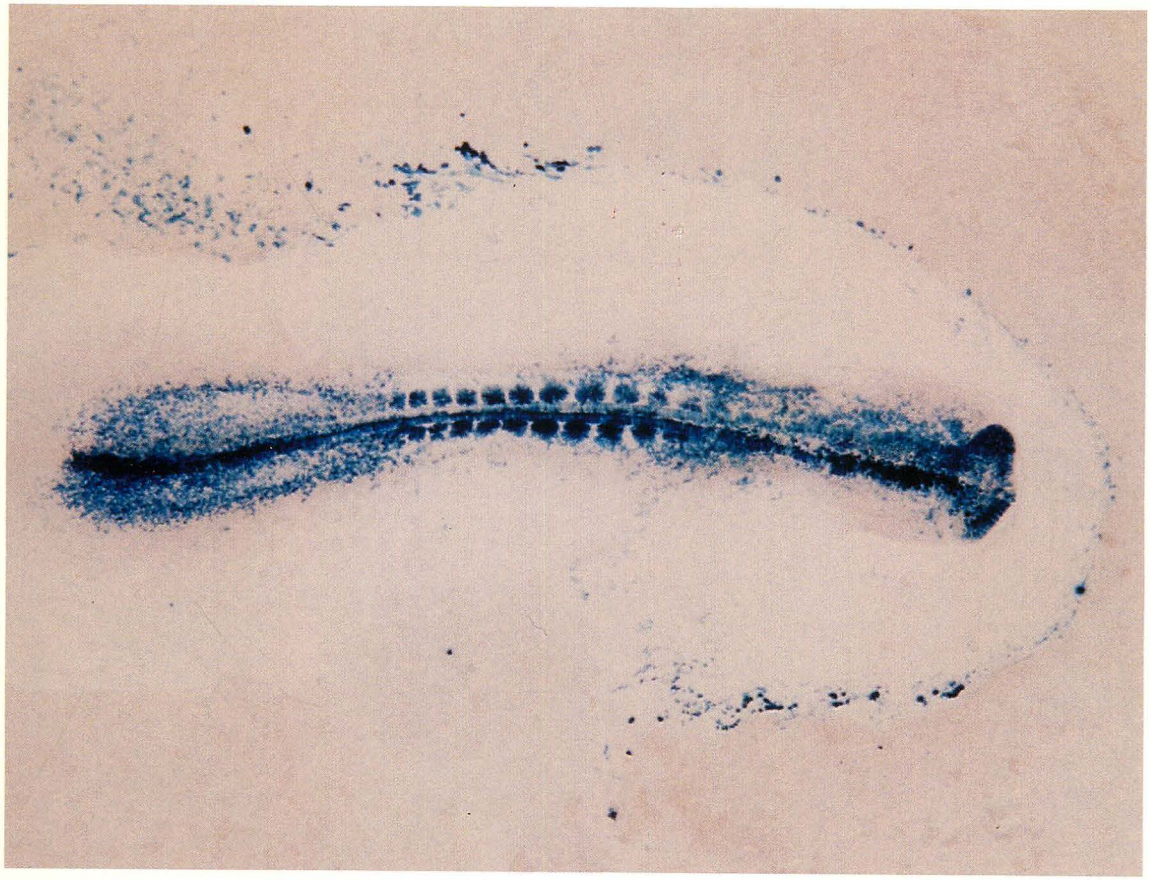
NATIONAL INSTITUTE FOR BASIC BIOLOGY

岡崎国立共同研究機構

基　礎　生　物　学　研　究　所



ANNUAL REPORT

1992

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 eluciate the fundamental mechanisms underlying various living phenomena at the molecular level.

Three new professors joined the NIBB during the past year. Prof. Kimiyuki Satoh of Okayama University filled the position of Professor of Biological Regulation (adjunct) to further molecular biological studies on the photochemical reaction centers involved in the primary processes of photosynthesis. Prof. Masatoshi Takeichi of Kyoto University was appointed to be in charge of Division of Behavior and Neurobiology (adjunct) to examine the role of cell recognition molecules in axon guidance and target recognition by using the neuro-muscular system of *Drosophila.* Prof. Masayuki Yamamoto of Tokyo University was nominated as Professor of Cell Proliferation (adjunct) to explore genes involved in regulation of meiosis in higher organisms by using homologs cloned from the fission yeast *Schizosaccharomyces pombe.* In addition, the Institute continued to be active in exchange of personnel: we appointed five post-doctoral fellows from other universities to research associates, while two research associates were promoted to an associate and a full professor of 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 "Biomembrane Research" and "Signal Transduction". Based on such programs, the NIBB held the 28th and 29th Conferences in 1992, entitled "Dynamics of thylakoid membrane assembly" (organized by Prof. Fujita) and "The visual system of *Drosophila*, from gene to structure and function" (organized by Prof. Hotta).

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 1992.

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:

Tasaka, M., Hasegawa, M., Nakata, M., Orii, H., Ozaki, T. and Takeuchi, I. (1992) Protein binding and DNase-I-hypersensitive sites in the *cis*-acting regulatory region of the spore-coat SP96 gene of *Dictyostelium*. Mechanisms of Development **36**, 105–115.

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

DEPARTMENT OF CELL BIOLOGY

Chairman: Goro Eguchi

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
NIBB Visiting Scientist: Kazimierz Strzalka[1)]
Graduate Students: Ryuji Tsugeki
Katunari Tezuka[2)]
Kaori Inoue
Akira Kato
Tomoo Shimada
Nagako Hiraiwa[3)]
Yasushi Shigemori[4)]
Technical Staff: Maki Kondo
Katsushi Yamaguchi

([1)]from Yagiellonian University)
([2)]from Nagoya City University)
([3)]from Aichi University of Education)
([4)]from Okayama University)

Higher plant cells contain several distinct organelles that play vital roles in cellular physiology. During the proliferation and differentiation of these cells, the organelles often undergo dynamic changes. The biogenesis of new organelles may occur; existing organelles may undergo a transformation of function; and other organelles may degenerate. Since the dynamic transformation of organellar function (differentiation of organelles) is responsible for differentiation – associated events in higher plant cells, the following two research topics are currently being studied in this division.

I. Functional transformation of microbodies

1. *Microbody transition during the greening and senescence of seedlings*

In many seeds, such as those of pumpkin and watermelon, oil is stored as reserve material in the cotyledons. During the course of germination, this reserve oil is utilized as the primary source of energy for the growth of young seedlings. When cotyledons emerge from the soil, the tissues become green and photosynthetically active upon exposure to light. Photosynthesis supplies most of the energy needed for the growth of the seedlings after greening.

During the greening process, a series of dramatic metabolic changes which underlies the shift from heterotrophic to autotrophic growth occur. Accompanying these metabolic changes, many constitutive organelles are functionally transformed. For example, etioplasts differentiate into chloroplasts and mitochondria acquire the ability to oxidize glycine. Glyoxysomes, which are microbodies engaged in the degradation of reserve oil via β-oxidation and the glyoxylate cycle, are transformed into leaf peroxisomes that function in several crucial steps of photorespiration. By contrast, the reverse transformation of microbodies from leaf peroxisomes to glyoxysomes occurs in the cotyledons during senescence. Since cell division does not occur in the cotyledons of these fatty seedlings after germination, all of these developmental events take place in a fixed number of preexisting cells. Therefore, these cotyledons are an excellent model system for studies aimed at elucidating the regulatory mechanisms responsible for the organelle differentiation.

As a step towards an understanding of the regulatory mechanisms that operate during the microbody transition in pumpkin cotyledons (*Cucurbita* sp., Amakuri Nankin), nine microbody enzymes, namely, three glyoxysomal enzymes, three leaf-peroxisomal enzymes and three enzymes present in both types of microbody have been purified and characterized. A cDNA clone for the leaf-peroxisomal enzyme, glycolate oxidase, has been screened and characterized. An analysis using the cDNA clone revealed that increases in

activities of leaf-peroxisomal enzymes during the greening of cotyledons are caused by the appearance and increases in levels of the mRNA (Tsugeki et al. Plant Cell Physiol., in press). With respect to the microbody transition during the senescence of the cotyledons, labeling experiment with proteinA−gold demonstrated that leaf-peroxisomal enzymes and glyoxysomal enzymes coexist in the microbodies of senescing cotyledons, indicating that leaf peroxisomes are transformed directly to glyoxysomes during senescence of the cotyledons (Fig. 1).

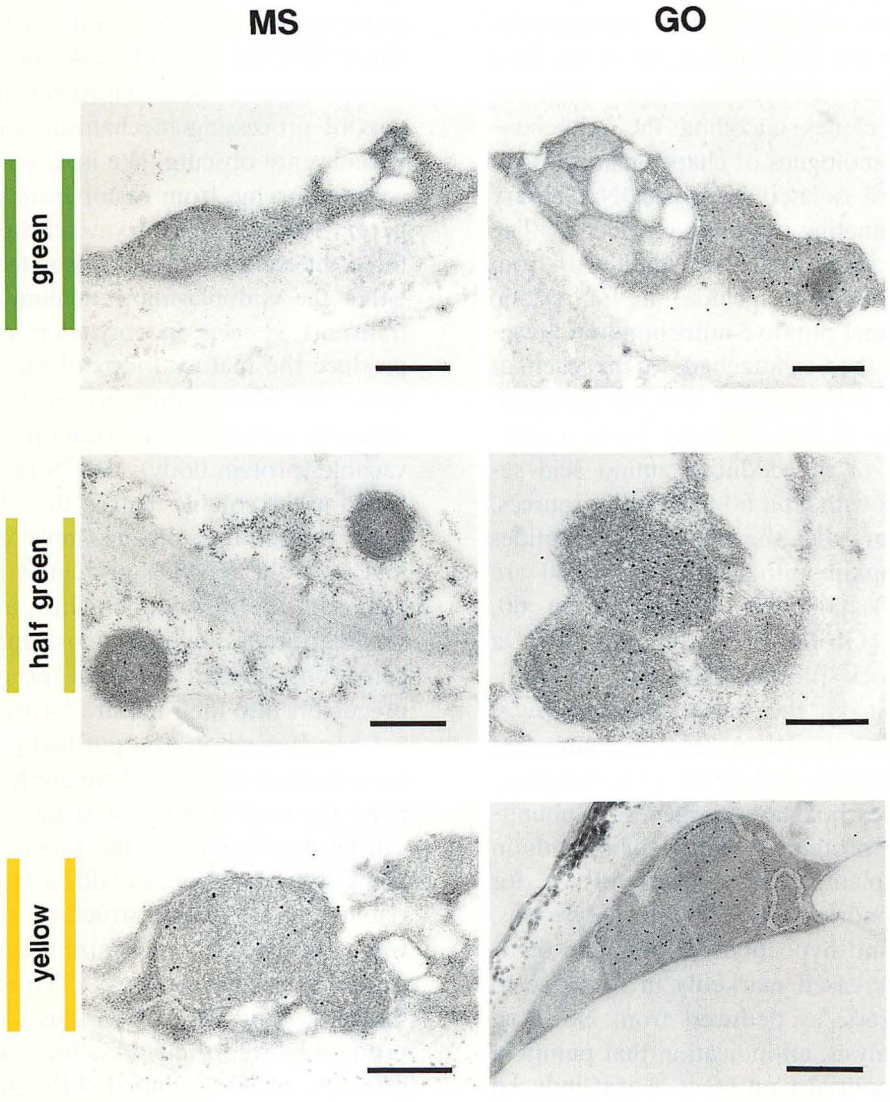


Fig. 1. Localization of glyoxysomal enzymes in senescing pumpkin cotyledons. Immunogold labeling experiments with green, half-green and yellow parts of senescing pumpkin cotyledons were performed using antibodies against malate synthase (MS; glyoxysomal enzyme) and against glycolate oxidase (GO; leaf peroxisomal enzyme). MS was detected in the half-green and yellow parts of the senescing cotyledons. Bars represent 0.5μm.

