemisegment, each of which extends the axon along the stereotyped pathway and innervates the specific region of the somites. In the last annual report, we reported the molecular cloning of the cDNA for zebrafish Isl-1. Its expression pattern in the ventral region of the spinal cord suggested that Isl-1 may be involved in the specification of primary motoneurons.

In collaboration with Ziyuan Gong and Choy Hew at University of Toronto, we have recently isolated two novel zebrafish cDNA clones which encode the proteins similar to the original Isl-1, using the salmon pituitary cDNA encoding a novel Isl-1-like protein as a probe. We named them ZISH (Zebrafish Isl-1 Homologue)-2 and -3, by counting the original zebrafish Isl-1 as ZISH-1.

The expression patterns of the original Isl-1 (ZISH-1) mRNA and ZISH-2 mRNA are almost complementary. ZISH-1 mRNA expression is at first observed in randomly distributed ventromedial cells and later restricted to RoP (or RoP and MiP) and the secondary motoneurons surrounding RoP. And the number of ZISH-1 mRNA-positive ventromedial cells increases in the tail region and in the *spaidtail* mutant where the somites surrounding the spinal cord are missing or defective. These data suggest that ZISH-1 mRNA expression is downregulated by the influence of the somites. In contrast, ZISH-2 mRNA is expressed only by CaP and VaP from the moment when its expression starts to be detected (around 15 hours after fertilization), just

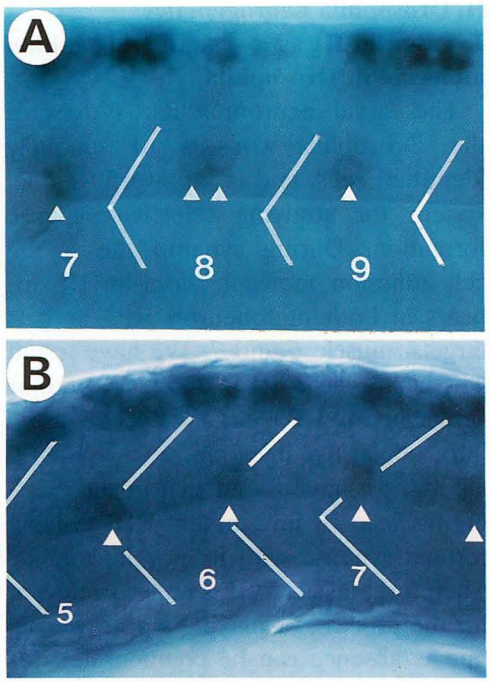

Fig. 1. The expression patterns of ZISH-2 and ZISH-1 mRNA in the spinal cord

Lateral views of the spinal cords of embryos hybridized in situ with ZISH-2 and ZISH-1 cRNA in (A) and (B), respectively. [18 hr embryo in (A), 16 hr embryo in (B)] ZISH-2 and ZISH-1 mRNA positive cells in the ventral regions of the spinal cord are indicated by arrowheads in (A) and (B), respectively. Somites are denoted by numbers, and the somite borders are indicated by white lines.

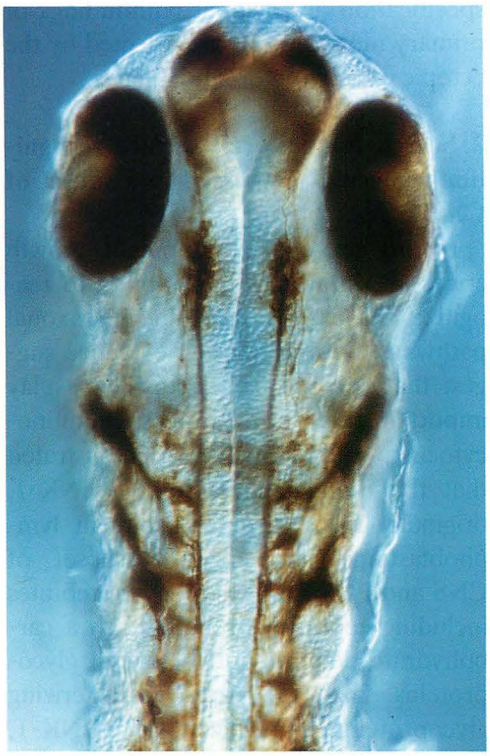

Fig. 2. The dorsal view of the brain of a 32 hr embryo stained with HNK-1 antibody

when the nascent CaPs are about to stop expressing ZISH-1 mRNA. ZISH-2 mRNA is not expressed in the caudal end of the spinal cord where the somites are not yet formed. These subtype specific expression patterns suggest that the Isl-1 (ZISH-1) and ZISH-2 genes may either react oppositely to the influence from the somites or regulate the expression of each other, and together be involved in the determination of cellular identities (specification) by motoneurons in embryonic zebrafish.

Although ZISH-3 mRNA is expressed only by Rohon-Beard cells but not by motoneurons in the spinal cord, its expression pattern is nonetheless very specific. In the body trunk, it is expressed intensely only in the ventral region of the axial muscle which is innervated by CaP. And in the brain, it is expressed both in the entire eye (including the retina and the lens) and in the nascent tectum. These data suggest that the ZISH-3 gene may regulate the expression of the molecules that help CaP and the retinal ganglion cells to correctly find their proper targets, the ventral axial muscle and the tectum respectively.

We are currently examining how the

specification and axonal pathfindings of primary motoneurons are affected by the ectopic expression of ZISH genes.

2. 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 pathways. 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, originally raised against 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 characterizing glycoproteins recognized by HNK-1, hoping to isolate new cell surface recognition molecules 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 affinity-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 family on Western blotting has been prepared. Immunization is now in progress.

II. Drosophila neurogenetics

1. 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 target 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 ectopically on all skeletal 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, PR3 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.

2. Analysis of A9 gene which affects larval neurogenesis

P-element insertion line A9 is a pupal

lethal mutant which has a diminished brain in the homozygotes. Using BrdU incorporation analysis, we found larval neuroblasts of this mutant are morphologically abnormal and showed almost no sign of proliferation.

We tried to clone this A9 gene and identified a 0.6 kb transcript around the P-element insertion site which was reduced in A9 homozygotes. The molecular cloning of the cDNA and the subsequent sequencing determination and in situ hybridization indicate that the putative product of this transcript is a secretory glycoprotein expressed in larval imaginal cells including those in CNS. These data suggest that the defect in the gene encoding this transcript is responsible for the A9 mutant phenotype. We are now trying to examine if A9 mutants can be rescued by P-element mediated transformation with this gene.

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DIVISION OF CELL FUSION (ADJUNCT)

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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 enhancer and promoter elements 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 three or four topographically distinct zones, where olfactory neurons expressing one particular kind of receptor are randomly distributed. Furthermore, it is assumed that a very limited number of the receptor genes (possibly one) is activated in each olfactory neuron.

We are interested in knowing how each neuron select a limited number of receptor genes which are to be expressed. One obvious regulatory mechanism is at the level of transcription. In this mechanism we may need a variety of transcriptional factors for both positive and negative regulations. In the mammalian immune system, somatic DNA recombination is utilized to activate one particular member of the receptor genes, and to exclude one of the two different alleles. It is of interest to study whether such a mechanism is also involved in the regulation of the olfactory system.

In order to study selective expression of the odorant receptor genes, we are characterizing the genomic structure of the murine receptor genes. Although many coding sequences have been reported for the odorant receptor genes, little is known about the 5' and 3' noncoding regions. These regions must contain important DNA elements that regulate the receptor gene expression. We have isolated genomic clones of the odorant receptor genes from the P1 phage library. We are currently analyzing the regulatory regions in these clones. We hope that these studies will reveal the molecular mechanisms for the selective and zonal expression of the odorant receptor genes.

We have made transgenic mice which are forced to express one particular member of the odorant receptors in every olfactory neuron. It is of interest to study whether the expression of the transgene represses the endogenous receptor genes. A plasmid construct contains the mouse receptor #28 gene under the control of OMP promoter. Since the OMP gene is expressed specifically in the olfactory epithelium, the #28 receptor is expected to be activated in all mature neurons in the transgenic mouse. These study will give us a new insight into the regulatory mechanisms of the odorant receptor genes.

When olfactory neurons form synapses with other neurons, they send their axons to the specific region called

glomeruli in the olfactory bulb. Each olfactory neuron sends only one axon to one of two thousand glomeruli. It is quite amazing that the olfactory neurons are able to find their right target glomerulus every time when they are regenerated. It is interesting to study how the neuronal activity affects the target specificity and selectivity. We are hoping that our transgenic approach will become a usuful clue to answer to these questions.

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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

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Yoshinao Katsu
Shinji Onoe
Daisuke Kobayashi
Xiao-Tian Chang
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Glen Van der Kraak[2)]
Noriyoshi Sakai[3)]
Michiya Matsuyama[4)]
Masamichi Nakashima[5)]
Jian-Quiao Jiang[6)]

[1)]from University of California
[2)]from University of Guelph
[3)]from Fukui Prefectural University
[4)]from Mie University
[5)]from Tohoku University
[6)]from Wuhan University

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 (P-450arom) and 20β-hydroxysteroid dehydrogenase (20β-HSD). These cDNA clones 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 increase in the amount of P450scc, 3β-HSD and P450c17 transcripts provide an explanation for the dramatic increase in 17α, 20β-DP production in follicles during naturally- and gonadotropin-induced oocyte maturation. In contrast, levels of mRNA for P450arom 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.

We have shown that 17α, 20β-DP acts via a receptor on the plasma membrane of oocytes. 17α, 20β-DP receptor concentrations increase during oocyte maturation. Our recent studies suggest that inhibitory G-proteins are involved in the signal transduction pathway of the maturational action 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). Goldfish immature oocytes contain 35-kDa inactive cdc2 kinase. Although immature oocytes contain mRNA for cyclin B, they do not contain cyclin B protein. 17α, 20β-DP induces oocytes to synthesize cyclin B. The preexixting 35-kDa inactive cdc2 kinase binds to *de novo* synthesized cyclin B at first, then is rapidly converted into the 34-kDa active form. Introduction of a bacterially produced goldfish cyclin B into immature goldfish oocyte extracts induces cdc2 kinase activation, concurrent with the shift in apparent molecular weight of cdc2 kinase from 35- to 34-kDa, as found in oocytes matured with 17α, 20β-DP. Phosphoamino acid analysis shows that threonine (Thr) phosphorylation of the 34-kDa cdc2 kinase and serine phosphorylation of cyclin B are associated with the activation. The same phosphorylation is found in oocytes matured by 17α, 20β-DP. Cyclin B-induced cdc2 kinase activation is not observed when threonine phosphorylation of cdc2 kinase and serine phosphorylation of cyclin B are inhibited by protein kinase inhibitors, although the binding of the 35-kDa cdc2 kinase to cyclin B occurs even in the presence of the inhibitors. In contrast, cdc2 kinase is activated by mutant cyclins that undergo no serine phosphorylation during the activation. The site of threonine phosphorylation on cdc2 kinase was mapped to residue Thr161. These findings indicate that the Thr161 phosphorylation of cdc2 kinase, but not serine phosphorylation of cyclin B, is required for cdc2 kinase (MPF) activation in goldfish oocyte (Fig. 1).