2. *Molecular chaperones in higher plant cells*

As part of our examination of the microbody transition, we started to study protein transport in higher plant cells, focussing on so-called "molecular chaperones". 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 structures. Two different cDNA clones encoding the mitochondrial homologues of chaperonin 60 (Cpn 60) were isolated from a cDNA library of germinating pumpkin cotyledons. The amino acid sequences deduced from these cDNAs include a 32-residue N-terminal putative mitochondrial presequence that is attached to the each of mature polypeptides, and the polypeptides are 95.3% identical. From a comparison of the deduced amino acid sequences with Cpn 60 from other sources, it appears that the mature polypeptides of pumpkin mitochondrial Cpn 60 are 44—59% identical to other Cpn 60, namely, GroEL of *E. coli*, the 60-kDa heat-shock protein (Hsp 60) of mitochondria in the yeast *Saccharomyces cerevisiae*, the P1 protein of mammalian mitochondria and ribulose-1,5-bisphosphate carboxylase/oxygenase subunit-binding proteins α and β of plastids in higher plants. The level of mRNA for mitochondrial Cpn 60 in cotyledons, hooks and hypocotyls of pumpkin seedlings increased markedly in response to heat stress, as deduced from Northern blot analysis, an indication that pumpkin mitochondrial Cpn 60 is a heat-induced stress protein. Chloroplast heat-induced protein 70 (C-Hsp 70) has also been isolated and characterized. Further investigations on the functions of these molecular chaperones in the transport of protein into organelles are in progress.

II. Biogenesis and transformation of protein bodies

1. *Processing of precursor proteins in vacuoles of developing seeds*

Proprotein precursors to vacuolar components are transported from the endoplasmic reticulum into vacuoles, where they are proteolytically processed to their mature forms. However, the details of processing mechanisms in plant vacuoles are obscure. We isolated a processing enzyme from castor bean endosperm. The purified enzyme can process three different proproteins isolated from either the endoplasmic reticulum or the transport vesicles in cotyledon cells to produce the mature forms of these proteins. The mature proteins are found at different suborganellar locations in the vacuole (protein body): the 2S protein is found in the soluble matrix; the 11S globulin is found in the insoluble crystalloid; and the 51-kDa protein is associated with the membrane. Thus, a single vacuolar processing enzyme is capable of converting several different proprotein precursors into their mature forms.

Characterization of a purified processing enzyme is required if we are to determine the way in which a single enzyme can be responsible for the processing of many vacuolar proteins with a large variability in molecular structure and the way in which it can recognize the numerous types of processing site. To identify the processing sites of various vacuolar proteins, several cDNAs for vacuolar proteins, which included 11S globulin, 2S albumin, membrane protein MP32 and trypsin inhibitor, were cloned and the primary structures of their precursors were deduced. Although processing of

each of these proteins occurs on the C-terminal side of an asparagine residue, there is no apparant homology between the respective primary and secondary structures of the polipeptides.

2. *Transport vesicles for precursors to vacuolar components*

We have chosen three proteins, located in different parts of protein bodies, and characterized their biosynthesis and intracellular transport. Although the three proteins are localized in different suborganellar parts of protein bodies, all three are synthesized on the endoplasmic reticulum as larger precursors and transported via transport vesicles to vacuoles in a similar manner. Thus, it appears that a single type of intracellular machinery plays a role in the targeting of these vacuolar proteins.

We have succeeded in isolating the transport vesicles from maturing pumpkin cotyledons. They contain large amounts of proprotein precursors. Double staining of immunoelectron microscopy demonstrated that the precursors to 11S globulin, 2S albumin and 51k-Da protein are located together in each vesicle. Further characterization of the isolated transport vesicles is required if we are to understand the details of the mechanisms for intracellular targeting of vacuolar proteins and the specific recognition processes in the ER, transport vesicles and vacuoles at the molecular level.

3. *Reversible transformation between vacuoles and protein bodies during seed maturation and germination*

Protein bodies are single membrane-bound organelles that accumulate seed storage proteins. The protein bodies are closely related to vacuoles which are the lytic compartments of plant cells. Protein bodies are formed by budding from the vacuoles during maturation of seeds, while vacuoles result from the fusion of protein bodies during germination of seed. Therefore, the protein body is a specialized form of vacuole in the dry seed. The dramatic transformation of protein bodies from storage to lytic compartments occurs during the maturation and germination of seeds. In order to clarify the regulatory mechanisms responsible for budding and the fusion of the membrane, we have focused on one membrane protein (MP32) of protein bodies which is rapidly degraded just after imbibition of seed. We have isolated protein bodies and vacuoles from maturing and germinating pumpkin seeds at various developmental stages. Using isolated protein bodies, we have recently established an *in vitro* system for reconstituting the process of fusion of the organelles. Experiments employing this *in vitro* system are currently underway as we attempt to elucidate the molecular mechanisms of membrane fusion during the germination of seeds.

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DIVISION OF BIOENERGETICS

Professor: Yoshihiko Fujita
Associate Professor: Shigeru Ito
Research Associates: Mamoru Mimuro
Katsunori Aizawa
*Visiting Scientists: Kaori Ohki**
*Anastasios Melis***
*Tiang-Qing Gu****
JSPS-Post-doctoral Fellow: Tohru Ikeya
*Visiting Fellow: Yuji Nakajima*****
Technical staff: Akio Murakami

*(*from College of Marine Sciences and Technology, Tokai University)*
*(**from Department of Plant Biology, University of California at Berkeley, U.S.A.)*
*(***from Institute of Botany, Academia Sinica, China)*
*(****from Advanced Technology Research Center, Mitsubishi Heavy Industries, LTD)*

Flexibility of autotrophic metabolism in algal organisms in response to growing environments has been studied. Special attention has been paid for photosynthesis and nitrogen-fixation. Mechanism for light-energy harvesting in photosynthesis has also been investigated.

I. Regulation of autotrophic metabolism in response to environments

1. *Regulation of PS I/PS II stoichiometry in cyanophycean thylakoid system*

In developing thylakoid system for light-energy conversion in oxygenic photosynthesis, PS I/PS II stoichiometry is adjusted in response to light regime so as to balance the two light reactions. We found that when the light excites preferentially PS I (PS I light) causing electron-poor state of thylakoid electron transport system (ETS), abandance of PS I is decreased, and it is increased under the light which excites preferentially PS II (PS II light) and causes electron-rich state of ETS (cf. Fig. 1). Based on previous results, we have proposed a work model for mechanism of regulation of PS I/PS II stoichiometry that imbalance of ETS between the two photosystems is monitored, and the signal induces regulation of synthesis of PS I complex during thylakoid development so as to adjust the stoichiometry best fit for the light regime.

To evaluate the model, we determined the rates of synthesis of photosystem polypeptides, PsaA/B, PsbA, and PsbC polypeptides. For this determination, we developed a pulse-labelling method with *Synechcocystis* PCC 6714 using immuno-precipitation. The developed method has permitted pulse-labeling as short as 2 min. Results indicated that (1) PS I, but not PS II, synthesis is regulated in response to the light regime; the rates of synthesis of PsaA/B polypeptides is two-fold faster under PS II light, where PS I/PS II ratio is higher level than that under PS I light where PS I/PS II ratio is low. However, rates of synthesis of PsbA and PsbC peptides remains constant under either PS I and PS II light. The life time of either PS I or PS II polypeptides, including PsbA polypeptide, is very long, and remains constant under either light regime, indicating that degradation of photosystem complexes is not involved in PS I/PS II regulation. Changes in rates of synthesis are fast: half time for both changes is around 4 min. Noteworthy is the effect of rifampicin, the inhibitor of transcription, on the synthesis of PsaA/B polypeptides. Rifampicin did not suppress the light regulation while the rates of PS I synthesis and the amount synthesized were decreased with time when rifampicin was present. This effect suggests that (1) regulation occurs at a step(s) after transcription though the translation strictly depends on transcription products, and (2) mRNA's of *psaA/B* are rather rapidly turing over.

We also found that regulation of Chl *a* synthesis occurs when PS I/PS II stoichiometry is regulated. PS I complex is

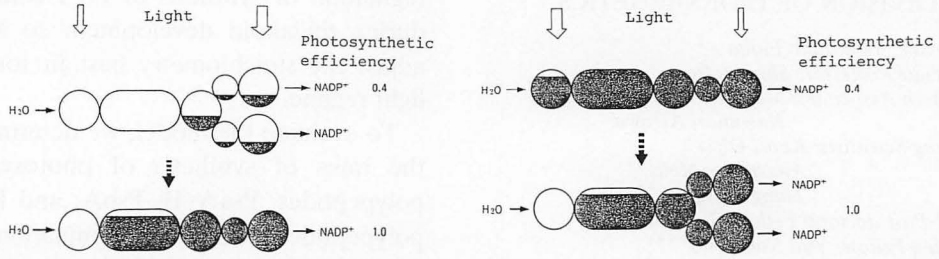

Fig. 1. Schematic presentation of regulation of PSI/PSII stoichiometry and electron transport state. Shaded area in each circle indicates level of reduced state of each component. Reduced PSII and oxidized PSI are photochemically inactive, respectively, and large occurrence of such states causes low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

Chl *a*-protein complex. Formation of stably assembled complexes requires not only synthesis of apoproteins but also synthesis and supply of prosthetic group such as Chl *a*. Thus, regulation of Chl *a* synthesis or supply to PS I assembly can cause regulation of PS I synthesis. As observed by Myers et al. for *Anacystis nidulans* Tx 20, *Synechocystis* PCC 6714 accumulated large amounts of Chl *a* precursors, protochlorophyllide (Pchlide) and Mg-protoporphyrin, in cells for the former and in the medium for the latter, under PS I light where PS I synthesis is suppressed, while such precursors were not accumulated under PS II light, where PS I synthesis is accelerated. A well correspondence between the two regulations, the regulations of PS I and Chl *a* synthesis, was found under various conditions. When PS I abundance is increased, levels of Pchlide always became insignificant, and it was accumulated when PS I synthesis is suppressed.

The step(s) in Chl *a* synthesis regulated was found to be located at the terminal of synthetic path or at the transportation process of Chl *a* to the site for PS I assembly, suggesting that regulation of PS I synthesis is achieved by control of Chl *a* synthesis or supply to the complex assembly. However, the fact that PsaA/B synthesis is strictly limited by abundance of mRNA suggests a possibility that synthesis of apoproteins such as PsaA/B polypeptides is also regulated in this acclimation. Further examination is under progress. Nevertheless, all results thus far obtained are consistent with our work model and can give more detailed insight view of mechanism for regulation of PS I/PS II stoichiometry.

2. *Mechanism of light-activation of nitrogenase in the cyanophyte Trichodesmium*

Nitrogenase in cyanophytes is easily inactivated by O_2 as similar to the enzyme in bacteria. However, nitrogen-fixation in *Trichodesmium* occurs actively in the same cells as those for and simultaneously with oxygenic photosynthesis. Mechanism of protection of nitrogenase from O_2 evolved by oxygenic photosynthesis has been studied, analyzing changes in nitrogenase proteins *in vivo*. Results have revealed that (1) nitrogenase is inactivated by modification of Fe-protein to inactive form in the dark, and (2) light causes reversed modification to the active form. Inactivation by O_2 has been found to be the same modification of Fe-protein as that in the

dark; phosphorylation/dephosphorylation of Fe-protein seems to occur while Fe-Mo protein remains unaltered. Regulation at transcription level seems not to occur in this case.

Activation by light has been found to involve protein synthesis. Fe-protein may be modified to active form by an enzyme which turns over rapidly; it will be degradated in the dark and synthesized in light. This light activation of Fe-protein may enables *Trichodesmium* to fix nitrogen simultaneously with and in the same cells as those for oxygenic photosynthesis.

II. Energy transfer processes in algal light-harvesting systems

Energy transfer processes from carotenoids to Chl *a* were analyzed experimentally and theoretically. We found a requisite for antenna function of carotenoids in terms of molecular structure (8 conjugated double-bonds and one keto carbonyl group) and optical properties (fluorescence from the forbidden S1 state with a long life time) which ensure a high energy transfer efficiency. The absorption band of the forbidden S1 state was directly found (Mimuro et al., in press), and the population dynamics in the excited states can be explained by the energy gap law of internal conversion. These fundamental properties of carotenoids in solution were also investigated in carotenoid-Chl *a* complexes. We also started to calculate the energy transfer matrix element. A most prominent result was the proof for involvement of the S1 state in energy transfer (Mimuro et al., submitted). This will lead to general and extended studies on the excited state of carotenoids and polyenes.

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DIVISION OF CELL PROLIFERATION (ADJUNCT)

Professor: Masayuki Yamamoto
Research Associate: Hidetoshi Iida
Graduate Student: Tomoko Ono (from Nara Women's University)
Visiting Scientist: Makiko S. Okumura

This division appointed the new Adjunct Professor, M. Yamamoto, in November 1992, and is currently setting up an experimental system to explore the regulation of meiosis in higher organisms. The major strategy going to be 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 by classical genetics, or by chromosome manipulation. It is therefore feasible to isolate homologs of these genes from plants and animals, by using either sequence similarity or functional complementation of the mutants. In preliminary experiments we have shown that *Arabidopsis thaliana* has a protein kinase family whose homolog in *S. pombe* affects the meiotic process. The search for the genes regulating meiosis will be carried out also in nematode, frog, fish and mammals.

Another line of research fulfilled in this division in 1992 was an extension of the projects run by the former Adjunct Professor Y. Anraku. We analyzed the mechanism of a rise in the cytosolic free Ca^{2+} concentration in response to the addition of glucose and other metabolizable hexoses in the budding yeast *Saccharomyces cerevisiae.* We previously developed a very sensitive system to measure the Ca^{2+} concentration using a jellyfish protein aequorin (Nakajima-Shimada et al., Proc. Natl. Acad. Sci. USA, 88, 6878–6882, 1991). Using this system, we could show that ATP is likely to be a determinant of the rise in the Ca^{2+} concentration. Furthermore, we isolated *S. cerevisiae* mutants apparently defective in the Ca^{2+} signalling, on the basis of our recent discovery that Ca^{2+} is essential for maintaining viability of the cells destined for mating (Iida et al., J. Biol. Chem., 265, 13391–13399, 1990).

(No publication to be reported)

DIVISION OF CELLULAR COMMUNICATION (ADJUNCT)

Professor: Yoshiki Hotta
Research Associate: Hitoshi Okamoto
Institute Research Fellows: Keiko Nakao
Akihiro Inoue
Graduate Student: Keita Koizumi
(from Univ. of Tokyo)

Brain has an integrated circuitry made of a complex network of neurons and their axons. One of the most intriguing questions in the contemporary biology is how this neural network is formed in the developing brain. Our research interest is currently focused on the two aspects related to this question, i.e. how neurons in the brain differentiate and how they send out their axons to their proper targets.

The latest explosive expansion of our knowledge in this field can be largely attributed to the recent tide of researchers to extensively apply genetics in combination with molecular biology to the proper model organisms. Based on our research experience using fruit fly (*Drosophila melanogaster*), which has been established as one of the most suitable material for developmental neurogenetics, we have started trying to extend our knowledge into the realm of vertebrates. Zebrafish (*Brachydanio rerio*) has been chosen as a new material for this purpose.

I. Molecular developmental neurobiology of zebrafish

Embryos of zebrafish stay transparent throughout the most period of their development, and many of their neurons in the central nervous system (CNS) are identifiable with respect to their shapes and positions. These features have made zebrafish embryos amenable to various modern approaches in cell biology, e.g. cell labeling by fluorescent dyes, cell ablation by laser beam and cell transplantation. These experiments have cast much light upon the mechanisms underlying neural differentiation and axonal pathfindings in zebrafish embryos. After such sophistications in cell biology have been made, we need to identify the molecules involved in such developmental events and to examine the functions of these molecules in vivo by genetic manipulations of embryos. In this context, we have set out to clone the genes whose products may control the differentiation of neurons together with the efforts to deveolp new technology for examing functions of such genes in vivo.

1. *Molecular cloning of the Isl-1 gene in zebrafish*

Isl-1 is a homeobox-containing DNA-binding protein having a LIM domain, a Cys-His rich metal binding motif structure. This has been known to be expressed in a subset of motoneurons in the spinal cord of chick and rat embryos. We have cloned the cDNA coding for the zebrafish counterpart of Isl-1 and determined the putative amino acid sequence. In situ hybridizations to the whole mount embryos have revealed neuronal type specific expressions of this gene together with a transient early expression in the prechordal mesoderm. Most intriguingly, this gene is expressed only in the most anterior one of the three primary motoneurons in most of the segments in the embryonic spinal cord (Fig. 1). We are carrying out the experiments such as the one described below to see if this gene is involved in the process of the final cell subtype specializations by the nascent primary motoneurons.

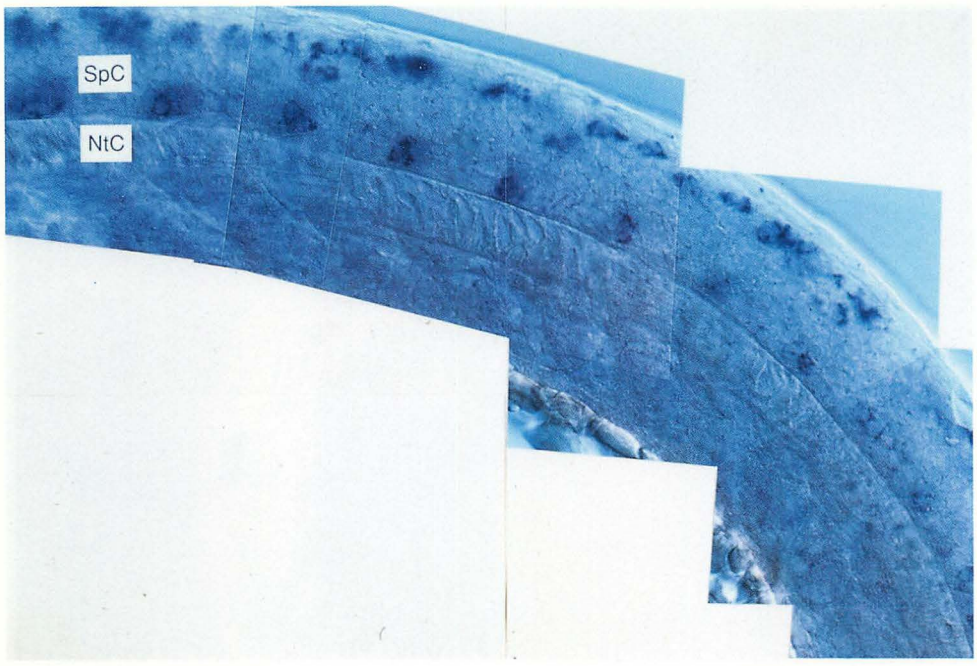

Fig. 1. The Isl-1 mRNA expression in the spinal cord of the zebrafish embryo.

2. *A new technique to achieve an ectopic expression of a foreign gene in zebrafish embryos*

One potent method to analyze the function of a cloned cDNA in vivo is to have it expressed in cells where the gene is normally never expressed and to examine how the developmental program is affected. To achieve this in the central nervous system of zebrafish embryos, we carried out the following series of the experiments.

DNA injected into a fertilized egg is partitioned unevenly in the cell divisions, resulting in a mosaic embryo with respect to the retention of the foreign gene by individual cells. When examined by using a recombinant plasmid carrying the lacZ gene under the control of the heat shock promoter, the expression was observed either almost ubiquitously in the entire body (in about 5% of the embryos) or only in a small number of the cells (in the rest of the embryos). In the latter case, the expression was mostly in muscles and was not observed in the central nervous system. Although the reason for this biased expression is not clear, we concluded that it is not feasible to get an ectopic expression of a foreign gene only in a subset of neurons in the central nervous system by injecting a simple construct containing cDNA under the direct control of the heat shock promoter.

We have succeeded in overcoming this problem by inserting an artificial transposon like element between the heat shock promoter and cDNA. The inserted element consists of the yeast FLP recombinase gene with the target FRT sequences on both ends of the element. In the embryos injected with this construct, the transcription of the cDNA in

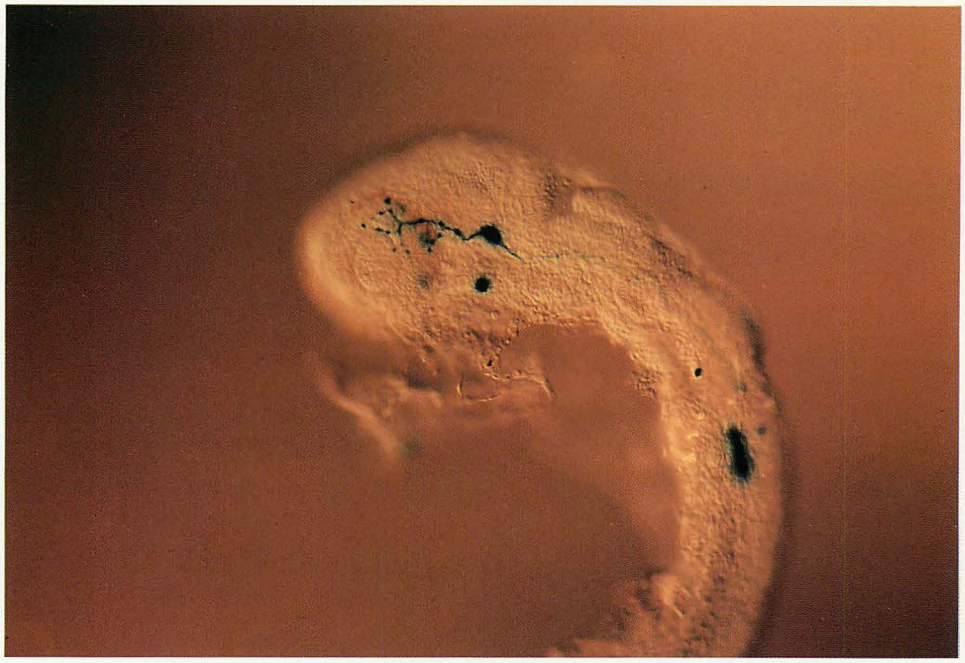

Fig. 2. Activation of the injected lacZ gene in a trigeminal neuron of the 24hr zebrafish embryo.

the heat shock condition is interrupted by the inserted element, and the FLP recombinase is transcribed instead. The induced FLP recombinase, then acts upon the FRT sequences and subsequently causes an excision of the element between the FRT's at a low frequency. In the cells where such an excision takes place, the cDNA becomes inducible by heat shock. Since the excisions happen purely stochastically and have no predilections to any tissues, we can expect activations of the injected gene in a randomly chosen one or a group of the cells that retain the injected DNA construct. Fig. 2 shows one of the results of a model experiment using the lacZ gene instead of cDNA. The expression of the lacZ gene in a single neuron was observed in about 2% of the injected embryos. In addition to the ectopic expression of a foreign gene, this technique has a potential for a wider variety of applications including the cell lineage analysis or the replacement of the reporter gene with an arbitrary foreign gene in an enhancer trapped transgenic zebrafish.