MPF activity decreases immediately after fertilization, coinciding with the degradation of cyclin B protein. Purified

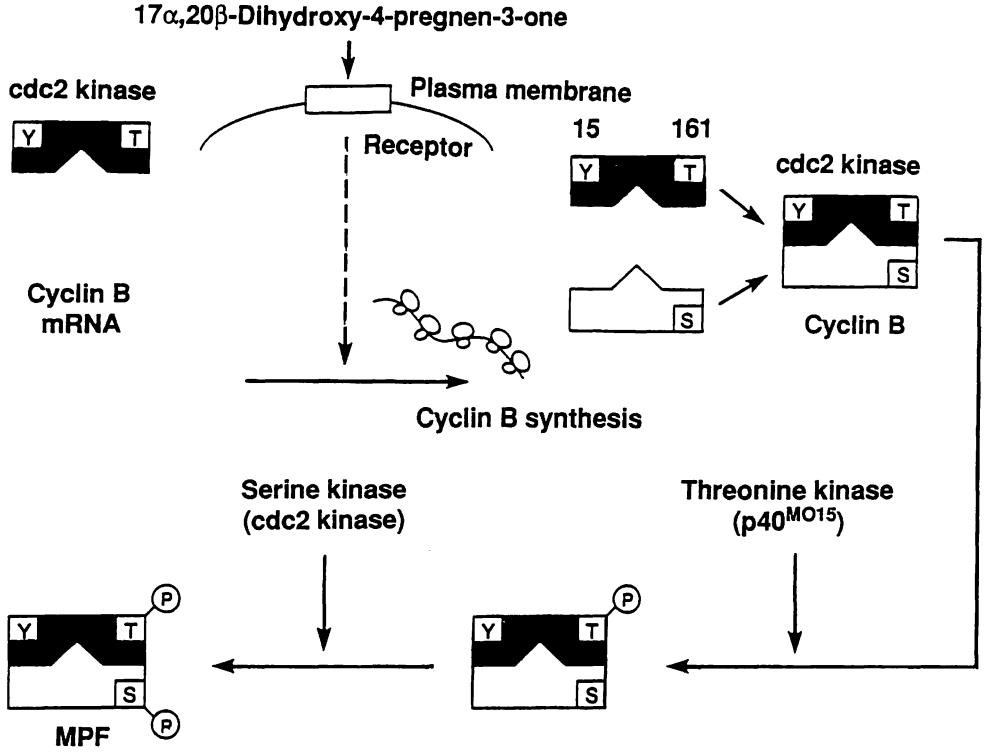

Fig. 1. Current model of the formation and activation of maturation-promoting factor (MPF) during 17α, 20β-DP-induced meiotic maturation of fish oocytes. S, serine; T, tyrosine; Y, threonine; P, phosphorylation.

goldfish 26S proteasome, an ATP-dependent protease, can digest a bacterially produced goldfish wild bype cyclin B, producing an intermediate cyclin B (42-kDa). In contrast, various cyclin B mutants lacking the first 42, 68, and 96 N-terminal amino acids are not digested by the proteasome. Amino acid sequence analysis of the 42-kDa intermediate cyclin B reveals that 26S proteasome cleaves the C-terminal piptide bond of lysine 57. This is the first evidence for the crucial role of 26S proteasome in cyclin B degradation.

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.

To isolate the genes that are expressed or suppressed in eel testes during HCG-induced spermatogenesis, we extracted mRNA from control testes and testes that had been given a single injection of HCG one day previously. Subtractive cDNA libraries were constructed to clone specific cDNAs expressed at each stage. So far, three cDNA clones have been isolated and characterized. From its deduced amino acid sequence, one of the up-regulated cDNAs was identified as coding for the activin B subunit. We used Northern blot analysis and *in situ* hybridization techniques to examine sequential changes in transcripts of testicular activin B during HCG-induced spermatogenesis. No transcripts for activin B 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 and there was a further sharp decrease by nine days. The HCG-dependent activin B 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. Taken together, these results suggest that activin acts as a regulator of spermatogonial proliferation.

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.

Our recently-initiated studies include work on the molecular mechanisms of (1) sex determination and gonadal differentiation, (2) meiosis initiation, and (3) vitellogenin uptake into the oocyte from the vascular system.

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DIVISION OF CELL DIFFERENTIATION

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Members of the Division have been involved in two well associated projects. One, which was 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 region 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 suppreses the transcription. The expression of the *POU-M1* gene has been analyzed in *Bombyx* embryos by *in situ* hybridization. To our surprise, the gene was expressed specifically for the first time at stage 18–19 (Fig. 1C) in the limited site of the labial segment where the silk gland is going to be formed by invagination (Fig. 1A). This location exactly matches with the site where the *Bombyx Scr* expression disappears specifically (Fig. 1B) which was detected in the entire labial segment in the preceding stage (see the section II). The *POU-M1* expression continues along with the silk gland development and is confined to the middle portion of the silk gland by late embryonic stages. It was late in the stage 22 when the *POU-M1* expression was also detected in the central nervous system as expected for a *Cf1-a* homologue. These observations suggest that the *POU-M1* gene may have multiple

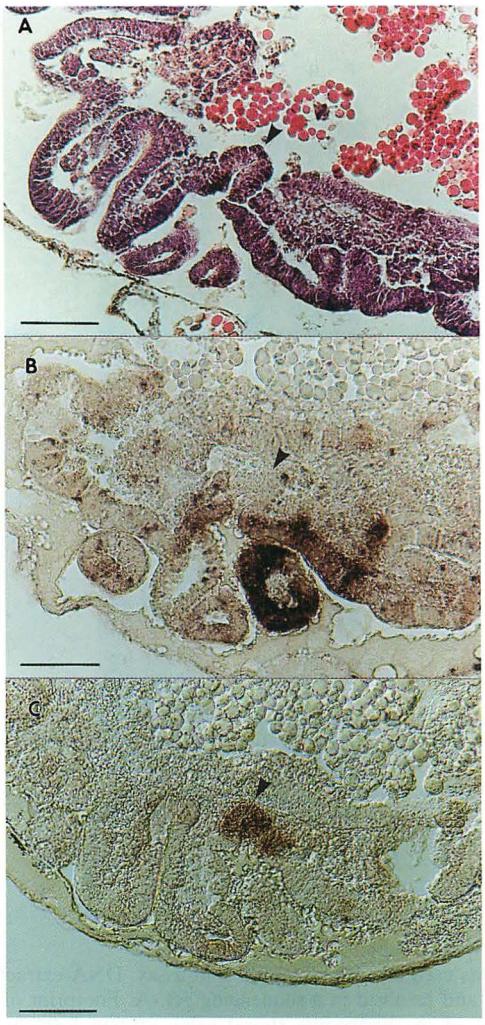

Fig. 1 Gene expression at the site of the silk gland invagination at stage 19.
A. A sagittal section stained with hematoxylin-eosine. B. Hybridization with a *Bombyx Scr* probe. C. Hybridization with a *POU-M1* probe. The arrowhead indicates an invagination of the silk gland. The bar indicates 50 um.

functions; (1) contribution to the commitment of the primordial silk gland cells, (2) roles in maintenance of the silk gland development, (3) roles in establishing terminally differentiated states as a positive transcription factor for the sericin-1 gene and a negative transcription factor for its own gene, and (4) contribution to the commitment of the nerve cells.

Silk gland specific transcription factor SGF-1 interacts with the SA, FA and FB sites localized upstream of the sericin-1 and fibroin gene promoters. Partial purification of this factor was achieved using SA site-DNA affinity resin. Competitive gel shift assay using proteins renatured from SDS-PAGE showed that SGF-1 corresponds to two polypeptides migrating on 42 and 43

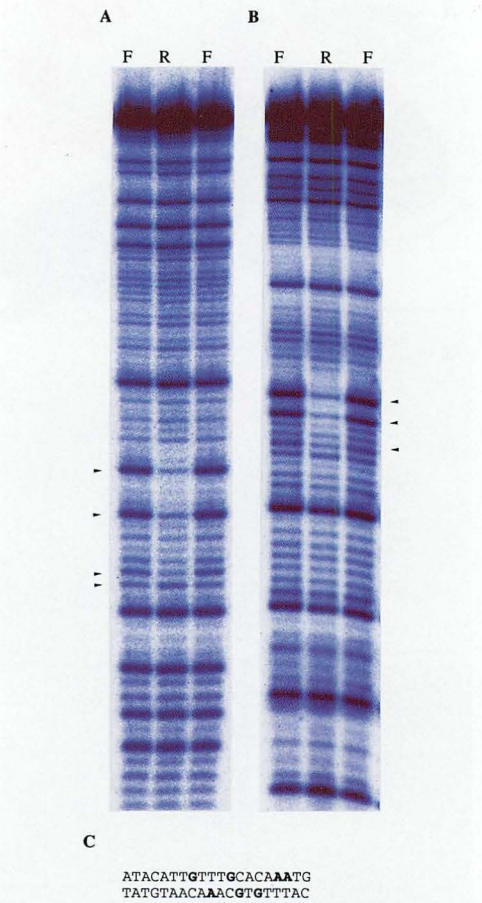

Fig. 2. SGF-1 interaction with SA site.

Mildly methylated DNA was used as a probe for gel shift assay. DNA extracted from free probe (F) or retarded band (R) was cleaved and resolved in a sequencing gel. A. Footprint of the coding strand using pure 42 kDa protein. B. Footprint of the noncoding strand using protein fraction obtained after DNA affinity resin. C. SA site in the extend previously identified by DNaseI footprinting. G and A residues the methylation of which actually affect SGF-1 binding are indicated in bold. These residues are marked by arrowheads in A and B.

kDa, respectively. In the next purification step, these two proteins were recovered in a single RPC fraction of more than 80% purity. The 42 and 43 kDa proteins were digested directly in SDS-PAGE gel, and peptides were eluted, fractionated and sequenced. Four of them yielded useful sequences, which should facilitate molecular cloning of SGF-1 gene. The pure 42 and 43 kDa proteins were used to study the SA binding site by the methylation interference assay (Fig. 2). Their footprints appear identical with each other and with the footprint obtained from the specific retarded band produced by the SA probe in a crude extract.

Intronic modulator (enhancer II; +156/+454) of the fibroin gene is composed of 6 octamer-like elements. The octamer binding factor-1 (OBF-1) binds to the elements at around +220 and

+290 in the enhancer II, and also to the element at around −130 in the upstream enhancer (enhancer I). The OBF-1 activity was detected only in the posterior silk gland abundantly at stages only when the fibroin gene was expressed. Purification of OBF-1 is in progress and 32 kDa protein recovered from SDS-PAGE showed the OBF-1 activity. The second octamer binding factor (OBF-2) binds strongly to the element at around +420 and weakly to other elements at around +220, +290 and +370. The OBF-2 binds also to the enhancer I weakly. Gel shift assay using a POU-M1 specific antibody showed that the OBF-2 is the POU-M1. The third octamer binding factor (OBF-3) binds to the elements at around +220 and +290, and also to an element at around −60. An oligonucleotide corresponding to the +290 element competed the transcription enhancement both by the enhancer I and II in the posterior silk gland extracts. Integration of these factors as well as FF1 and 2 which were purified before will be important for regulation of the fibroin gene transcription.

II. Genes involved in the *Bombyx* body plan

We have isolated a *caudal* (*cad*) homologue from a cDNA library of *Bombyx mori* embryos. The *Bombyx cad* transcripts were firstly accumulated in the nurse cells and transferred into the oocyte in a definite period during oogenesis. The maternal transcripts formed a concentration gradient spanning anteroposterior axis during the gastrulation stage and were restricted to the anal pad after 2 days of embryogenesis (*Development* **120**, 277−285 (1994)). This observation gives a sharp contrast with the *Drosophila cad* expression pattern which reveals the corresponding expression profile during the syncytial blastoderm stage. The *Bombyx* cad protein was not detected in the ovary and early 9 hrs of eggs, but was first detected evenly during cellular blastoderm stage. It was during gastrulation when *Bombyx* cad protein concentration gradient shifted along the anteroposterior axis. The observed distinct timing and conservation on mRNA as well as protein gradients formation between *Drosophila* and *Bombyx* might contribute to realize differences in the body plans and give some clues to elucidate the mechanism and function related to mRNA and protein concentration gradients.

To understand how the labial segment identity is determined and the silk gland development is controlled, we have begun characterizing expression patterns of *Bombyx Sex combs reduced (Scr), Deformed (Dfd), fork head (fkh),* and *POU-M1.* The *Bombyx Dfd* was expressed in the mandibular and maxillar segments but not in the labial segment where the *Bombyx Scr* was specifically expressed. As described in the previous section, the *Bombyx Scr* expression was eliminated in the invagination site where the silk gland development takes place. This elimination was complemented with the specific expression of *POU-M1.* During the silk gland development the *POU-M1* expression was detected in the entire region of the gland in the early phase, and restricted to the anterior portion and middle portion of the gland and finally to the middle portion, while the *Bombyx fkh* expression was detected in the middle and posterior portions in the middle phase of development. These observations may outline the framework of the hierarchy in the silk gland development and differentiation.

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 a 270 kDa protein (p270) is expressed specifically in the abdominal legs. We have purified the p270 from embryos, and prepared a specific antibody against the p270. Using the antibody no p270 was detected by Western blot analysis in the homozygous E^{Ca}/E^{Ca} embryos which do not carry the *Bombyx abd-A* gene. It is likely that the p270 expression is under the control of the *Bombyx abd-A* gene. Immunohistochemical analysis indicated that the p270 is localized in restricted cells of the wild type abdominal legs but not detected in the late embryonic stages. To study a role of the p270 in morphogenesis of embryonic abdominal legs, molecular cloning of p270 cDNA is being planned.

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There is a mode of reparative regeneration, in which the lost tissue or organ can be compensated by cellular metaplasia (transdifferentiation) of once specialized tissue cells. In the newt and some other limited species of the vertebrate, the lens and neural retina can be completely regenerated through the 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 transdifferentiaton of PECs of vertebrates has been studied in *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 including human.

Our studies have been conducted to clarify the molecular mechanism controlling the lens transdifferentiation of 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, particularly focussing on genes which have been predicted to have essential roles in regulation of the differentiated state of PECs and also of lens transdifferentiation of them. The followings are abstracts of investigations and results obtained in 1993 through studies of these major projects and the following additional subjects which have been conducted as collaborative works with scientists from the outside: (1) Pattern formation of Lepidopteran wing and (2) Basic analysis of biocompatibility of hydroxyapatite as a biomaterial.

I. Analysis of functions of genes responsible for the lens transdifferentiation of PECs

It has been predicted by analysis thus far that products of two genes, tentatively designated as pP344 and pP64 must function to regulate the differentiated state and lens transdifferentiation of PECs *in vivo* and *in vitro*. In this year, we have extended our study to analyze functions of these two genes. We analyzed pP344 gene expression during chicken eye development by RT-PCR and *in situ* hybridization and also characterized the pP344 protein using anti-peptide antibodies. The time course of expression level showed two peaks; the first peak occurred around the 10th day similarly to the expression of melanosome-related genes, while the second peak occurred just after hatching when PECs had completely differentiated, sug-

gesting that pP344 gene may be related to the function of fully differentiated PECs. Anti-synthetic peptide antibodies detected pP344 protein in the culture medium of the PECs but not within the cells, strongly suggesting that pP344 gene product is a secreted protein. The pP64 gene produces two different transcripts, 5.0kb and 6.0kb mRNAs, whose products are TGFβ-binding proteins, and fully differentiated PECs express 5.0kb mRNA as a major product in addition to 6.0kb mRNA but both multipotent dedifferentiated PECs and transdifferentiated lens cells express only 6.0kb mRNA. It has been clearly shown that protein produced by 5.0kb mRNA is secreted by PECs but protein produced by 6.0kb mRNA is trapped by the extracellular matrix of PECs in the *in situ* eye. These results must be the fundamental information for our further studies on the molecular regulation of the lens transdifferentiation of PECs.

II. Analysis of transcriptional regulator in pigmented epithelial cells

The product of mouse microphtalmia (*mi*) gene is thought to be a class of basic helix-loop-helix-zip transcriptional factor which may regulate the directions of differentiation of some types of cells including pigmented epithalial cells, because some mutations in *mi* gene cause transformation of pigmented epithelium to neural retina in mice. To analyze the molecular mechanisms in differentiation and transdifferentiation of cultured pigmented epithelial cell, avian homologs of *mi* gene were isolated from cDNA libraries of chicken and quail pigmented epithelial cells. Nucleotide and amino acid sequences of *mi* are well conserved between aves and mammals. The products of *mi* genes have a basic helix-loop-helix-zip domain similar to ubiquitous transcriptional regulators TFE3, TFEB and TFEC, showing possible interactions of *mi* product and some factors relating the TEFs in gene regulation. Northern blotting shows activation of *mi* gene during differentiation of pigmented epithelial cells and inactivation of it in dedifferentiated state of cells suggesting the key role of *mi* gene in differentiation of pigmented epithelial cells. The possibility in which transdifferentiation of pigmented epithelial cells may be regulated by activity of *mi* product is now analyzed by genetic manipulation of *mi* gene in cultured cells.

III. Expression of connexin in the pigmented epithelial cells

The morphological change of the gap junction in the course of the transdifferentiation of PECs of the chick embryo was previously studied. In essence, although the gap junction is abundantly found in the PECs cultured *in vitro*, it is temporally lost in the dedifferentiated PECs but reappear in the lentoids or redifferentiated PECs. Molecular biological analysis showed that the gap junction in the PECs is made up of chicken connexin 43, which is one of the genes for the main component of the gap junction. We are preparing probes for other connexin genes in order to clarify the expression pattern of connexin genes in the course of transdifferentiation. Here we summarize some preliminary data on connexins.

Polyclonal antibodies against connexin 43 were raised against synthetic peptides. One of the antibody against a cytoplasmic loop reacted specifically and can serve as a good marker. Another antibody against an extracellular loop reacted from outside of the cell. This reac-

tion was shown by directly applying the antibody to living cells, and then detecting the bound antibody with fluorescent-labeled second antibody. This result suggests that it may be possible to modify the gap junctional cell-to-cell communication from outside of the cell, thus enabling the inhibition of gap junction in large population of cultured cells.

The pattern of connexin 43 gene expression was examined in the eyes of the 9-day-old chick embryo by *in situ* hybridization technique. Although the expression is seen throughout the pigmented epithelium, the expression is conspicuously strong in the iris and the ciliary pigmented epithelium. The immunofluorescent staining with anti-connexin 43 peptide antibody also showed high level of connexin 43 protein in the iris and ciliary pigmented epithelium. The expression pattern of a PEC-specific gene, pP344, which was found in our laboratory, showed an opposite gradient of expression, *i.e.* highest expression is seen in the retinal PE. These genes can be excellent markers for the regional specificity of the pigmented epithelium and also it is possible that they bear some roles in the physiological activity of each epithelium.

IV. Analyses of the process of programmed cell death in the pupal wing of *Pieris rapae*

The shape of the wings of butterflies are formed by an extensive cell death at the margin of the wings during the pupal period. We have suggested, through ultrastructural observations of the pupal wings of *Piris rapae*, that the cell death observed here resemble the apoptosis, which is a characteristic form of programmed cell death reported to occur throughout the development of vertebrates and invertebrate.

One of the critical symptom of the apoptosis is the fragmentation of the DNA in the nucleus prior to the death, but examination of such fragmentation by standard method using the electrophoresis must be difficult because the amount of the tissue is very limited. We recently adopted the TUNEL method (TdT-mediated dUTP-biotin nick end labelling) to detect the fragmentation of DNA in *in situ* tissue. This procedure adds biotinylated dUTP at existing 3′ ends by an enzyme terminal deoxynucleotidyl transferase. The nuclei containing DNA with many breaks has much more number of 3′ ends than other nuclei, so that much more biotinyl residues are incorporated. The biotinyl residue is detected by avidin-biotinyl-horse raddish peroxidase complex (ABC) yielding staining on the nuclei with DNA fragmentation. We applied this method to the whole mount preparation of the pupal wing and showed that nuclei with positive staining are observed at the period and the area of extensive cell death formerly observed ultrastructurally (Fig.). Although this method provides no information on the length of the DNA fragments, this result strongly suggests that there works a mechanism of cell death including DNA fragmentation resembling the apoptosis in the process of the formation of the wing of butterflies.

We also re-examined ultrastructural data and concluded that cells with a morphology of macrophages are abundantly seen near the area of cell death, suggesting that the dead cells are actively phagocytosed by these macrophages.

We have concluded at present that the loss of the margin of the pupal wing proceeds by the programmed cell death of the epithelial cells at the margin and by

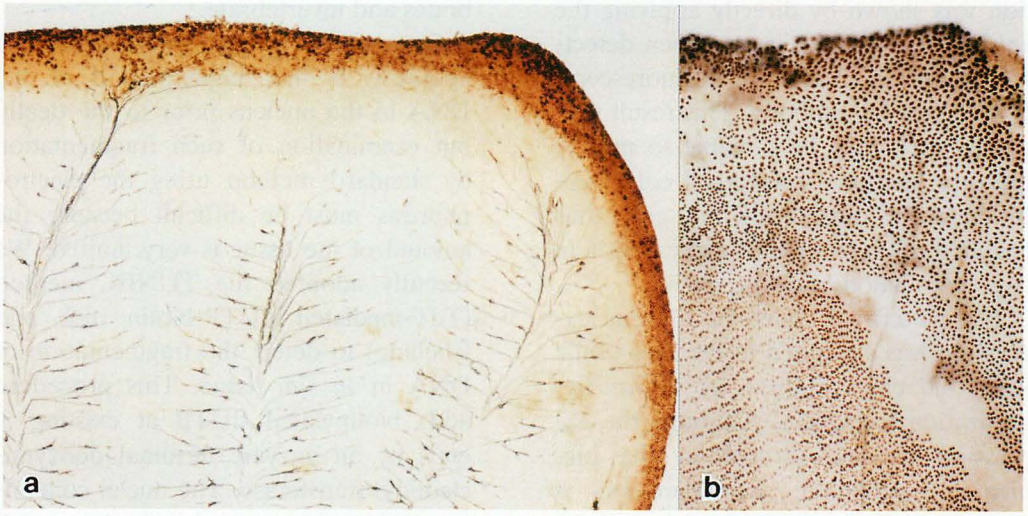

Fig. The nuclei in which fragmentation of DNA occurrs are stained by TUNEL method (a). They are found only in the margin of the pupal wing. In the control which were pretreated with DNase I, all the nuclei are stained (b).

the removal of cell debris through phagocytosis by neighboring epithelial cells and by macrophages. The molecular mechanism determining the area to be removed and how this determination is realized as cell death are two important aspects of further studies.

V. An attempt to improve biocompatibility of hydroxyapatite as a biomaterial

Based on findings of cell biological analysis conducted thus far, we have attempted to improve biocompatibility of hydroxyapatite using *in vitro* culture system of human gingival cells established in our laboratory. Adhesion, spreading and growth of gingival cells, epithelial cells, and connective tissue fibroblasts, cultured on the hydroxyapatite can be dramatically enhanced when the hydroxyapatite surface is modified by coating with type I collagen molecules after ion-etching under condition of 10^{-2} mmHg with an ion-sputtering equipment, suggesting a strong possibility to improve biocompatibility of hydroxyapatite materials by biochemical modification of its surface.

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DIVISION OF DEVELOPMENTAL BIOLOGY (ADJUNCT)

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Research Associates: Koji Mikami
Takefumi Kawata
JSPS Postdoctoral Fellow: Hisabumi Takase
Visiting Scientist: Shweta Saran (from University of Delhi, India)
Graduate Students: Takuya Ito (from Kyoto Univercity)
Ken-ichiro Taoka (from Kyoto Univercity)

Cell division is the most fundamental event in cell growth and cell differentiation during development. One of powerfull approaches for understanding of the molecular mechanisms of cell division is to investigate the gene expression regulated in a cell cycle-dependent manner. Representative examples of eukaryotic cell cycle-dependent genes are histone genes, expressed mainly in the S phase during the cell cycle. We have identified the *cis*-acting hexamer (ACGTCA) motif involved in S phase-specific transcription of the wheat histone H3 (TH012) gene, and bZIP-class *trans*-acting factors specific to the hexamer motif. Of bZIP proteins, HBP-1a(17) and HBP-1b(c38) are known to be essential for controlling periodic transcription of the H3 gene, because genes for two proteins are also regulated in a cell cycle-dependent manner. It is, therefore, likely that HBP-1a(17) and HBP-1b(c38) function as regulators of cell division in plants. In 1993, we focused our attention on the functional significance of bZIP proteins during plant development (summarized in the sections I and II).