II. Drosophila neurogenetics

A lethal mutant l(2)KM13 was isolated on the basis of having excess neurons along the ventral midline in 13hr embryo of Drosophila melanopgaster. The primary culture derived from a single mutant embryo revealed the defects of this mutant in the differntiation of both neurons and the mesoderm, especially the muscle pioneers. An enhancer trap line Fz13 was identified as an allele of l(2)KM13. In the heterozygotic Fz13, the reporter lacZ gene is expressed in neuroblasts and in the muscle pioneers during the embryonic period

and in neuroblasts during the larval period. To identify new genes required for the de novo neurogenesis during the larval period, we screened for the mutations that alter the expression of the lacZ gene in the larva of the heterozygotic Fz13. A9 is the pupal lethal mutant obtained as a result of this screening. The larva homozygous for this mutation has a significantly smaller brain than normal and a reduced lacZ expression in the CNS in the heterozygous Fz13 background. To analyze the defects in the larval neurogenesis in A9, proliferating neuroblasts in the larva of the A9 homozygotes were labeled with BrdU. They were abnormal in morphology and showed almost no sign of proliferation. No abnormality in the cellular proliferation was observed in the larval discs or in the embryos of the A9 homozygote including embryonic nervous system. These data suggest that the A9 gene is required specifically for the proliferation of the larval neuroblasts. Molecular characterization of this gene is now in progress.

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DIVISION OF CELL FUSION (ADJUNCT)

Professor: Hitoshi Sakano
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Fumikiyo Nagawa
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Graduate Student: Setsuyuki Aoki
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In the mammalian immune system, somatic DNA recombination plays key roles in generating functional immunoglobulin (Ig) and T cell receptor (TCR) genes. This recombination, known as V-(D)-J joining, generates a diversity of receptor genes by combining multiple gene segments and by modifying the junctional sequences. DNA rearrangement is also an effective mechanism for activating a particular member of a multigene family by bringing the promoter and enhancer elements into close proximity. It is of interest to study whether such gene rearrangement is restricted to the immune system, or can occur in other tissues during development.

I. DNA rearrangement in the immune system

V-(D)-J type recombination takes place between two recombination signal sequences (RSS's) which are each composed of a heptamer (CACAGTG), and a nonamer (ACAAAAACC) (Sakano et al., Nature, **280**, 288, 1979). The length of the spacer separating the two consensus sequences is always either 12bp or 23bp, and recombination takes place between two heptamer-nonamer pairs only when one pair is separated by a 12bp spacer and the other by a 23bp spacer. Although the heptamer-nonamer signals are generally well conserved, substantial deviation is found in many naturally occurring RSS's. Mutation analysis with an artificial substrate has revealed that only a few nucleotides in the consensus sequences are essential (Nagawa et al., Mol. Cell. Biol., submitted). In the heptamer, three nucleotides adjacent to the recombination site have to be conserved, whereas others can be changed without loss of substrate activity. In the nonamer, two residues in the middle of the A-rich core seem to be essential.

The 12/23bp spacer rule is not as strict as was previously thought. Under certain circumstances, recombination can occur between one complete RSS and a heptamer alone. Since the recombinase cannot regulate V-(D)-J joining to preserve the reading frame of the genetic code, out-of-frame V-(D)-J structures are generated in two cases out of three. To rescue out-of-frame V-(D)-J structures, cells undergo V gene replacement, which is a correction mechanism to replace a non-functional V sequence with a new germline V gene. In collaboration with Dr. Takemori at the National Institute of Health in Tokyo, we have identified circular DNA excised by V gene replacement (Usuda et al., EMBO J. **11**, 611, 1992) showing that replacement occurs by deletion type recombination. A heptamer-like sequence embedded in the V coding sequence is used as a truncated RSS, and recombined with the 3′-RSS of the germline V gene. This variant type of V-(D)-J joining between one complete RSS and a heptamer alone is resposible for some chromosomal abnormalities in lymphocytic tumors.

For the V-(D)-J recombinase, at least three activities have been postulated: a DNA binding activity, an endonucleolytic activity, and a ligase activity. In an attempt to isolate cDNA clones for RSS-binding proteins, we screened a λgt11 phage library of a pre-B cell line with

RSS probes (Shirakata et al., Mol. Cell. Biol. **11**, 4528, 1991). Among positive clones, one type of cDNA, T160 appears to be promising. It codes for 81kDa protein that binds to the wild-type 12bp-RSS but not to an RSS in which the heptamer sequence has been changed. The T160 protein contains a DNA binding domain in the C-terminal region whose sequence is related to a nuclear protein HMG1. In collaboration with Dr. Huppi at NIH, we recently found that a gene coding for the RSS binding protein T160 (designated as rsb-1 for recombination signal binding) is tightly linked to the recombination activating genes, rag-1 and rag-2, both in the mouse (chromosome 2) and in the human (chromosome 11) (Huppi et al., Immunogenetics, **37**, 288, 1993).

Both Ig and TCR gene rearrangements appear to follow the same 12/23bp spacer rule probably using the same recombinase. However, V-(D)-J joining is strictly regulated in a tissue-specific manner and in a time-ordered fashion. Complete Ig gene rearrangement occurs only in B cells, while TCR genes rearrange only in T cells. Furthermore, V-(D)-J joining takes place sequentially during lymphocyte development. For example, in B cells, the heavy-chain gene rearranges before a light-chain gene does. What discriminates between Ig and TCR gene rearrangement has been a puzzling question. Recently, in collaboration with Dr. Yamamura at Kumamoto University, we have obtained data indicating that the 3′Cκ enhancer region may contain DNA element(s) necessary to achieve cell-type specificity and stage specificity of recombination (Akagi et al., Cell, submitted). When the 1.2kb-3′Cκ region (XhoI-EcoRI) was deleted, Vκ-Jκ joining occurred not only in B cells, but also in T cells. Even in B cells, the κ gene rearrangement took place at both the pro-B and the pre-B stages. In contrast, the substrate with the 3′Cκ enhancer region underwent rearrangement only in B cells at the pre-B stage. Thus, the 3′Cκ region appears to contain a suppressive DNA element(s) which restricts κ gene rearrangement to the pre-B stage of B cells.

II. Somatic DNA changes in CNS

The brain, like the immune system, recognizes and memorizes many different external signals. Comparison of the immune system with the central nervous system yields interesting parallels. An increasing number of cell-surface markers and differentiation factors have been found to be common to both systems.

In order to examine a possibility of somatic DNA rearrangement in the central nervous system, we generated transgenic mice carrying a recombination reporter gene (Matsuoka et al., Science, **254**, 81, 1991). Our recombination substrate contained the lacZ gene in a reverse orientation with respect to a ubiquitous promoter from the β-actin gene, such that rearrangement results in β-galactosidase (β-gal) expression. In the construct, sufficient space was maintained in front of the lacZ gene to allow for flexibility of the recombination site, because the site specificity of recombination in the brain might not be as strict as in V-(D)-J joining in lymphocytes. In the transgenic mice, we found that not only lymphatic tissues, but certain areas of the brain were stained blue with X-gal, a chromogenic substrate of β-gal. Since similar regions in the brain were stained in two independent founders, detection of β-gal-positive cells was not an accidental event resulting from transcriptional

activity near the integration site of the transgene (Matsuoka et al., Science, **257**, 408, 1992).

To determine whether our substrate was actually rearranging, we amplified DNA sequences surrounding the recombination junctions by PCR. Rearranged transgenes were not found in DNA from the tail or kidney. Brain samples produced multiple bands, each of which differed in size from the 400bp band. In contrast to the signal joint found in the lymphatic tissues, the recombination breakpoints we found in brain were not adjacent to RSSs. Recombination junctions were located at various distances away from RSSs. In the brain, the joining detected in the reporter gene thus is imprecise in site specificity. At this point, the recombination we observed in the brain appears to be distinct from V-(D)-J joining. Since the reporter gene system does not identify rearranging genes in the brain, a new approach should be taken when one tries to identify them. For deletion-type recombination, isolation of circular DNA has been instrumental in the study of antigen receptor gene rearrangement in lymphocytes. Characterization of brain circular DNA may allow us to identify the rearranging genes in the central nervous system.

Besides DNA recombination, gene conversion is another somatic event which often occurs in multigene families. In chicken immunoglobulin genes, gene conversion is exploited to diversify the primary sequences of variable region genes. It is of interest to study gene conversion activity in the brain, e.g., in an odorant receptor multigene family. We have made transgenic mice carrying a substrate for detection of the gene conversion activity. The substrate contains a mutated lacZ gene connected to the β-actin promoter/enhancer complex. A two-base insertion was generated by filling in the ClaI site within the lacZ. This insertion causes frame-shift in the downstream region. We also included a donor sequence, the PvuII-AvaII fragment of the wild-type lacZ. From the reporter construct, β-gal can be produced when the mutation is corrected by the gene conversion mechanism, replacing the mutated ClaI site with the wild-type sequence from the donor segment. We are currently analyzing both brain and lymphatic tissues by staining with X-gal.

III. Summary

In the central nervous system, there are several potential functions for somatic DNA changes. As in the immune system, recombination and gene conversion could be used to generate diversity by shuffling gene sequences. DNA rearrangement is also a convenient mechanism for the generation of an active gene whose protein product consists of two distinct domains, one containing a variable interaction site for potential ligands, and the other having a fixed biological effector function. Another possibility is that recombination or gene conversion could be used to insure the expression of one member of a multigene family. Identification and isolation of the rearranging genes in nerve cells should provide a new insight into the role of somatic DNA changes in the development and assembly of the central nervous system.

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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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Sachiko Fukada

([1])from Institute of Developmental Biology, Beijing, China)
([2])from Fukui Prefectural University)
([3])from Mie University)
([4])from Tohoku University)
([5])from Wuhan Univeristy)

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.

As an initial step to address this question, 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). The deduced amino acid sequences of these fish steroidogenic enzymes are found to have 45–55% similarity with those of mammals. The fish cDNA inserts were confirmed to encode each steroidogenic enzyme by introducing it into non-steroidogenic mammalian COS-1 cells.

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. In order to define the molecular basic of this stage- and gonadotropin-specific regulation of fish P450arom, we have isolated and characterized the gene encoding medaka P450arom. A full-length rainbow trout cDNA was used as a probe. The medaka P450arom gene exists as a single copy gene and its coding region is composed of nine exons; human P450arom gene also contains nine exons. Although the intron/exon boundary sequences of medaka P450arom gene are exactly the same as those of mammals, introns are much shorter in medaka than mammals. Primer extension and S1 mapping experiments identified at least two transcriptional initiation sites. Although we have previously shown that the gonadotropin-induced increase in P450arom activity in medaka ovarian follicle mediated through the action of cAMP, no classical cAMP responsive elements (CREs) have been identified in the 5' region of medaka P450arom gene. Instead, we found two ad4 consensus sequences located 5' upstream of the putative promoter I.

We have previously shown that 17α,20β-DP acts via a receptor on the oocyte surface membrane and not through cytoplasmic or nuclear receptors. The concentration of 17α,20β-DP binding sites was significantly elevated during oocyte maturation. It was also shown that gonadotropin increased 17α,20β-DP binding sites; this gonadotropin-induced increase in 17α,20β-DP binding sites coincided with the development of oocyte maturational competence. 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 laboratories 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. To understand the molecular mechanisms of MPF formation and activation following the MIH reception on the oocyte surface, we purified MPF from mature oocytes of carp (*Cyprinus carpio*) and showed that it consists of 34-kDa cdc2 and 46- and 48-kDa cyclin B proteins. Immature oocytes contained a 35 kDa cdc2. Thus, it is concluded that the 34-kDa- and 35-kDa cdc2 proteins are active and inactive forms,

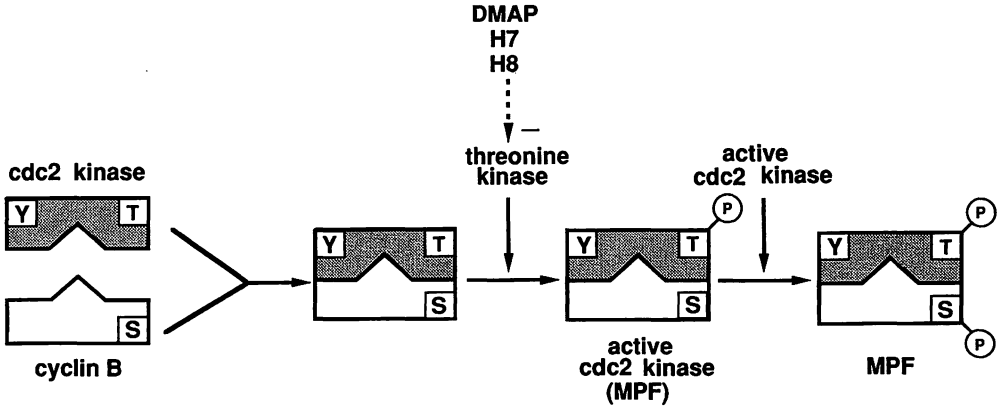

Fig. 1. Current model of the formation and activation of MPF during fish oocyte maturation.

respectively. The 34-kDa active cdc2 appeared in accordance with the onset of germinal vesicle breakdown (GVBD). Cyclin B was absent in immature oocyte extracts and appeared when oocytes underwent GVBD, coinciding with the appearance of the 34-kDa active cdc2. Cyclin B that appeared during oocyte maturation was labelled with [^{35}S]methionine, indicating its *de novo* synthesis. Precipitation experiments with p13^{suc1} and anti-cyclin B antibody revealed that cyclin B that appeared during oocyte maturation formed a complex with cdc2, as soon as it appeared. Therefore, it is most likely that the 35-kDa inactive cdc2 preexisting in immature oocytes forms a complex with *de novo* synthesized cyclin B at first, then is immediately converted into the 34-kDa active form, which triggers all changes that accompany oocyte maturation, such as GVBD, chromosome condensation and spindle formation.

Phosphoamino acid analysis showed that threonine phosphorylation of the 34-kDa cdc2 and serine phosphorylation of cyclin B were associated with the activation of cdc2. However, it was shown that cdc2 was activated by mutant cyclin B that underwent no serine phosphorylation during the activation. These results indicate that the threonine phosphorylation of cdc2, but not serine phosphorylation of cyclin B, is required for cdc2 activation. Taken together, it is strongly suggested that 17α,20β-DP induces oocytes to synthesize cycline B, which in turn activates preexisting 35-kDa cdc2 through its threonine (probably Thr161) phosphorylation, produce the 34-kDa active cdc2. These mechanisms of MPF activation in fish apparently differ from those in *Xenopus* and starfishs, in which cyclin B is present in immature ooctyes and forms a complex with cdc2 (pre-MPF).

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 (*Anguilla 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.

Our current research focuses on the isolation and characterization of genes expressed or suppressed in eel testes during the HCG-induced spermatogenesis. So far, three cDNA clones (activin B, 3β-HSD and an unidentified protein) have been isolated and characterized. Northern blot analyses of testes during the HCG-induced spermatogenesis *in vivo* have shown that activin B mRNA was nondetectable in testes prior to the treatment, but could be detected in testes for 1–6 days with HCG. The HCG-dependent activin B mRNA expression was confirmed by *in situ* hybridization; the site of activin B mRNA hybridization was restricted only to Sertoli cells in testes treated with HCG for 1–6 days. HCG treatment also caused a rapid rise in the abundance of testicular 3β-HSD

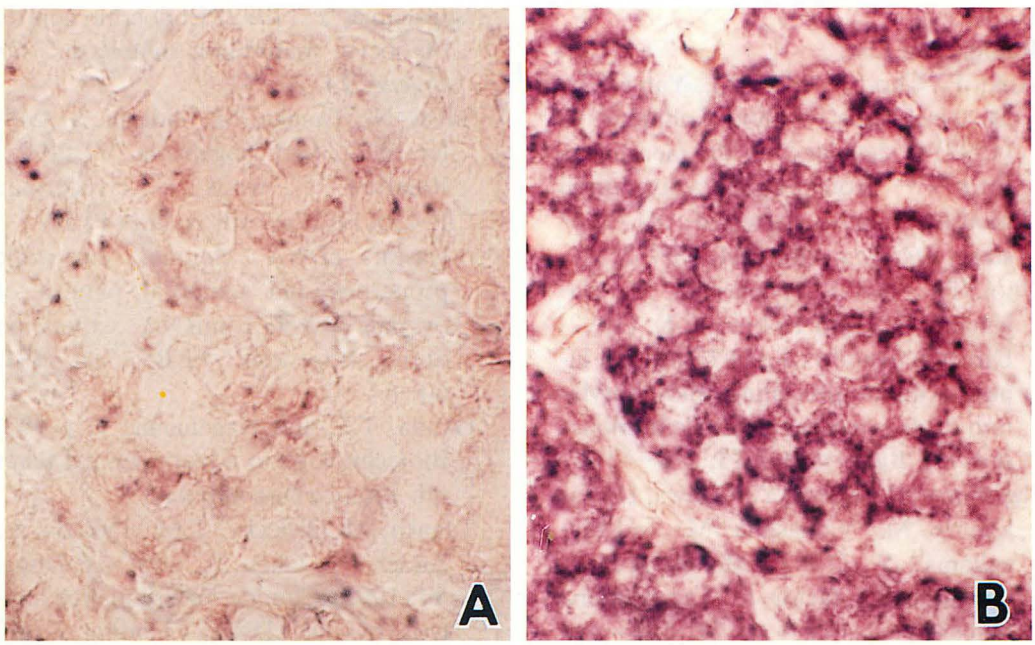

Fig. 2. *In situ* hybridization pattern of activin B mRNA in eel testis. A, uninjected testis; B, testis 1 day after HCG injection showing high levels of activin B mRNA in Sertoli cell cytoplasm.

mRNA. In contrast, levels of mRNA for the unidentified protein were abundant in testes prior to HCG treatment, but became nondetectable by 1 day after the treatment.

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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Kensuke Suzuki (from Nagoya City University)
Technical Staff: Miyuki Ohkubo
Chikako Inoue

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 like homeobox genes regulate a set of target (structural) 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 the part of networks. In complementing this approach, a top-down type approach referred in the next section 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 silk gland is originated, and nearby regions of *Bombyx* embryos.

Among many factors proposed to bind and control the fibroin and sericin-1 genes, one candidate factor has been cloned and characterized, which is a POU-domain-containing protein named as POU-M1. The POU-M1 accommodates a POU-domain identical to *Drosophila Cf1-a*, is expressed abundantly in the larval middle silk gland where the sericin-1 gene is specifically expressed, and binds to the SC region of the sericin-1 gene, a key element for transcription enhancement accommodating an octamer consensus sequence. To characterize the transcription regulation of the POU-M1 gene, a genomic DNA fragment encompassing the whole coding region and its flanking sequences has been cloned. This gene does not contain any intron. The 5′-flanking region of the gene contains several interesting motifs (see Fig. 1), such as homeo-domain binding motifs (PB region), sequences resembling the transcriptional factor Spl-binding site (positions −102 to −38), and TGTTT motifs (PA and PC regions), but lacks some of typical transcriptional regulatory sequences such as TATA and CAAT boxes. Transcriptional analysis of a series of deletion

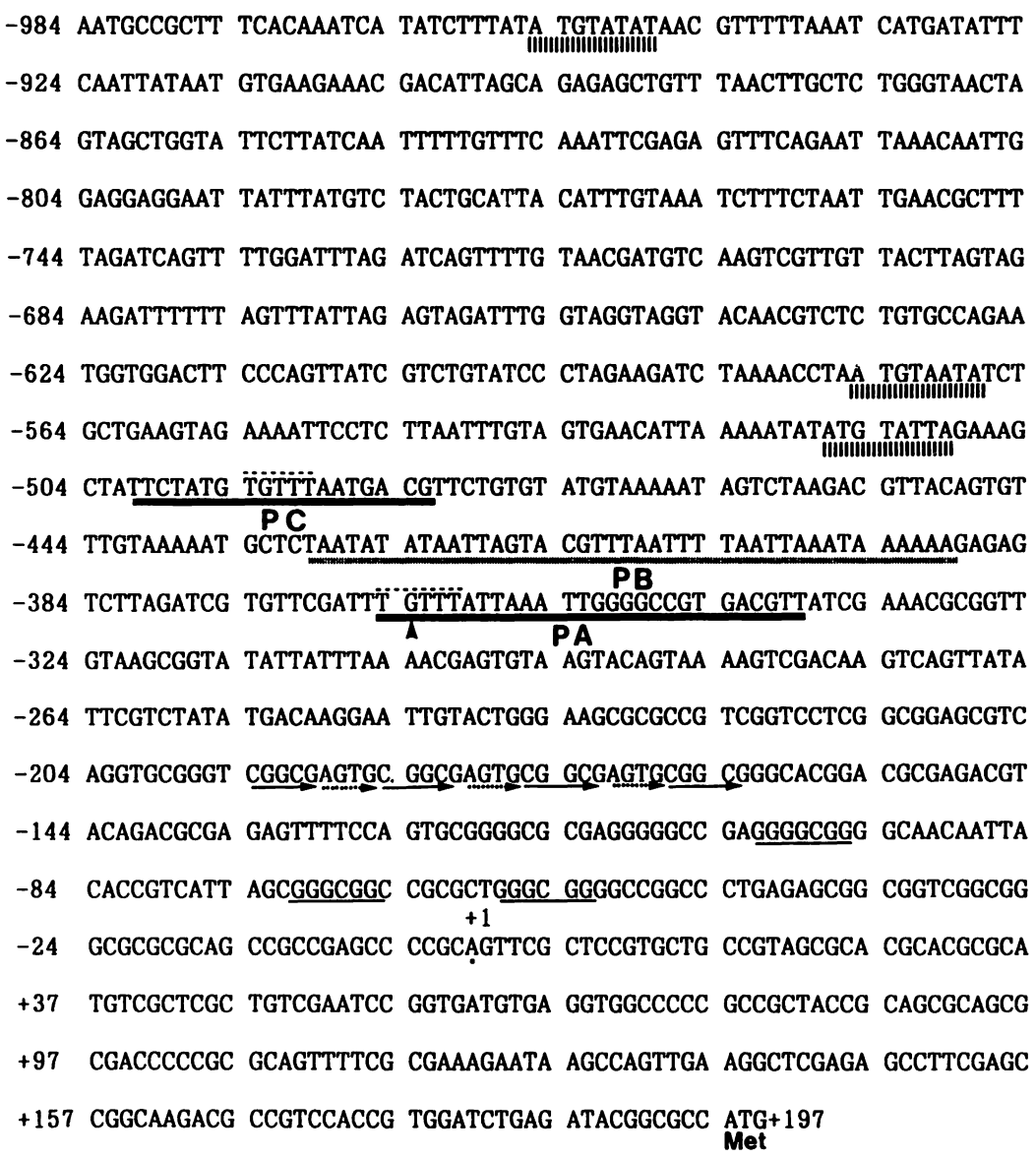

Fig. 1. DNA sequence of the POU-M1 promoter and its surrounding regions. +1 marks the transcription initiation site.

mutants of the gene in the nuclear extracts from the middle silk gland of 2-day-old fifth instar larvae revealed the presence of multiple *cis*-regulatory elements located both upstream and downstream of the transcription initiation site. One of these elements, the homeodomain-binding element (PB), was identified to mediate negative regulation; deletion of this element resulted in higher levels of transcription (compare lane 3 with lane 5 in Fig. 2). By mobility shift assay using the POU-M1 specific antibodies, we found that this negative