We also have been continuously studying the transcriptional regulation of wheat histone genes from a different viewpoint. Based on the function in chromatin formation, histones are classified into core (H2A, H2B, H3, and H4) and linker (H1) proteins. Despite differences in their functions, core and linker histone genes are coordinately expressed in S phase during the cell cycle. To understand how a coordinate expression of core and linker histone genes is regulated, we investigated the regulatory mechanisms of the wheat histone H1 (TH315) gene (surmmarized in the section III).

I. Regulation of a gene encoding transcription factor HBP-1a(17) in transgenic *Arabidopsis*

To analyze how the HBP-1a(17) and HBP-1b(c38) genes are regulated *in vivo*, we have produced transgenic *Arabidopsis* plants carring chimeric genes which are composed of the HBP-1a(17) or HBP-1b(c38) promoter and the β-glucronidase (GUS) coding sequence. The expression patterns of the chimeric genes were histochemocally analyzed during development from germination to seed production. Unfortunately, the HBP-1b(c38) promoter/GUS chimeric gene was not expressed in any transgenic plants. We, therefore, examined the spacial and temporal expression pattern of the HBP-1a(17) promoter/GUS fusion gene.

In germinating seedlings, the HBP-1a(17) promoter/GUS fusion gene was expressed in only cotyledons and thereafter in cotyledons and first leaves (Figs. 1A and 1B). At eight days after germination, GUS activity was detected mainly in first and second leaves, although weak GUS activity was still observed in expanded cotyledons (Figs. 1C). The gene expression seemed to decrease as the leaves became older. As plants progressed from vegitative to productive growth, GUS activity was not

detected in floral organs, stems, cauline leaves and axillary buds (Figs. 1D and 1E), indicating that the HBP-1a(17) promoter is activated in only developing young leaves in the vegitative growth stage.

We next investigated the effect of light on the HBP-1a(17) expression during germination. When seven-day-old seedlings grown in light were placed in the dark for a week, GUS activity in young leaves was decreased (Fig. 1F). Since dark-grown seedlings showed no GUS activity in any organs (data not shown), the above result indicated that the expression of the HBP-1a(17) gene is controlled by light. This conclusion is supported by the fact that the HBP-1a(17) promoter has several characteristic sequences such as boxII, I-box, G-box and GATA motif, all of which are known to be involved in the light-regulated transcription. Since HBP-1a(17) can specifically interact with the G-box, we supposed that HBP-1a(17) is implicated in the transcriptional regulation of light-inducible genes in the early stage of photomorphogenesis as well as cell cycle-dependent gene expression. This possibility is beeing checked using plants produced by mating HBP-1a(17)-expressing transgenic *Arabidopsis* with several phytochrome-deficent *Arabidopsis* mutants.

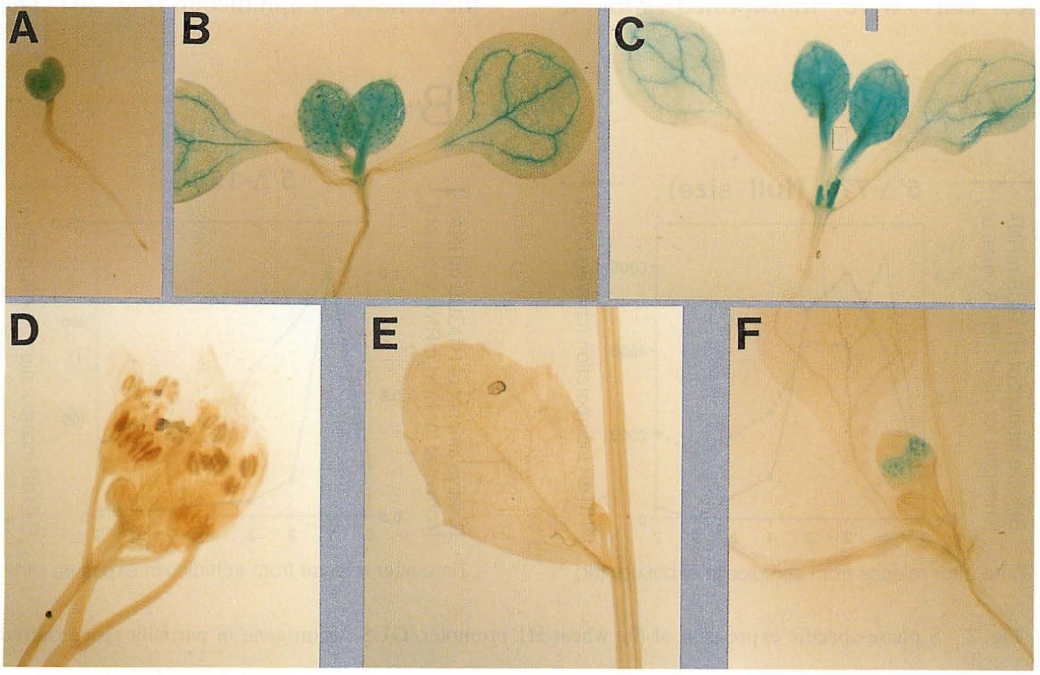

Fig. 1. Vegitative leaf-specific expression of the HBP-1a(17) promoter/GUS fusion gene in transgenic *Arabidopsis* during development.

The spacial and temporal expression pattern of the cheimeric gene was analyzed by histochemical assays for GUS expression. A, Three-day-old seeding; B, Seven-day-old seeding; C, Eight-day-old seeding; D, Floral organ (mature flower); E, Stem, cauline leaf and axillary bud; F, Plant grown in the dark for a week after growing in light for a week.

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

When the HBP-1b(c38)-expression plasmid was co-transfected into tobacco BY-2 cells with reporter plasmids containing the binding sites of HBP-1b(c38), expression of the reporter gene was repressed. This suggests that HBP-1b(c38) may act as a transcriptional repressor. To elucidate the functional roles of HBP-1b(c38) in plant development, we have been trying to produce transgenic *Arabidopsis* which can overexpress *Arabidopsis* HBP-1b(c38)-homologue or its antisense RNA.

III. *Cis*-control elements for S phase-specific expression of the wheat histone H1 gene

To study cell cycle-dependent transcription of the wheat H1 gene, cultured rice cells were transformed with a chimeric gene which consists of the GUS coding sequence and the 5' upstream sequence of the H1 gene spanning from −771 to +74. When cell cycle progression of the stable transformants was partially synchronized by aphidicolin treatment, the GUS mRNA level was peaked at 2hr after the removal of drug and then gradualy decreased (Fig. 2A). Considering a result of DNA synthesis rate in aphidicolin-treated cells, we concluded that the wheat H1 gene expression is regulated in an S phase-specific manner in rice cells.

The H1 promoter that was 5'-deleted to the position −153 still had the ability to regulate S phase-specific transcription, although the level of the GUS mRNA transcribed from the 5'-deleted promoter appeared to be maximum at 1hr after the removal of aphidicolin (Fig. 2B). The

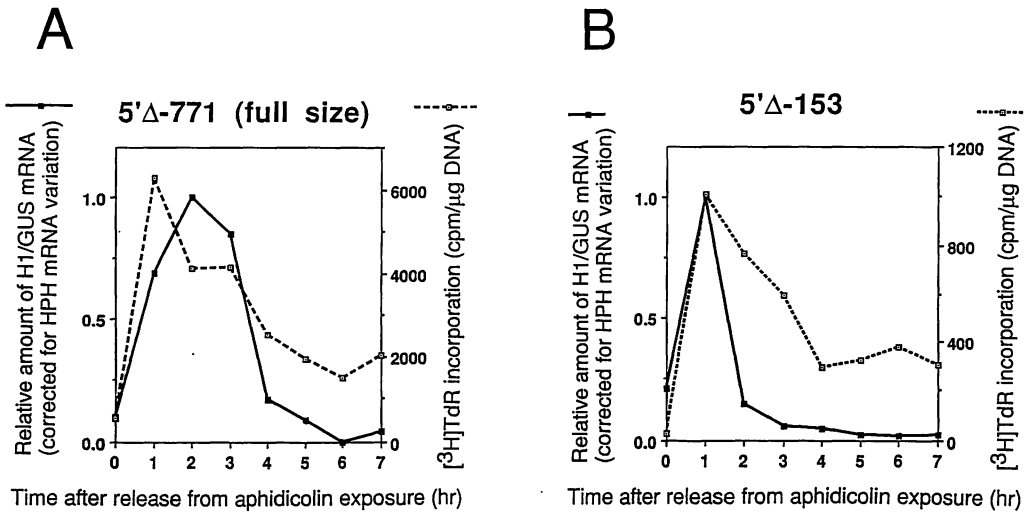

Fig. 2. S phase-specific expression of the wheat H1 promoter/GUS fusion gene in partially syuchronized rice cells.

Transformed rice cells were partially synchronized by aphidicolin treatment and then released into the S phase. Accumulation and turnover of the mRNA transcribed from the wheat H1 promoter/GUS fusion gene was analyzed by the S1 protection assays. Quantity of mRNA was corrected for that of constitutively transcribed HPH(hygromycin phosphotransferase) mRNA. DNA synthesis rates were monitored in parallel by measuring the incorporation of [^{3}H]TdR. A, accumulation and turnover of the transcript from the 5'Δ-771 (full size) promoter/GUS fusion gene; B, accumulation and turnover of the transcript from the 5'Δ-153 H1 promoter/GUS fusion gene.

proximal promoter region (−153 to +74) contains several characteristic sequences, conserved in the *Arabidopsis* H1 promoters as well. We are now doing more detailed experiments to identify the *cis*-element directing S phase-specific transcription of the wheat H1 gene.

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Minami, M., Huh, G. -H., Yang, P. and Iwabuchi, M. (1993) Coordinate gene expression of five subclass histones and the putative transcription factors, HBP-1a and HBP-1b, of histone genes in wheat. *Plant Mol. Biol.* **23**, 429–434.

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DEPARTMENT OF REGULATION BIOLOGY

Chairman: Norio Murata

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

DIVISION OF MOLECULAR NEUROBIOLOGY

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Jun-ichi Yuasa
*Visiting Fellow: Haruyuki Matsunaga**
Technical Staff: Tomoko Mori
Shigemi Ohsugi

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

The principal interest of this division is molecular mechanisms that govern the development of the vertebrate central nervous system. Our efforts are currently focused on projects to reveal the molecular bases of neuronal cell migration and neuronal network formation.

I. Molecular basis of neuroblast migration

In the developing central nervous system, migration of post-mitotic neurons is the key process through which laminated and/or nucleated structures form out of the uniform neuroepithelium. Thus, to establish the functional neuronal network, the course of neuroblast migration must be precise with the combination of radial and tangential movement. Although many adhesion molecules are known to be expressed in the neural tissue, the molecular basis of neuroblast migration remains to be elucidated.

T61 antigen is a good candidate of the molecule which is responsible for the neuroblast migration in the radial direction. Monoclonal antibody T61 was originally characterized by its inhibitory activity on neurite outgrowth from chick retinal explants *in vitro*. However, it turned out recently that neuroblast migration was inhibited when the T61 producing hybridoma was injected in the ventricle of the developing midbrain of chick embryos. As far as we know, this is the first example in which an antibody inhibited neuroblast migration *in vivo*.

The expression of T61 antigen is restricted to the neural tissue, and the distribution within the tissue is rather uniform. Immunocytechemistry of primary culture cells showed that only neuronal cells express the antigen. The finding that T61 antigen is concentrated at the filopdia of growth cone is consistent with the presumptive function of this molecule in the cell migration. The T61 detects several bands including a major 440kD band by Western blotting and immunoprecipitation. These results suggest that T61 antigen is a novel molecule. Screening of λgt 11 library prepared from chick brain with T61 gave rise to many positive signals with various intensity. Nucleotide sequence analysis revealed that these clones are derived from several different mRNAs. We are now trying to identify the authentic cDNA clone from them.

II. Proteoglycan and brain development

Proteoglycans have been recognized to play important roles in the regulation of cell growth, differentiation and adhesion. The vertebrate brain also contains many kinds of proteoglycans, which are considered to be involved in the neuronal migration, axon guidance and axonal outgrowth.