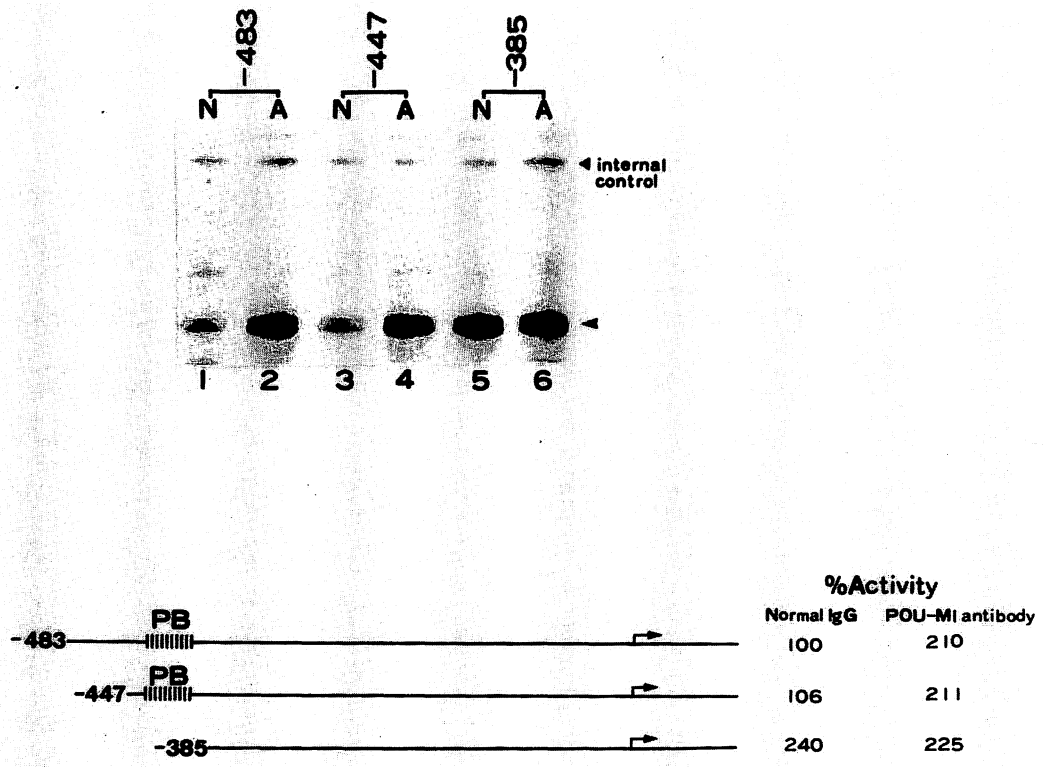

Fig. 2. Effect of immunodepletion of POU-M1 protein on the POU-M1 gene transcription. Affinity purified anti-POU-M1 C1 peptide IgG (lanes 2, 4 and 6) or normal IgG (lanes 1, 3 and 5) was incubated with the middle silk gland nuclear extract of 2-day-old fifth instar larvae. Protein A-Sepharose was then added and incubated for 1 hr on ice. The mixture was centrifuged and the supernatant was assayed for transcription.

element interacts with the POU-M1 protein. Transcription analysis *in vitro* using one of the POU-M1 antibodies (see Fig. 2) indicated that POU-M1 can negatively autoregulate its own gene expression as a consequence of binding to the homeodomain-binding element with a weaker affinity than the binding of the protein to the SC site of the sericin-1 gene. Several other elements including the TGTTT motifs, the possible Spl-binding sites and others appear to be activation elements, and an additional negative element as well as a few more activation elements in the downstream of the intiation site were detected.

The protein FF1 in the presence of another protein FF2 binds to the enhancer element I of the fibroin gene. FF1 was purified to the homogeniety but only a partial purification was attained for FF2. Use of polyclonal antibody against the FF1 suggested the FF1 is probably a transcription factor involved in the enhancement of the fibroin gene transcription in the posterior silk gland. Recently, several monoclonal antibodies against FF1 have been obtained, and characterization of FF1 as a transcription factor and cloning of FF1 are being planned. Another factor OBF-1 binds to the enhancer element II in the intron at around +220 and +290 regions and to the enhancer element I at around −130

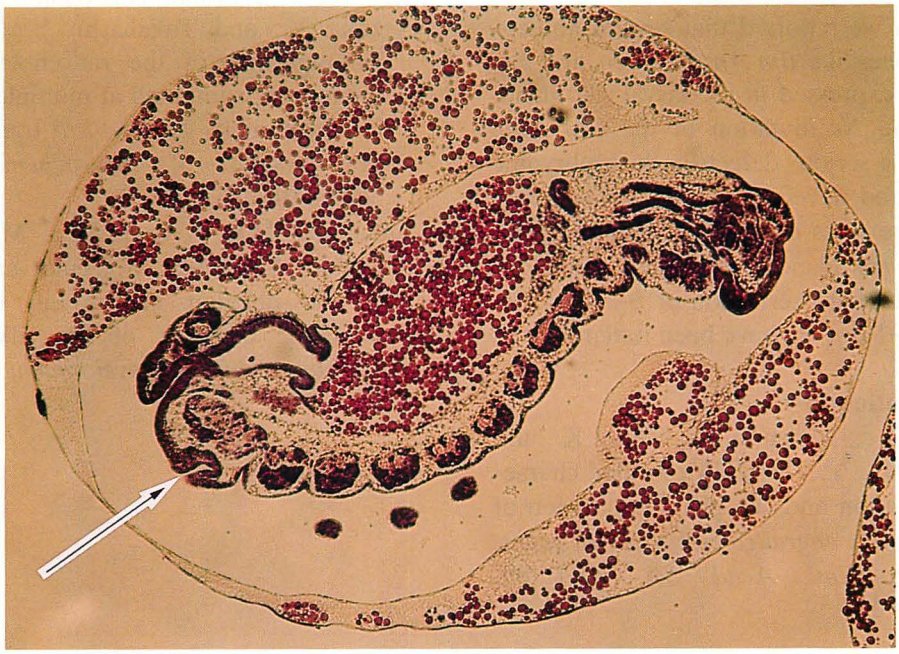

Fig. 3. Initiation of the silk gland development by the invagination of a part of the labial segment. The section was derived from stage 21B embryo. The arrow indicates the invagination of the silk gland.

region. The oligonucleotide corresponding to the +290 region competed the transcription enhancement both by the enhancer I and the enhancer II. Purification and cloning of the OBF-1 are also being planned.

II. Genes involved in the *Bombyx* body plan

To know the roles of the *Bm Ubx*, *Bm abd-A*, *Bm Abd-B*, *Bm Antp*, *Bm en*, *Bm in*, *Bm Wnt-1*, and *Bm cad* genes on the body plan, we have analyzed the expression of these genes in the wild type and mutant embryos. *In situ* hybridization to the wild type embryo sections revealed that the expression of the *Bm Antp* was detected in the first to the third thoracic segments. This transcript was strongly detected in three pairs of thoracic legs and the caudal legs at stage 20. In the embryos homozygous for the E^N mutation which lacks both the *Bm Ubx* and *Bm abd-A* genes the transcript of the *Bm Antp* was detected in the thoracic-type legs and the epidermis from the first thoracic segment to the seventh abdominal segment. The transcript of the *Bm Abd-B* was detected from the sixth abdominal segment to the telson both in the wild type and E^N/E^N embryos. From these results we speculate that functional deficiency of the *Bm Ubx* and *Bm abd-A* genes in the E^N/E^N embryos may have caused derepression of the *Bm Antp* gene and transformation of the first to the seventh abdominal segments into the thoracic-type segments.

Development of the silk gland is initiated in the labial segment at the embryonic stage 18 or 19 (see Fig. 3), and must be under the control of genes that

specify the labial segment identity. Previously we reported that several homeobox genes like *Bm Antp*, *Bm en*, and *Bm in* are expressed in the larval silk gland, and the *Nc* mutation of the *Bm Antp* causes a serious defect on the embryonic silk gland development. To analyze further the roles of other homeobox genes on the silk gland development, the cloning and characterization of *Bm Dfd*, *Bm Scr*, and *Bm lab* have been initiated.

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DIVISION OF MORPHOGENESIS

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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 vertebrate species, 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 useful model for studying stability and instability in differentiation of tissue cells. From this view point, lens transdifferentiation 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 by the vertebrate species including human.

We are studying in order to answer the questions why the PECs can transdifferentiate into lens cells and why the pigmented epithelia of the species other than the newt and so forth never compensate the lost lens in the *in situ* eye. Itoh and Eguchi previously established a unique culture system for the lens transdifferentiation using PECs from chick embryos. In this system the differentiated states of PECs are completely regulated by manipulation of the culture condition. Thus, molecular biological approaches have become possible using cultured chick PECs. We are also trying to establish transgenic systems of birds in order to examine directly the functions of individual genes closely related to control of differentiation and transdifferentiation of tissue cells. In addition to these major projects, the following problems have been recently 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 biomaterials. The investigations and the results achieved in 1992 by the members of this division are as follows.

I. The extracellular factors modulating the lens transdifferentiation of the PEC in vitro

Itoh and Eguchi have established that the lens transdifferentiation from PECs of chick embryos in vitro is achieved by phenylthiourea (PTU) and testicular hyaluronidase, which are added to the culture medium. In 1991, we established that FGF is one of key factors to regulate the lens transdifferentiation of PECs through the analysis of crude testicular hyaluronidase. On the basis of this finding, we have studied the mode of actions of FGF and of PTU in the process of the lens transdifferentiation of PECs in vitro and the following results were obtained. FGF can effectively induce collagenase production by PECs in culture when added to the culture medium. The zymographic analysis of culture medium using gelatin as a substrate has revealed that

FGF increases production of gelatinase (= latent 75kDa type IV collagenase) and activates gelatinase as well. In addition, the analysis of ECM has revealed that thick fibrous distributions of tenascin in extracellular matrix (ECM) of PECs cultured without both PTU and FGF dramatically altered to scattered fine network by addition of PTU, although distribution of fibronectin is not affected. Since PECs themselves synthesize type IV collagen to form the rather thick ECM consisting mainly of striated collagen fibers and lens transdifferentiation of this cell type can be suppressed by an artificial collagen substrate, these results are strongly suggesting that FGF might be one of key factors which regulate the differentiated state of PECs and the process of this lens transdifferentiation through cooperative functions with ECM in the presence of PTU.

On the basis of these results, we are now extending our analysis of roles of both FGF and PTU in regulation of the lens transdifferentiation of PECs, particularly focussing on the functions of ECM.

II. Analysis of functions of pP344 gene responsible for the lens transdifferentiation of PECs

By 1992, it has been established that PEC-specific pP344 gene is one of key genes, whose products must function to regulate the differentiated state and lens transdifferentiation of PECs in vivo and in vitro. In this year, we have concentrated to analyze the function of pP344 gene and the following results have been obtained. In situ hybridization has revealed that this gene is only expressed in the retinal pigmented epithelium but neither in iris pigmented epithelium nor ciliary epithelium. Analysis of a molecule produced by pP344-transfected Cos cells using an antibody against a peptide representing a distict region of this gene has revealed that pP344 gene encodes for a secreted 70kDa protein. We could purify 70kDa protein from the conditioned culture medium of PECs and the amino and sequencing showed that this protein was definitely the pP344 gene product. Moreover, we have confirmed that this 70kDa protein exhibited a strong inhibitory activity on trypsin. Based on these findings we are now bending our efforts on our studies of the regulatory mechanism of the lens transdifferentiation of PECs.

III. Development of avian transgenic systems

In addition to chick transgenic system, we have tried to establish the transgenic system of the quail. We firstly established the technique for complete cultivation of quail embryos from single cell stage to hatching. By this technique we can obtain a high hatchability of 25%. The β-actin-lacZ hybrid gene (Miw Z) was microinjected to the fertilized ova to be cultured. Seven out of 17 survived embryos from 85 to 90 h after injection exhibited the lacZ gene expression in their embryonic tissues. Thus, our culture technique will serve as a powerful tool for the production of transgenic quails.

As to the development of transgenesis in chick, we have developed a new method for microinjection of DNA to be transfered to a chick ovum. Although we should reserve the description of the technique in detail at the present stage of our study, we have obtained dramatically high efficiency in expression of the transferred gene. In three out of nine survived

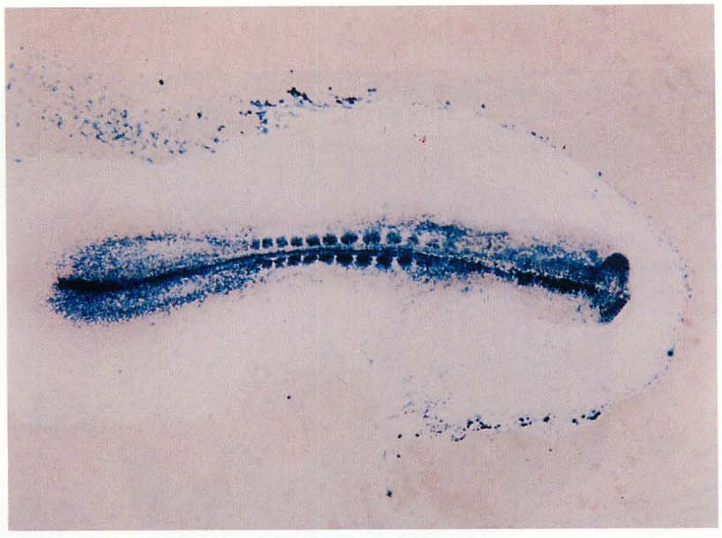

Fig. 1.

embryos, strong and homogeneous expression of transferred β-actin-lacZ hybrid gene (Miw Z) was achieved as Fig 1 shows. In all of the rest six embryos, the transferred gene was found to be more or less expressed in the embryonic tissues. Thus, our technique for microinjection of DNA to be transferred to fertilized chick ova is reaching to the goal.

IV. Pattern formation of butterfly wings through programmed cell death

The wings of butterflies are formed by vigorous extension of the imaginal disc epithelium at pupation. However, the pupal wing usually has a different outline shape from that of an adult butterfly wing, i.e. the latter often has projections and indentations at its margin, while the former has a round outline. This difference was reported to occur through the death of wing epithelial cells at the margin during pupal period. We precisely observed this process in *Pieris rapae* pupa and found that (1) the extensive cell deaths occur in the period of about half a day (from 3.5th to 4th day at 20°C), and that (2) dying cells show a ultrastructure characteristic to apoptosis (programmed cell death). Figure 2 shows a crossection of the wing tip on the 4th day where extensive cell deaths are observed at the very tip. Preliminary study suggested that cells die after S-phase. This system is geometrically very simple since all the cells in a certain area of a simple epithelium die. We believe this system is suitable for analyses of the mechanisms controlling cell death in general.

V. Cell biological analysis of biocompatibility of hydroxyapatite as a biomaterial

We have firstly established a human gingival fibroblast line, HGF-22 and a gingival epithelial cell line, HGE-15.I. Both of the established cells grow actively and maintain their cellular characteristics with high stability. HGE-15.I

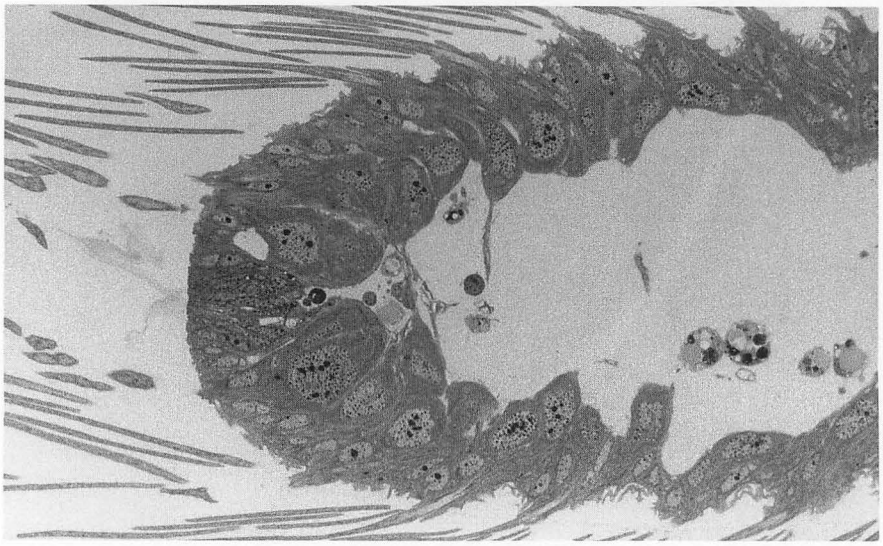

Fig. 2

cells conserve high activity to synthesize keratin molecules. Using these cell lines, we have quantitatively analyzed their cellular activities represented by adhesion, spreading, growth and differentiation by culturing cells on the surface of a hydroxyapatite disc, which is thin enough to observe living conditions of cells by a phase contrast microscope. Every examined activity expressed by both HGF-22 and HGF-15.I cells cultured on a hydroxyapatite disc was much reduced in comparison with cells cultured on the surface of commercial plastic cell culture dishes, strongly suggesting that the biocompatibility of hydroxyapatite is not always so high as thought thus far.

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Supplements to 1991

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DIVISION OF DEVELOPMENTAL BIOLOGY (ADJUNCT)

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Takahiro Morio (from Kyoto University)
Masa-aki Katsura (from Kyoto University)
Ayako Sakamoto (from Kyoto University)

This division is devoted to the studies on transcriptional regulation of genes whose expression depends on cell proliferation and differentiation in higher plants and a cellular slime mold. We have conducted researches on the following two aspects.

I. *Trans*-acting factors involved in transcriptional regulation of cell cycle-dependent genes in higher plants

Histone genes are mainly expressed in the S phase during the cell cycle, coupling with DNA synthesis. The molecular mechanisms of S phase-specific gene expression are still unknown. We have focused our attention on transcription of histone genes as a model system for the cell cycle-dependent gene expression in higher plants. The S phase-specific transcription of a wheat histone H3 gene (TH012) is regulated by a proximal promoter region up to −184 (relative to the transcriptional initiation site, +1). *In vivo* transcription experiments with deletion and site-directed mutations revealed that a type I element (CCACGTCANCGATCCGCG) (Mikami and Iwabuchi (1993) in: *Control of Plant Gene Expression*, ed. by D.P.S.Verma, CRC Press, Boca Raton, pp51–68) may be a *cis*-acting element controlling the periodic transcription of the H3 gene. This is supported by evidence that the type I element is highly conserved in promoters of many plant histone genes and that nuclear factors specifically to the type I element as wheat HBP (histone promoter-binding protein)-1a and HBP-1b have been identified in a variety of plant species.

Wheat bZIP proteins encoded by different five cDNA clones bound specifically to the type I element as the nuclear factors HBP-1a and HBP-1b. We concluded that five bZIP proteins constitute one family, designated HBP-1 family. By differences in overall structure and binding specificity, the HBP-1 family was grouped into HBP-1a and HBP-1b subfamilies. All members of the HBP-1a subfamily had a potential for homo- and hetero-dimerization, and members of the HBP-1b subfamily were supposed to form heterodimers because of their structural similarity. The binding sequences to bZIP proteins are present in promoters of plant genes other than histone genes. The various combinations among bZIP proteins in heterodimerization may generate a relatly expanded repertoire of their regulatory potential. No heterodimers could be observed between HBP-1a and HBP-1b subfamily members. We proposed that HBP-1a and HBP-1b subfamilies might mediate different regulatory processes.

The heterodimerization between different members of the HBP-1 family provides the complexity in addressing the functional properties of the HBP-1 family in the S phase-specific transcription of the H3 gene. From the above view-point, we have performed several experiments. To investigate regulatory mechanisms of HBP-1a and HBP-1b

gene expression, the promoter regions of these genes were fused to a bacterial GUS gene and resulting constructs were stably introduced into rice cells. The cell cycle-dependent expression of the fusion genes in transformed rice cells have been analyzed. All bZIP proteins of the HBP-1 family have consensus motifs for phosphorylation by various protein kinases; for example, casein kinase II, MAP (mitogen-activated protein) kinase and cdc2 kinase. So, we have started the study on relationship between protein kinases and HBP-1a and HBP-1b activities. In addition, we isolated several cDNA clones encoding proteins analogous to some members of the HBP-1 family from a *Arabidopsis* cDNA library. Functional analyses of the *Arabidopsis* bZIP proteins are in progress by a genetical approach. These efforts may make it possible to demonstrate that at least some members of the HBP-1 family are *trans*-acting factors involved in S phase-specific transcription of plant histone genes.

II. Function and expression of Dp87 gene in *Dictyosterium discoideum*

Cells of *Dictyosterium discoideum* aggrigate to form a multicellular organism and differentiate into prespore and prestalk cells which are the precursors of respective spore and stalk cells in a fully developed fruiting body. To elucidate the mechanisms of cell differentiation, we have studied the regulation of a prespore-specific gene Dp87.

1. *Expression of Dp87 protein during development*

The Dp87 protein shows a structural similarity to some spore cort proteins. However, timing of transcription of the Dp87 gene was earlier than that of any other prespore-specific gene so far examined, including spore coat protein genes. We compared expression profiles of the Dp87 and spore coat proteins immunologically. At late aggrigation stage, a 81kD protein was initially identified in many small particles, dispite a molecular weight of the protein was estimated about 58kD from a primary amino acid sequence. At slug stage, the 83kD protein, slight larger than the product at the aggrigation stage, was observed in a prespore vacuoles (PSVs) of prespore cells as is the case for spore coat proteins. In this stage, the Dp87 protein is concentrated at the center of PSV, while spore coat proteins were localized near its inner surface. After formation of a mature fruiting body, 83kD protein was dispersed in the sorus matrix and on the stalk sheath surface, but not on the spore surface where spore coat proteins localize. These results suggest that the Dp87 protein is a novel sorus matrix protein which is modified post-translationally at different two stages.

2. *Function of Dp87 protein*

By homologous recombination, we made a mutant which lack the Dp87 gene. All of independent mutants showed normal development. This suggest that the Dp87 is not essential for life of *Dictyosterium* or, even if it is important, some other genes can be replaced its function.

3. *Cis-acting regions important for prespore-specific transcription of the Dp87 gene*

Chimeric constructs composed by fusion of various parts of the 5′ upstream region to reporter gene were introduced into *Dictyosterium* cells. Expression of these constructs suggested that at least three parts of the upstream region affected positively and negatively to the efficient transcription of the Dp87 gene.

We speculated that the combinations among these *cis*-acting regions may be required for prespore-specific transcription of the Dp87 gene.

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DEPARTMENT OF REGULATION BIOLOGY

Chairman: Yoshiaki Suzuki

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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Research Associates: Nobuaki Maeda
Shinji Hirano
Technical Staff: Sonoko Ohsawa
Tomoko Mori

This division is devoted to the study of molecular mechanisms that govern the development of the vertebrate central nervous system. We are currently involved in projects to reveal the molecular bases of neuronal cell migration and neuronal network formation.

I. Molecular basis of neuronal cell migration

In the development of laminated structures, e.g. the cerebral cortex, of the higher vertebrate brain, later generated neurons must pass by the earlier generated ones so as to form an inside-out sequence in the radial dimension. This migration of post-mitotic neurons to their correct location within the developing brain is a key event in the cellular differentiation that leads to the functional neuronal network.