6B4 proteoglycan is a brain specific large chondroitin sulfate proteoglycan with a 300-kDa core protein. This proteoglycan has the soluble and membrane-bound forms, and its expression is dynamically regulated during development of the brain. In the adult rat hindbrain, 6B4 proteoglycan is selectively expressed around the neurons constituting the cerebellar mossy fiber system. In the

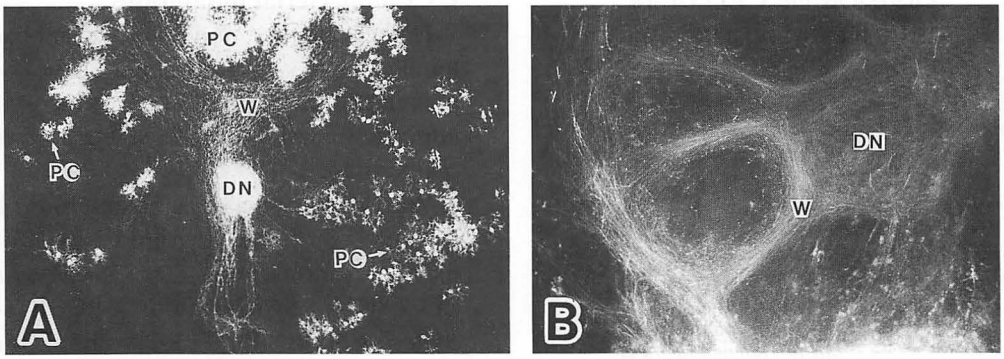

Fig. 1. Cocultures of slices from cerebellar cortex and pontine nuclei. Fluorescent micrographs to illustrate Purkinje cells (A) and fibers from pontine nuclei (B) in cerebellar slices. PC, Purkinje cell; DN, deep cerebellar nuclei; W, white matter.

early postnatal animals, the expression of 6B4 proteoglycan is highly correlated with the synapse formation of this system, suggesting that 6B4 proteoglycan plays important roles in the cerebellar circuit formation.

To investigate this possibility, we developed an *in vitro* reconstitution system of cerebellar circuits, in which slices of cerebellum and pontine nuclei were cocultured. The latter is a major precerebellar nuclei from which many mossy fibers project to the cerebellum *in vivo.* During long-term culture (2–3 weeks), Purkinje cells developed well-arbored dendrites and projected to the deep cerebellar nuclei as revealed by anti-InsP3 receptor monoclonal antibody staining (Fig. 1A). On the other hand, the pontine fibers projected to the cerebellar granule cell layer as shown by DiI staining (Fig. 1B). Such a projection pattern is very similar to that observed in vivo. We are now studying the function of 6B4 proteoglycan in the fiber projection and the neural circuit formation using this system.

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DIVISION OF CELLULAR REGULATION

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The research effort of this division is directed toward the understanding of tolerance and adaptation of plants to temperature extremes, with particular emphasis on the molecular mechanisms by which plants acclimate or tolerate these temperature conditions. In 1993, several significant advances were made in the area of temperature response and related areas in the study of cyanobacteria.

I. Cyanobacterial desaturases.

Higher plants, and most cyanobacterial strains, contain high levels of polyunsaturated fatty acids, which are important in their response to ambient temperature.

We isolated the *desA* genes for Δ12 desaturases of the acyl-lipid type from the cyanobacteria, *Synechocystis* PCC6803, *Synechocystis* PCC6714, *Synechococcus* PCC7002, and *Anabaena variabilis* and found four conserved sequence domains. We over-expressed the *desA* gene of *Synechocystis* PCC6803 in *Escherichia coli* using the bacteriophage T7 polymerase system. The Δ12 desaturase, thus over-expressed in *E. coli*, was active *in vitro* when reduced ferredoxin was added as an electron donor. This result indicates that the cyanobacterial desaturase is similar to the plastidial desaturases in terms of the electron-donating system, but is dissimilar to the cytoplasmic desaturases that use cytochrome b_5 as an electron donor.

We isolated the *desB* and *desC* genes of *Synechocystis* PCC6803, which encode the ω3 and Δ9 desaturases, respectively, of the acyl-lipid type. We transformed another cyanobacterial strain, *Synechococcus* PCC7942, with the *desA* and *desB* genes. This strain contains only the Δ9 desaturase. The mode of fatty acid desaturation in the transformed cells demonstrates that the ω3 desaturase can introduce a double bond at the ω3 position of fatty acids that contain an unsaturated bond at the Δ12 position.

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

To understand the roles of unsaturation of membrane lipids, we transformed *Synechococcus* PCC7942 with the *desA*

gene. This transformation greatly modified the extent of unsaturation of the fatty acids of membrane lipids. In the wild-type strain, only monounsaturated lipid molecules existed; in the transformant, each of the monounsaturated and diunsaturated lipid molecules contributes to about 50% of the total membrane lipids. This change in the unsaturation of membrane lipids greatly reduced susceptibility to low temperature.

These results suggest that diunsaturated fatty acids play an important role in protection against damage to photosynthetic processes by low temperature. By contrast, photosynthetic electron transport, measured at various temperatures, and susceptibility to high temperature were not affected by changes in the extent of unsaturation of the fatty acids.

III. Regulation of the expression of the *desA* gene by changes in the fluidity of the plasma membrane.

Living organisms can maintain the molecular motion, or "fluidity", of membrane lipids by regulating the level of unsaturation in fatty acids. For example, cyanobacterial cells respond to a decrease in temperature 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 fatty acids of membrane lipids is regulated at the level of the expression of the desaturase genes.

To study the mechanism of the low temperature-induced regulation of the expression of the *desA* gene in greater detail, we examined the effect of membrane fluidity on the gene expression by catalytic hydrogenation of the unsaturation bonds of fatty acids of membrane lipids. A 4-min hydrogenation of the *Synechocystis* PCC6803 cell increased the level of fully saturated lipids at the expenses of diunsaturated lipids in plasma membrane, but had no such effect in thylakoid membranes. After this hydrogenation, the level of mRNA of the *desA* gene increased 10-fold within 30 min. This increase in the mRNA level after hydrogenation resembles that following a decrease in ambient temperature. These results suggest that the decease in fluidity of plasma membrane is the first response to temperature change.

IV. Heat stability of photosynthesis.

We also focused on the response of plants to high-temperature stress. Since photosynthesis is the physiological process most susceptible to heat stress in plants, its heat stability is an important factor in the tolerance of plants to high temperature.

We studied a component responsible for the heat stability of photosynthesis in the cyanobacterium, *Synechococcus* PCC7002. When 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 with Triton X-100, we purified a protein that increased the heat stability of oxygen evolution by 4°C. The protein was identified as a low redox potential cytochrome *c*-550 having a molecular mass of 16 kDa. We isolated the gene encoding this cytochrome from *Synechococcus* PCC7002, and determined its nucleotide sequence. The deduced amino-acid sequence revealed that the gene product consists of a transit

peptide of 34 residues and a mature protein of 136 residues. 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.

V. Two *groEL*-homologous genes in cyanobacterial cells.

There are a wide variety of heat shock proteins, most of which are highly conserved in both prokaryotes and eukaryotes. Cellular levels of these proteins dramatically increase upon heat shock or other environmental stresses. Some heat shock proteins act as molecular chaperones that assist in the folding and assembly of other proteins.

To examine the role of heat shock proteins in acclimation to high-temperature stress, we attempted to isolate the genes for the heat shock protein *groEL* from *Synechococcus* PCC7002. We discovered that there are two *groEL*-homologous genes. One of the *groEL*-homologous genes is accompanied by the *groES* gene, and the *groES* and *groEL* genes constitute the *groESL* operon as the *groESL* operon in *E. coli.* The other *groEL*-homologous gene is not accompanied by the *groES* homologous gene. However, this *groEL*-homologous gene retains the carboxyl-terminal repeat of Gly-Gly-Met, which is typical in the *groEL* gene of *E. coli.* Heat shock increased the levels of mRNAs of both

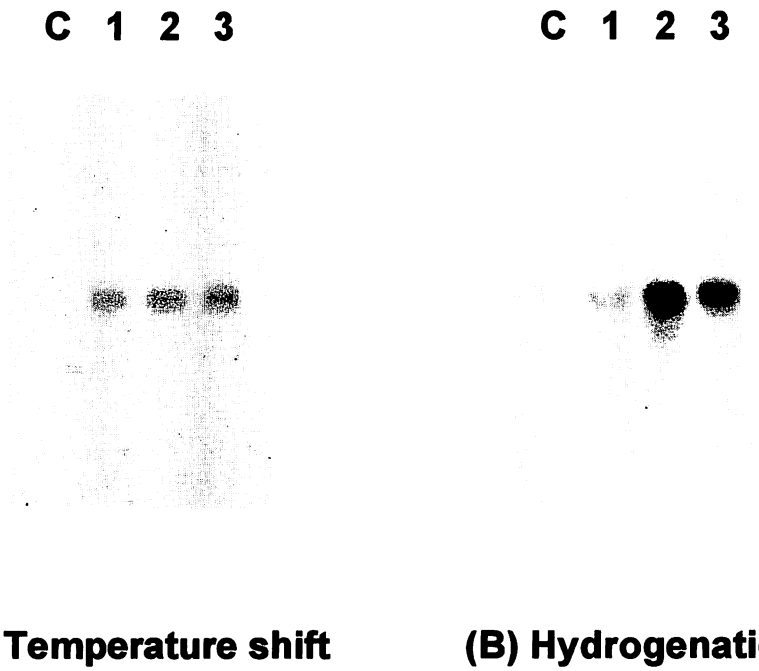

Fig. 1. Regulation of the *desA* gene expression in *Synechocystis* PCC6803 analyzed by RNA-DNA blot hybridization. (A) Temperature-induced regulation. Cells grown at 36°C were subjected to a temperature shift to 22°C, and incubated in the light for 15 minutes (1), 30 minutes (2), and 60 minutes (3). Control (c) corresponds to cells before the temperature shift. (B) Hydrogenation-induced regulation. Cells grown at 36°C were subjected to hydrogenation for 4 min to partially hydrogenate the lipids of plasma membrane, and incubated at 36°C for 15 minutes (1), 30 minutes (2), and 60 minutes (3); Control (c) corresponds to cells which were chydrogenated but before the incubation.

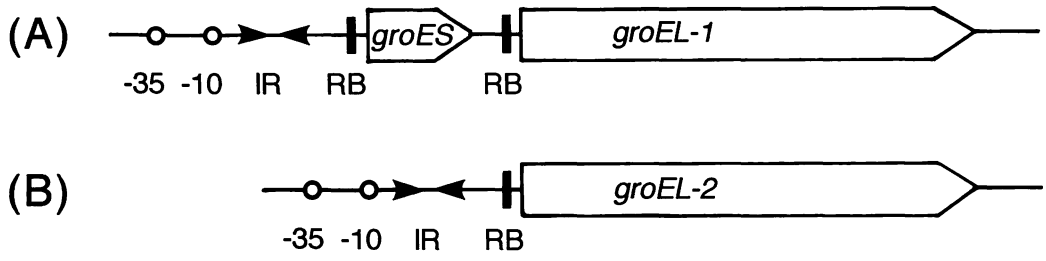

Fig. 2. Organization of two *groEL*-homologous genes in *Synechococcus* PCC7002. (A) The *groESL* operon consisting of a *groEL*-homologous gene (*groEL*-1) and a *groES* gene. (B) The other *groEL*-homologous gene (*groEL*-2) which is not accompanied by the *groES* gene. RB, the ribosome binding site; IR, a 9-bp inverted repeat found in the promoter region; -10 and -35, -10 and -35-like sequences, respectively.

groEL-homologous genes. To understand the function of the product of the *groEL*-homologous genes, we have knocked out one of the *groEL* homologous genes by insertional disruption with a kanamycin resistance gene cartridge. We are currently investigating the responses of these mutants to high-temperature stress.

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(1) Original papers

Allakhverdiev, S.I., Hayashi, H., Fujimura, Y., Klimov, V.V. and Murata, N. (1993) Inactivation of photosynthetic oxygen evolution by *o*-phenanthroline and $LiClO_4$ in photosystem 2 of pea. *Photosynth. Res.* **35**, 345–349.

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DIVISION OF BIOLOGICAL REGULATION AND PHOTOBIOLOGY (ADJUNCT)

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*Tatsuya Tomo**

*(*from Okayama University)*

The efficient energy transformation in the primary processes of photosynthesis is ensured by the highly ordered organization of molecules in the photochemical reaction centers, 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 water molecules as electron donor.

In the first approach, the basic molecular structure of the reaction center, which has been isolated in our study, will be analyzed by several methods which include crystallographic analysis, chemical modification and cross-linking analysis and physical methods. Structure-functional analysis will also be conducted on the reaction center proteins using random and site-directed mutagenesis for transformable algae, *Synechocystis* PCC 6803 and *Chlamydomonas reinhardtti.* The target of these analyses is the structure and molecular environment of P-680, the primary donor, which determine the redox potential of this system.

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 reaction center, the D1 protein. The process involves some of apparently unique steps; *i. e.*, light-regulated gene expression at the translational level, post-translational cleavage of the C-terminal extension of protein 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 (P-680) 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 both the keto and carbomethoxy C=O groups form hydrogen bonds, while in the other chlorophyll 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 P-680 structure and its interactions with apoproteins has been proposed (collaborative research with Drs Noguchi and Inoue, RIKEN). Site-directed modification of the protein subunits responsible for this hydrogen-bonding interaction (Ser-191 and Thr-192) 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 constituent subunits and amino acid residues in cross-contact in the reaction center complex.

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

The D1 subunit of photosystem II reaction center has a C-terminal extension consisting of 9-16 amino acid residues. Post-translational removal of this extension is absolutely required for constituting the machinery of oxygen evolution in photosystem II. The enzyme involved in this processing has been purified and the N-terminal partial amino acid sequence was determined.

The recognition signal was analyzed for the enzyme using substituted synthetic oligopeptides corresponding to the C-terminal sequence of precursor protein. A series of systematic substitutions around the cleavage site (between Ala-344 and Ala-345) were synthesized to analyze the function of specific amino acid and the sequence in the recognition. The efficiency of these oligopeptides as a substrate and their effectiveness as an inhibitor were examined. The conclusion from this analysis is that the secondary structure formed by the presence of specific amino acids around the cleavage site, *i. e.*, Asp-342 and Ile-343, is important in the recognition.

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 in the dark. The result of analysis suggested that the translation and/or stable accumulation of D1 protein require(s) factor(s) caused by illumination, in addition to energy supply by ATP.

III. Biophysical analyses on the molecular event during photo-transformation of phytochrome.

Phytochrome is a photoreceptor in green plants responsible for a variety of morphogenetic responses including light-regulated gene expression. Molecular event during its phototransformation from an inactive to an active forms, Pr and Pfr, respectively, was studied by using three different biophysical techniques. (1) Primary event on absorption of quanta is proposed to be isomerization of the chromophore composed of an open-tetrapyrrol. We had detected a photoproduct from Pr with rise time of 24ps by sub-picosecond flash photolysis, which has been proven to be a novel one formed directly from the excited state and probably resulted from isomerization (collaborative research with Drs Kandori and Yoshihara, IMS). (2) As the second event, we are proposing proton migration from the chromophore to the protein moiety based on resonance Raman scattering, which may be a trigger of the conformational change. We found that a proton associates with the C-ring nitrogen of the chromophore in Pr dissociates prior to the formation of a meta-intermediate by low-temperature resonance Raman scattering (collaborative research with Drs Mizutani and Kitagawa, IMS. Mizutani et al., 1994, published). (3) Ultraviolet resonance Raman scattering was measured to figure out the details in the conformational change of the protein moiety. Hydrophobicity around (a) Trp(s) in the chromophoric domain, possibly around Trp^{365} and (or) Trp^{567}, increases on phototransformation. The content of

α-helices and nonregular structure which are less populated in the chromophoric domain than the other domains, are almost unchanged by the phototransformation (collaborative research with Drs Mizutani, Kaminaka and Kitagawa, IMS).

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DIVISION OF BEHAVIOR AND NEUROBIOLOGY (ADJUNCT)

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Institute Research Fellow: Tomoko Tominaga
Visiting Scientist: Tatsuo Umeda

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 neuro-muscular 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 neuro-muscular 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 but also on the axons and growth cones of the very motoneurons which innervate these muscles (Figure 1). Its specific expression both in presynaptic motoneurons and postsynaptic muscles, and its function as a homophilic cell adhesion molecule in vitro strongly suggested that connectin play a role in the neuro-muscular specificity. We are currently study-

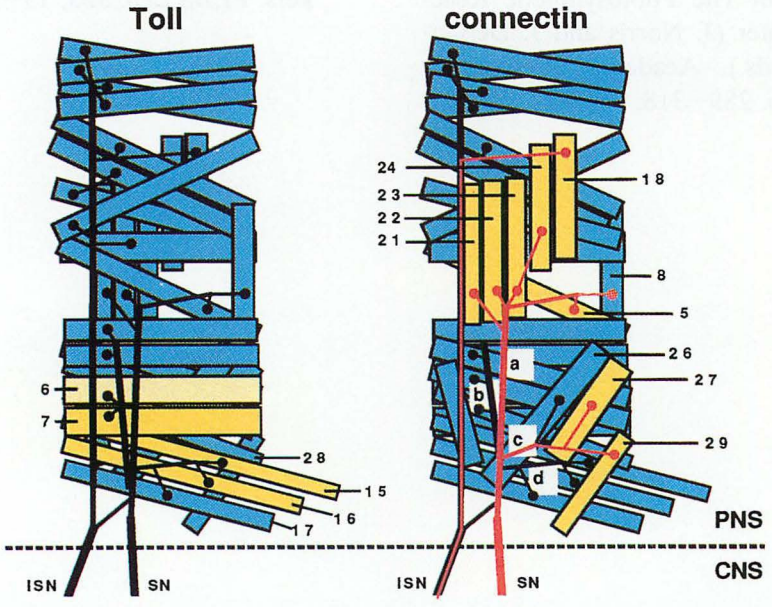

Fig. 1. Expression pattern of connectin and Toll. Schematic diagrams showing expression of connectin and Toll (yellow) on the surface of subsets of muscle fibers and expression of connectin on the subset of motoneurons that innervate the connectin-positive muscles (red). (from Nose et al., Cell 70, 1992)

ing the function of connectin by molecular genetic methods and also trying to clone novel genes implicated in the neuro-muscular connectivity.

I. Molecular genetic analysis of the function of connectin.

To study the role of connectin in vivo, we misexpressed connectin on muscle fibers that normally do not express the molecule by using Toll promoter. As shown in Fig. 1, Toll is expressed on ventral muscles #6, 7, 14–17 and 28, a different subset of muscle fibers from those expressing connectin. We used a 7 kb Toll upstream sequence sufficient for the muscle expression to misexpress connectin on these muscle fibers in P-element mediated transgenic flies.

The analysis of the transgenic flies (Toll-connectin) showed that the development of a motor nerve (SNb) is abnormal. After leaving the CNS, SNb normally grows directly into the ventral muscles and makes synaptic contact with the target muscles by st17. In Toll-connectin, the SNb axons often take abnormal trajectory and fail to make the right synaptic contact by this stage. They often grow dorsally along another nerve (ISN) or take an independent path below the ventral muscles. The results suggest that ectopically expressed connectin on the ventral muscles (#6, 7, 14 and 28) prevent the SNb from growing into these muscle fibers, pointing to connectin's role as a inhibitory signalling molecule in the formation of neuro-muscular connectivity. Thus in addition to its possible role as an attractive homophilic recognition molecule (for the motoneurons and their target muscles both expressing the molecule), connectin may serve as an inhibitory recognition molecule for other motoneurons that do not express the molecule (probably via heterophilic interaction).

We are currently trying to misexpress connectin in yet different subsets of muscle fibers by using GAL4 system, to further analyse connectin's role in vivo.

II. Cloning of novel genes implicated in the neuro-muscular connectivity in Drosophila

1. *Search for novel connectins.* An interesting possibily is that connectins constitute a LRR subfamily which are expressed on different subsets of motoneurons and muscles. We are trying to isolate novel connectins by using PCR and will study their expression pattern and function.

2. *Cloning and characterization of other enhancer trap lines*

We are conducting molecular and genetic analysis of two other enhancer trap lines that are expressed in specific subsets of muscles and/or motoneurons.

rQ224 expresses the reporter gene (ß-gal) in a small subset of neurons including a motoneuron RP3 but not RP1. These two motoneurons take the same peripheral pathway as they exit the CNS and send axons via motor nerve SNb. However, despite the similarlity of their trajectories, once they reach the target region, they show distinct behaviors: the RP3 growth cone projects onto the cleft region between muscle #6&7 while the RP1 growth cone goes past 6&7 to innervate muscle #13. Expression of rQ224 only in RP3 but not in RP1 suggests its possible role in such specific aspects of target recognition. The cDNA cloning and partial sequence analysis showed that the ORF contains a signal sequence. rQ224 product thus is probably a surface or secreted molecule with

potential roles in recognition. The sequencing of the complete cDNA is now in progress.

The other line AN34 expresses ß-gal in a single muscle fiber (#18) 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 protein shows extensive amino acid similarity to rat F-spondin, a secreted molecule expressed at high levels in the floor plate that has been shown to promote neural cell adhesion and neurite extension in vitro.

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 neuro-muscular development.

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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

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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 500 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 responses toward physical stimuli such as gravity, light or touching.

I. Development and morphogenesis of flowers

Mutants with abnormal floral morphology could be divided into the following categories on the basis of the stages of floral development where the genetic defects were presumed to occur; namely, stage 1: transition from vegetative to reproductive growth (mutants with delayed transition or earlier transition), stage 2: elongation of inflorescence axis (mutants with short inflorescence axis, dwarfs), stage 3: formation of floral meristem (mutants lacking floral meristem at the top of the inflorescence axis), stage 4: formation of floral organ primordia (mutants with increased or decreased numbers of floral organs, or aberrant positions), stage 5: fate determination of the floral organ primordia (homeotic mutants: mutants where some floral organs are replaced by other organs), and stage 6: development and morphogenesis of floral organs (mutants with organs of aberrant structure and function). Most of the mutants have been shown to have single, recessive, nuclear mutations (Komaki *et al.* (1988) *Development,* **104**, 195—203; Okada *et al.* (1989) *Cell Differ. Dev.*, **28**, 27—38).

The *AGAMOUS* gene of *Arabidopsis 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. Using the MADS domain of AGAMOUS protein overproduced in *E. coli*, we have shown that the consensus sequence of the high-affinity binding sites of the AGAMOUS MADS domain was 5'-TT(A/T/G)CC(A/T)$_6$GG(A/T/C)AA-3'. Comparisons with the binding-site sequences of other MADS-box proteins revealed that the MCM1 binding-sites show similarities with the binding-site sequence of the AGAMOUS MADS domain (Shiraishi *et al.* (1993) *Plant J.*, **4**, 385—398).

The mutant *apetala3* of *A. thaliana* and the mutant *deficiens* of *Antirrhinum majus* have a homeotic conversion of petals to sepals and stamens to carpels. We have isolated a homologous gene to the *DEFICIENS* from *A. thaliana* and shown complete complementation of *apetala3* mutation by introducing the isolated gene. These results show that the *APETALA3* is a homologue of *DEFICIENS* structurally and functionally. The 5'-upstream region of *APETALA3* gene contains three SRE-like sequence, where MADS box-containing proteins are assumed to bind and regulate expression in tissue- and stage-specific manner (Okamoto *et al.* (1994) *Plant Mol. Biol.*, in press). Now we are trying to isolate the target genes, of which the expression is regulated by the MADS-box proteins, AGAMOUS, APETALA3 or PISTILLATA.

Attempts were also made to isolate cDNAs which are specifically expressed in floral organs. From a cDNA library of young floral buds of *A. thaliana*, we isolated two cDNA clones whose amino acid sequences are highly homologous with the known amidophosphoribosyltransferase cDNAs. Northern blot analysis showed that one gene is expressed in flowers and roots, while the other gene is mainly expressed in leaves (Ito *et al.*, submitted).

II. Stimulus-response interactions in root

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). Using a newly devised system which provides a constant obstacle-touching stimulus to root tips on agar plate, mutants which show abnormal responses to obstacle-touching stimulus were isolated (Okada & Shimura (1990) *Science*, **250**, 274–276). Gravitropic and phototropic responses were also analyzed using agar plates. Young seedlings grown on vertical agar plates have roots which grow straight downward on the agar surface. When the plates were put aside, roots bend 90 degrees and grow to the new direction of gravity. If the plates were covered with black cloth and illuminated from the side, roots grow to the opposite direction of incoming light. Using these systems, mutants which show abnormal graviresponse or photoresponse were isolated (Okada & Shimura (1992) Aust. J. Plant Physiol., 19, 439–448). 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 (Okada & Shimura (1992) *Cell*, **70**, 369–372).

Physiological and biochemical aspects of stimulus-response reaction in roots were also analyzed. Proteins newly synthesized in cells of root tips of *Arabidopsis* seedlings after gravistimulation and photo-induced tactile stimulation were separated by two-dimensional gel electrophoresis. Intensities of 14 protein spots were shown to increase after continuous rocking treatment for 24 hours. Analysis of [^{32}P]-labeled proteins revealed that the continuous rocking enhanced the phosphorylation of proteins in two spots. When the seedlings in flasks were illuminated from the front, and the roots bent towards the back wall of the flasks, total of 12 spots were

newly appeared or enhanced (Sakamoto *et al.* (1993) *Plant Cell Physiol.*, **34**, 297–304).

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* and of transgenic plant regeneration. We have tested several combinations of *A. thaliana* ecotypes and *Agrobacteria* strains and established an efficient system (Akama *et al.* (1992) *Plant Cell Rep.*, **12**, 7–11). We also 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 thought that problems caused by somaclonal variation are avoided. About four hundred of transformants were generated and screened.

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Complementation of *apetala3* mutation. A homeotic mutant *apetala3* of *A. thaliana* (right) was completely complemented (left) by introducing the wild type *APETALA3* gene.

DIVISION OF GENE EXPRESSION AND REGULATION II

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Homologous recombination on the chromosome is often uniform, however, in both procaryotes and eucaryotes, there are specific regions or sites, named "hotspots", where homologous recombination occurs at a higher rate. DNA replication origin in procaryotes (phage) is one example. Another example is the "*HOT1*" site in yeast which has activity to stimulate recombination, homologously, in adjacent regions. As molecular mechanisms involved in enhancing homologous recombination are not fully characterized, related studies, in an attempt at elucidation, are ongoing in our laboratory. Special focus has been directed to termination processes, and homologous recombinations.

I. Analysis of *E. coli* recombinational hotspots, Hot.

In *E. coli*, there are replication fork blocking event-dependent recombinational hotspots. In *E. coli* RNase H defective (*rnh*$^-$) mutants, we found specific DNA fragments, termed Hot DNA, when DNA in the ccc form is integrated into the *E. coli* genome by homologous recombination to form a directly repeated structure, a strikingly enhanced excisional recombination between the repeats occurs. We obtained 8 groups of such Hot DNA, 7 of which were clustered in a narrow region, called replication terminus region (about 280 kb) on the circular *E. coli* genome. A terminus site (*Ter*) can impede the replication fork in a polar fashion. The six *Ter* sites are located approximately symmetrically in terminus and its surrounding region. To block the fork at the *Ter* site, another protein factor, Ter binding protein encoded in the *tau* (or *tus*) gene is required. In *tau*$^-$ cells, Hot activity of HotA, B and C DNAs disappears, thereby indicating that the Hot activity is fork arrest-dependent. In *rnh*$^-$ cells, an alternative new replication origin(s) other than an ordinary replication origin (*oriC*), located at the terminus region is activated so that a newly initiated fork is immediately blocked at one of the *Ter* sites and consequently, there is an accumulation of stalled forks. It seems fairly certain that the *rnh*$^-$ specific accumulation of the fork (which we confirmed) is the cause of the Hot activity at the nearby site. In addition, at least for HotA activity, the presence of a Chi, an *E. coli* recombinational hotspot sequence, properly oriented on HotA DNA itself or somewhere between HotA and *Ter* site, is required. We prepared a putative model (Figure 1), in which the following events may occur; (a) Chromosomal structure of a HotA DNA transformant, in which repeated HotA DNAs flanks a Km^r fragment. (b) When the DNA replication fork proceeds from left to right (this can occur under *rnh*$^-$ conditions), there is an efficient block against the fork at the *TerB* site and the resulting Y-shaped molecules accumulate most in the *rnh*$^-$ strain, less in wild type and not at all in the *tau*$^-$ strain. (c) A ds-break is introduced, probably by nicking at a single stranded DNA complementary to the newly synthesized lagging strand. (d) A Chi responsible enzyme, RecBCD, enters the duplex DNA through the ds-break, and travels to the Chi site with concomitant degradation of the newly synthesized

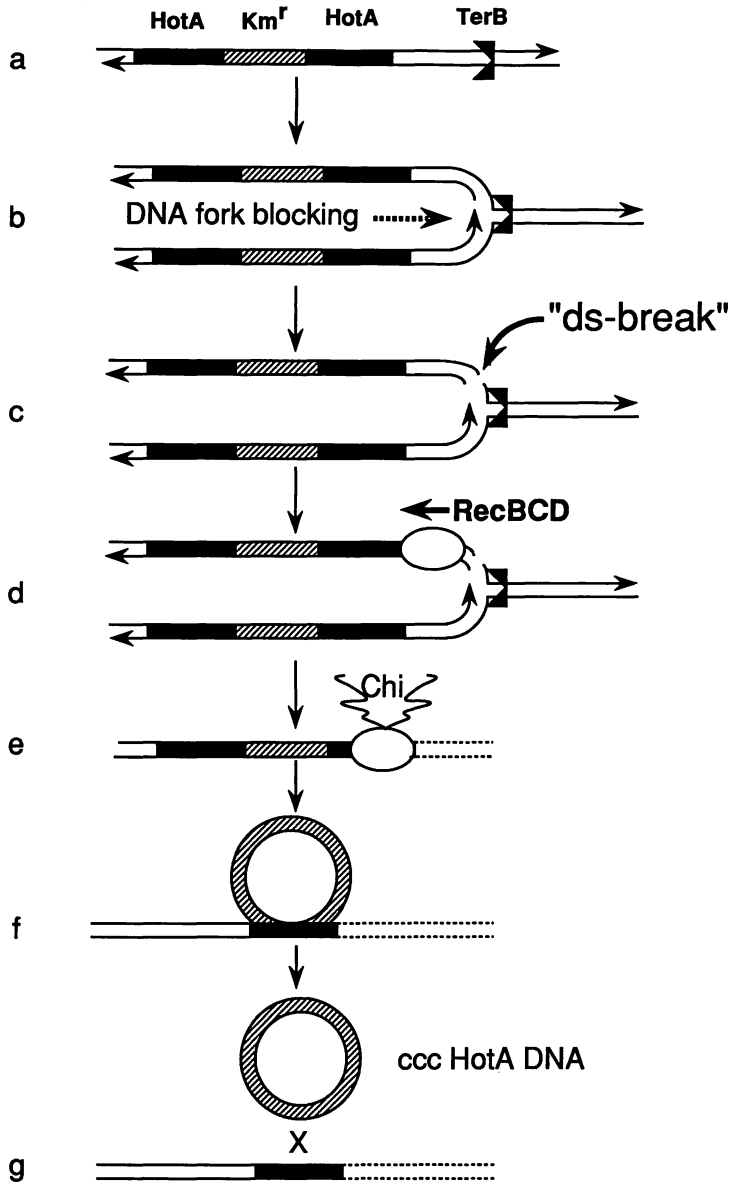

Figure 1. A model for enhancement of excisional recombination between HotA DNAs (see text).

ds-DNA molecules, by exonucleolytic activity. (e) The Chi sequence modulates the exonucleolytic activity. (f) The resulting enzyme stimulates excisional homologous recombination between repeated HotA DNAs, resulting in production of the ccc Hot-Km^r DNA molecule.

II. Analysis of a yeast recombinational hotspot, *HOT1*.

In yeast, *Saccharomyces cerevisiae*, there are also DNA replication fork blocking sites in rRNA repeated genes

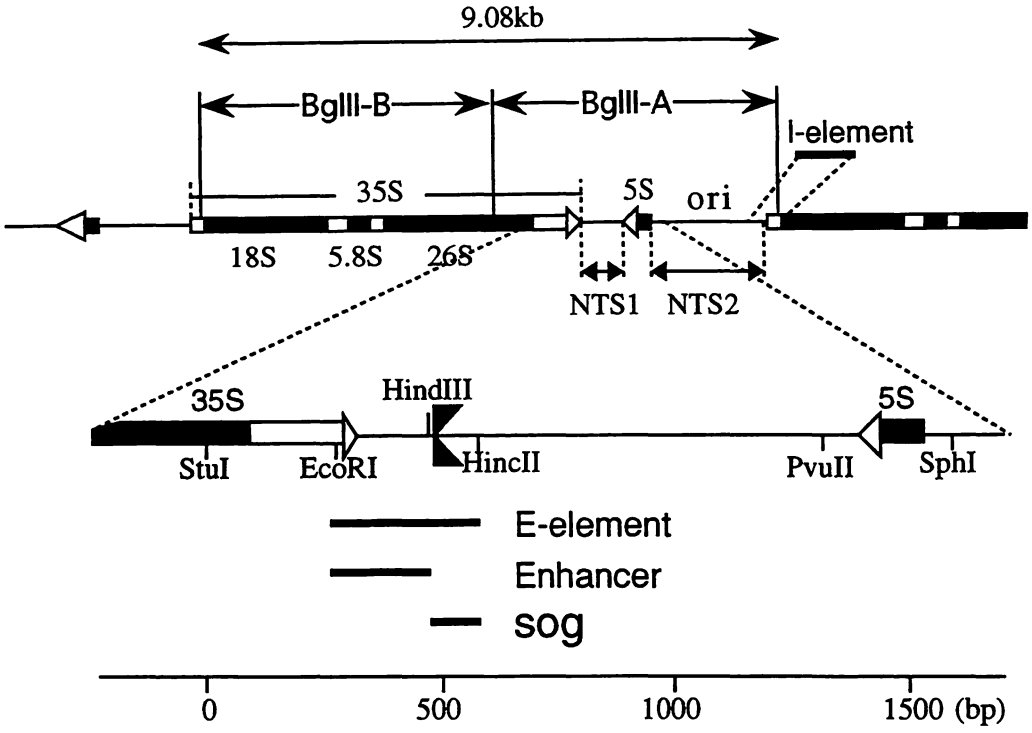

Figure 2. Physical map of the rRNA gene repeat and location of the replication fork block (*SOG*) site. The direction of transcription of the 35S and 5S rRNA genes (open arrow), the mature 18S, 5.8S, 26S and 5S rRNAs (closed bar), the introns (open bar), the two non-transcribed spacer regions (NTS1 and NTS2), ori (replication origin) and I-element (a component of *HOT1* DNA) are indicated. *Bgl*IIA and B DNA fragments are produced when rDNA is digested with *Bgl*II restriction enzyme. NTS1 and its surrounding region are expanded (Bell et al., 1977), under which three solid bars represent the E-element (another *HOT1* component), enhancer (enhancer element of 35S rRNA transcription) and *SOG* (replication fork blocking site; in this case the *SOG* can inhibit the fork traveling from the right side only). The relevant restriction sites are also shown.

(about 140 copies) on chromosome XII. A single repeat unit consists of two transcribed 35S and 5S rRNA genes and two non-transcribed regions, NTS1 and NTS2 (Figure 2). The 35S rRNA gene is transcribed by RNA polymerase I, a polymerase specific for 35S rRNA transcription, and transcription of the 5S RNA gene, the direction of which is opposite that of the 35S rRNA, is carried out by RNA polymerase III, another polymerase specific for 5S rRNA and tRNA production. The NTS1 has a site, termed *SOG*, at which the replication fork is blocked. We obtained evidence that fork blocking activity at the *SOG* site, termed *SOG* activity, is expressed not only on the genome but also on the plasmid, suggesting that the *SOG* site functions in any context (Kobayashi et al., 1992). 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 the replication fork advancing in a direction opposite

that for transcription. The *SOG* sequence is unique and has no characteristic structure, such as 2-fold symmetry, repeated structure etc., hence, a 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.

HOT1 is a yeast mitotic homologous recombinational hotspot, identified by Keil and Roeder (1984). *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 *Bgl*II fragment (Fig. 2) and 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 the enhancer and initiator of the 35S rRNA transcription, respectively, they suggested that transcription by RNA polymerase I, initiated at the 35S rRNA promoter site may stimulate recombination of the downstream region, thereby reveal *HOT1* activity. However, our finding that this E region contains the *SOG* site may give another interpretation.

To examine the functional relationship between *SOG* and *HOT1* activities, *HOT1* defective mutants were isolated and their fork blocking activities were subjected to 2D agarose gel electrophoresis. One was *rad52* mutant defective in a gene included in homologous recombination. The remaining *HOT1* mutants are being examined to determined whether or not their fork blocking activity is active.

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DIVISION OF SPECIATION MECHANISMS

Professor: Tetsuo Yamamori (from early 1994)

TECHNOLOGY DEPARTMENT

Head: Hiroyuki Hattori

Common Facility Group
Chief: Kazuhiko Furukawa

Research Support Facilities
Mamoru Kubota
Chieko Nanba
Toshiki Ohkawa
Tomoki Miwa
Kimiko Yamamiya
Sonoko Ohsawa
Takeshi Mizutani

Radioisotope Facility
Yoshimi Matsuda

Center for Analytical Instruments
Hisashi Kojima
Yukiko Kabeya
Yumiko Makino

Glassware Washing Facility
(Kazuhiko Furukawa)
(Toshiki Ohkawa)

Research Support Group

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

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

Regulation Biology Group
Shoichi Higashi
Miki Ida
Tomoko Mori
Shigemi Ohsugi

Gene Expression and Regulation Group
Yoko Fujimura (Subunit Chief)
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 to 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, biochemical 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 various technical problems. The Proceedings 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
Mariko Saitoh (—May 30)
Kimiko Yamamiya
Takeshi Mizutani
Sonoko Ohsawa (Aug. 4—)

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 is dedicated to cooperative use under the NIBB Cooperative Research Program for the Use of the Okazaki Large Spectrograph.

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 tropical sunlight at noon (Watanabe et al., 1982, Photochem. Photobiol., 36, 491—498).

A tunable two-wavelength CW laser irradiation system is also available as a complementary light source to OLS to be used in irradiation experiments which specifically require ultra-high fluence rates as well as ultra-high spectral-, time- and spatial-resolutions. It is composed of a high-power Ar-ion laser (Coherent, Innova 20) (336.6—528.7 nm, 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 μm 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 levels. 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 (VAX11/780 and micro VAX II), UNIX workstations (SPARC stations IPC, IPX, and 10; DEC station 2100), and personal computers (PC9801, IBM compatible machines, and Macintosh). Through Ethernet or CDDI, all of them are linked to the backbone FDDI of our institute, which is further linked to the new hyper multimedia network of Okazaki National Research Institutes

(the ORION network). Each laboratory has at least two computers connected to the network and all sites linked to the Internet are accessible from there. In addition, NetWare server machines (Quarter L and DECpc) works as file servers and printer servers. Latest databases and softwares of various kinds are also maintained.

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 experimenatal cultures of plant and microbial cells.

7. *Molecular Biological Analysis Laboratory*

In order to facilitate molecular biological analyses, high performance equipments such as DNA sequencers (ABI 370A; 373A-18), peptide synthesizers (ABI 430A and 431A), a nucleotide synthesizer (ABI 381A), imaging analyzers (Fuji BAS 2000—3060 and —3080; PDI Discovery Series) and a glycoprotein analysis system (Takara Glyco-Tag) are provided.

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 by measuring computerized-videomicroscopically the motile behavior of the cells at the cellular and subcellular levels. 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. Currently, there are two projects. One is to develop an expert system to predict various protein localization sites from amino acid sequence data. The other is to construct a prediction system of mature mRNA sequence from their precursors. The former work is done in cooperation with Dr. A. Goffeau (Univ. Catholique Louvain, Belgium) et al. of yeast genome sequencing group and Dr. T. Shimizu (Hirosaki Univ.). Currently, we are trying to find a reliable way to predict transmembrane segments of membrane proteins correctly. The latter work is a cooperation with Dr. H. Sakamoto (Kobe Univ.) and we are now focusing

on the study of aberrant and alternative splicing events: we have constructed a novel databese containing aberrant splicing mutations of mammalian genes (Nakai and Sakamoto, Gene, 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.

Publication List:

I. Faculty

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II. Cooperative Research Program for the Okazaki Large Spectrograph

Goto, N., Yamamoto, K. T. and Watanabe, M. (1993) Action spectra for inhibition of hypocotyl growth of wild-type plants and of the *hy*2 long-hypocotyl mutant of *Arabidopsis thaliana* L. *Photochem. Photobiol.* **57**, 867–871.

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Sasaki, M., Takeshita, S., Sugiura, M., Sudo, N., Miyake, Y., Furusawa, Y., and Sakata, T. (1993) Ground-based observation of biologically active solar ultraviolet-B irradiance at 35 N latitude in Japan. *J. Geomag. Geoelectr.* **45**, 473–485.

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RADIOISOTOPE FACILITY (managed by NIBB)

Head of Facility: Takashi Horiuchi
Techinical Staffs: Kazuhiko Furukawa
Yoshimi Matsuda

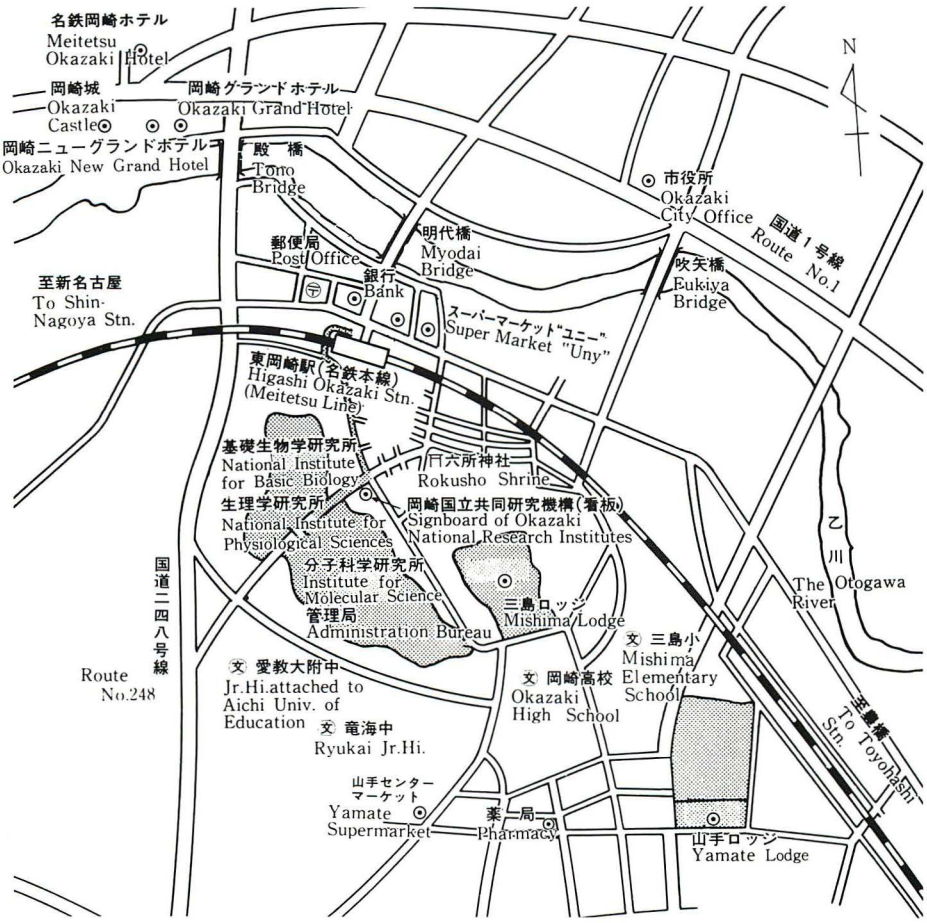
The facility is composed of a main center and two subcenters, one in the NIBB and the other in the NIPS. The facility is being used for molecular analyses of organisms. At the center, a variety of radioisotopes including ^{3}H, ^{14}C, ^{22}Na, ^{32}P, ^{35}S, ^{45}Ca, ^{125}I and various species of beta and gamma-ray emitting nucleides are handled. A laboratory facilty for recombinant DNA research is installed in the center. At the subcenters, only a limited number of radioisotopes such as ^{3}H, ^{14}C, ^{32}P and ^{35}S in the NIBB, and ^{3}H, ^{14}C, ^{32}P, ^{35}S, ^{45}Ca and ^{125}I in the NIPS, are processed. 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.

CENTER OF FACILITY FOR ANALYTICAL INSTRUMENTS (managed by NIBB)

Head of Facility: Mikio Nishimura
Technical Staffs: Hisashi Kojima
Yukiko Kabeya
Yumiko Makino

The Center of Facility for Analytical Instruments consists of the following five sections. (1) Chemical Analysis, (2) Preparation of Biological Materials, (3) Spectroscopic Analysis, (4) Physical Analysis, and (5) Microscopic Analysis. Each section is equipped with instruments for general use as listed below.

1. Section for Chemical Analysis
   - Amino-Acid Analyzer
     - HITACHI 835
   - Gas Chromatograph
     - SHIMADZU GC-7APTF
     - SHIMADZU GC-14APFSC
   - HPLC
     - SHIMADZU LC-6AD
     - SPECTRA-PHYSICS SP-8700
   - Ion Chromatograph
     - DIONEX QIC
   - Spectrophotometric HPLC Detector
     - SHIMADZU SPD-M10AV
   - Peptide Synthesizer
     - BECKMAN 990C
2. Section for the Preparation of Biological Materials
   - Coulter Counter
     - COULTER ZB
   - Isotachophoresis System
     - LKB 2127 TACHOPHOR
   - Preparative Ultracentrifuge
     - BECKMAN L8-80
   - Two-Parameter Cell Sorter
     - BECTON-DICKINSON FACS-II
3. Section for Spectroscopic Analysis
   - Atomic-Absorption Spectrophotometer
     - PERKIN-ELMER 603
   - Differential Refractometer
     - CHROMATIX KMX-16
   - Dual-Wavelength Spectrophotometer
     - HITACHI 557
   - Inductively Coupled Plasma Spectrometer
     - SEIKO SPS 1200A
   - Infrared Spectrophotometer
     - JASCO A-302
   - Laser-Raman Spectrophotometer
     - JASCO R-800
   - Light-Scattering Photometer
     - CHROMATIX KMX-6DC
   - Microplate Reader
     - CORONA MTP-120
     - CORONA MTP-100F
   - Spectrofluorometer
     - HITACHI 850
     - HITACHI MPF-4
     - SIMADZU RF-5000
   - Spectrophotometer
     - HITACHI 330
   - Spectropolarimeter
     - JASCO J-40S
4. Section for Physical Analysis
   - Analytical Ultracentrifuge
     - HITACHI 282
   - Differential-Scanning Calorimeter
     - SEIKO DSC100
   - EPR Spectrometer
     - BRUKER ER 200D
   - GC-Mass Spectrometer
     - HITACHI M-80
   - GC/LC-Mass Spectrometer
     - JEOL DX-300
   - NMR Spectrometer
     - BRUKER AMX 360wb
   - Viscometer
     - CONTRAVES RM-30
5. Section for Microscopic Analysis
   - Film Data Analysis System
     - NAC MOVIAS GP-2000
   - Image Analyzer
     - KONTRON IBAS-I & II
   - Microscope Photometer
     - CARL ZEISS MPM 03-FL
   - Two-Dimension Microdensitometer
     - JOYCE LOEBL 3CS



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