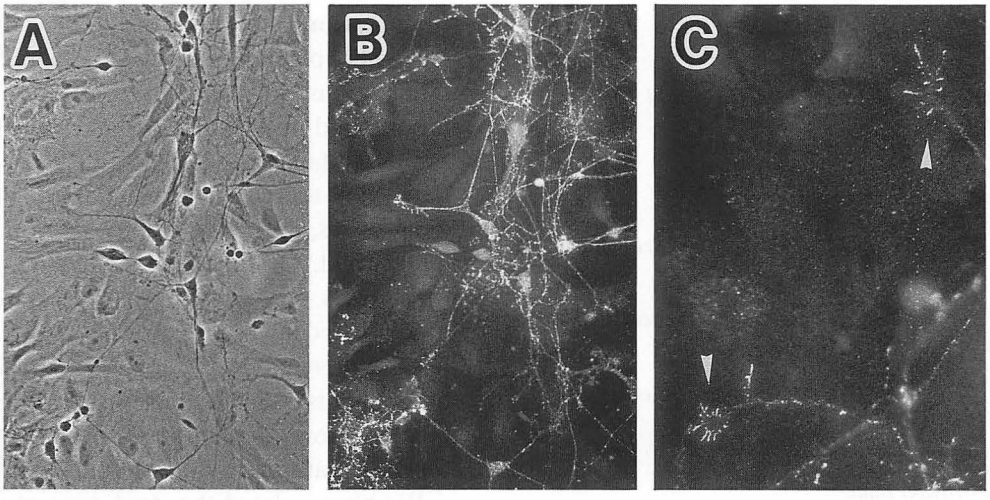
To elucidate the basic molecular mechanism of neuronal migrations, our study is now focused on the characterization of T61 antigen. Monoclonal antibody T61 was originally isolated with its inhibitory activity on neurite outgrowth from chick retinal explants *in vitro*. Recently, it turned out that T61 also inhibits the neuroblast migration, which results in aberrant laminar structure of the optic tectum, when the T61 producing hybridoma is injected into the ventricle of the developing midbrain of chick embryos. From immunoprecipitation analysis, an antigen molecule of 440 kD was detected as a major component. T61 antigen is distributed broadly in the developing brain, being concentrated on the surface of neuronal cells (Figure). Purification and molecular cloning of T61 antigen are now in progress.

II. Molecular basis of neuronal network formation

The development and maintenance of diverse neuronal systems in the vertebrate brain depend on various cell-cell and cell-substratum interactions. Such interactions require varieties of genetic and epigenetic information. Recently, it has been recognized that the vertebrate brain contains a diverse set of proteoglycans, which are supposed to play important roles in the cell-cell interactions in the nervous system.

Proteoglycans are a group of molecules composed of glycosaminoglycan chains covalently bound to a protein core. Glycosaminoglycans are very complex polysaccharides classified into chondroitin sulfate, dermatan sulfate, heparan sulfate, heparin and keratan sulfate. The structure of glycosaminoglycans is highly variable according to cell types, the environment of cells, and stages of their differentiation. The core proteins also show the structural diversity. We presume that this structural variability makes these molecules potent carriers of the information that is defined genetically and epigenetically, and accordingly, that proteoglycans can be a key regulator in the brain development.

In order to examine such possibility, we first prepared monoclonal antibodies against brain proteoglycans. One of the monoclonal antibodies we isolated (MAb 6B4) recognized a 600 to 1,000 kD chondroitin sulfate proteoglycan with a core protein of 250 kD (6B4 proteoglycan). This proteoglycan bears



Distribution of T61 antigen in the primary cell culture from E10 chick brain. (A) Phase-contrast photomicrograph. (B) Immunofluorescence of neuronal cells stained with T61 antibody. (B) is the same field as in (A). Note that glial cells are not stained. (C) Higher magnification of growth cones (arrow heads). T61 antigen is concentrated at the tips of growth cones.

chondroitin sulfate, keratan sulfate and HNK-1 epitope.

The distribution of 6B4 proteoglycan in the adult rat brain is highly unique. In the hindbrain, 6B4 proteoglycan is expressed on the cerebellar Purkinje cells and Golgi cells, and neuronal cell bodies at particular nuclei, including the pontine nuclei and lateral reticular nucleus. Almost all of these nuclei are connected to the cerebellum through the mossy fiber system.

The expression of 6B4 proteoglycan shows characteristic features during the formation of the cerebellar mossy fiber system. The mossy fibers from the pontine nuclei express 6B4 proteoglycan transiently from embryonic day 20 (E20) to postnatal day 30 (P30), during which period the axonal outgrowth and glomerular synapse formation occur. The Purkinje cells, glomeruli, and Golgi cells start to express 6B4 proteoglycan from P10, P16, and P20, respectively, in good accordance with the onset of their synapse formation. These results suggest the possibility that 6B4 proteoglycan is closely involved in the development of the cerebellar mossy fiber system. Molecular cloning and analysis of the polysaccharide portion of 6B4 proteoglycan are also in progress.

DIVISION OF CELLULAR REGULATION

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Technical Staff: Shoichi Higashi
Miki Ida

The research effort of this division is directed toward the understanding of tolerance and adaptation of higher plants and microbial plants to temperature extremes and high-salt stress, with particular emphasis on the molecular mechanisms by which plants acclimate or tolerate these conditions.

I. Genetic manipulation of fatty-acid desaturation in cyanobacteria

Higher plants, and most cyanobacterial strains, contain high levels of polyunsaturated fatty acids. While polyunsaturated fatty acids are thought to be important to the function of biological membranes, their roles are not fully understood. A fuller understanding of these roles will develop as we become more skilled at modifying the extent of unsaturation of the fatty acids of membrane lipids. This requires the genetic manipulation of the genes responsible for the desaturation of fatty acids.

We have isolated the *desA* gene of the cyanobacterium, *Synechocystis* PCC6803. This gene is responsible for the desaturation of fatty acids at the Δ-12 position. We constructed a disrupted *desA* gene, which was interrupted by the kanamycin-resistance gene cartridge, in order to manipulate the extent of desaturation of fatty acids in the membrane lipids of *Synechocystis* PCC6803. The wild type contained mono-, di-, and triunsaturated lipids. The mutant, which we designated Fad6, contained mono- and diunsaturated lipids; and a transformant of Fad6, with a disrupted gene for the desaturation which we designated Fad6/*desA*::Km^r, contained only monounsaturated lipids (Fig. 1).

We used mutants and transformants of *Synechocystis* PCC6803 to examine the effects of the unsaturation of fatty acids in membrane lipids on the low-temperature photoinhibition of photosynthesis. The strain most susceptible to low-temperature photoinhibition was Fad6/*desA*::Km^r, whereas Fad6 and the wild type showed the same level of sensitivity to photoinhibition. This suggests that diunsaturated fatty acids play an important role in protection against low-

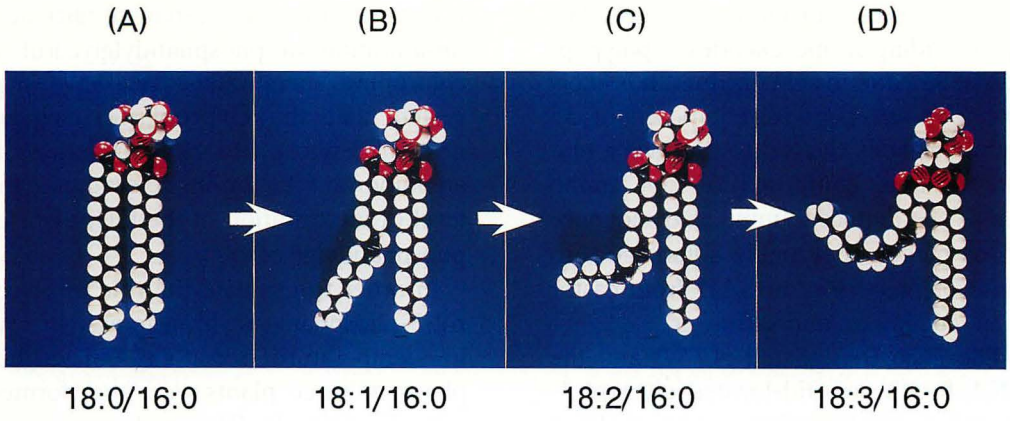

Fig. 1. The molecular structure of membrane lipids in the cyanobacterium *Synechocystis* PCC6803 grown at 34°C. Arrows indicate the pathway of biosynthesis. As major molecular species wild type contains (B), (C) and (D); Fad6 mutant contains (B) and (C); Fad6/desA::Km^r double mutant contains only (B). By comparing these strains, it is possible to study the biological functions of lipid unsaturation.

temperature photoinhibition. The unsaturation of fatty acids also affected photoinhibition at room temperature, although much less than at low temperature. By contrast, photosynthetic electron transport, measured at various temperatures, was not affected by changes in the extent of unsaturation of the fatty acids.

II. Temperature-induced desaturation of fatty acids is regulated at the level of gene expression

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 the temperature-induced decrease in the molecular motion of membrane lipids. Desaturases are responsible for the introduction of these specific double bonds.

To study the mechanism of low-temperature induced desaturation of membrane lipids in greater detail, we examined the effect of low temperature on the expression of the *desA* gene from *Synechocystis* PCC6803. The level of *desA* transcript increased 10-fold within 1 hour as temperature decreased from 36°C to 22°C. This suggests that the low-temperature-induced desaturation of membrane lipid fatty acids is regulated at the level of the expression of the desaturase genes. The accumulation of the *desA* transcript depends on the extent of temperature change exceeding a threshold level, and not on the absolute temperature.

III. Biochemical and molecular characteristics of enzymes involved in lipid synthesis

cDNAs and isomeric forms of enzymes involved in lipid synthesis were isolated and characterized in order to understand the molecular mechanisms of lipid synthesis in higher plants.

Stearoyl-(acyl-carrier-protein) desaturase (SAD) introduces the first double bond into C18 fatty acids in higher plants. We isolated a cDNA for this

enzyme from spinach cotyledons. The open-reading frame encodes a polypeptide of 399 amino acid residues. It is likely that a transit peptide, composed of 35 amino acids, is cleaved to produce a mature protein, composed of 364 amino acids. The deduced amino acid sequence of the precursor is highly homologous to those of precursors to SAD from castor bean, cucumber, and safflower.

The gene (designated *ATS1*) and the RNA for the plastid-located glycerol-3-phosphate acyltransferase were isolated from *Arabidopsis thaliana*. The nucleotide sequences of the gene and the cDNA were determined, and the 5′ end of the RNA was mapped by primer extension. The sequences similar to the TATA box, and the polyadenylation and intron-splicing sequences were found at the expected locations. The amino acid sequence deduced from the open-reading frame was highly homologous to those of precursors to plastid-located glycerol-3-phosphate acyltransferase from squash and pea.

Acyl-(acyl-carrier-protein) hydrolase releases fatty acids from the end product of fatty acid synthesis in plastids for the subsequent synthesis of glycerolipids in the cytoplasm. Isoelectric focusing of chloroplast stroma proteins from squash cotyledons suggested that there were at least three isomeric forms of acyl-(acyl-carrier-protein) hydrolase. The pI 4.5 form was isolated after a 100,000-fold purification from squash cotyledons. The enzyme is specific for long-chain-acyl-(acyl-carrier-protein).

IV. Genetically-engineered alteration of fatty-acid unsaturation modifies the chilling sensitivity of higher plants

The chilling sensitivity of higher plants is correlated with the extent of fatty-acid unsaturation of phosphatidylglycerol in chloroplast membranes. We have suggested that the chloroplastic enzyme, glycerol-3-phosphate acyltransferase, is an important factor in determining the level of unsaturation of the fatty acids of phosphatidylglycerol.

In order to evaluate the role of glycerol-3-phosphate acyltransferase in the low-temperature sensitivity of higher plants, tobacco plants were transformed with Ti-plasmids. These plasmids were constructed with cDNAs encoding the precursor protein of this enzyme, which were isolated from a low-temperature-sensitive plant, squash, and from a low-temperature-resistant plant, *Arabidopsis*. The introduction of squash cDNA decreased the content of unsaturated fatty acids in the phosphatidylglycerol, while the introduction of the *Arabidopsis* cDNA caused a small but significant increase in these fatty acids.

The chilling sensitivity of transformed plants was assayed by the inactivation of photosynthesis in excised leaves. The wild-type tobacco plants showed some sensitivity to chilling, but this sensitivity increased markedly with the introduction of the cDNA for squash glycerol-3-phosphate acyltransferase. In contrast, the introduction of the cDNA for the *Arabidopsis* enzyme decreased its chilling sensitivity. These remarkable differences correlated well with the extent of fatty-acid unsaturation of phosphatidylglycerol. These results demonstrate that it is possible to regulate the chilling sensitivity of higher plants solely by a genetically-engineered alteration of fatty-acid unsaturation of phosphatidylglycerol, with the introduction of an appropriate acyltransferase.

V. Heat shock proteins of cyanobacteria

The two most abundant HSPs, HSP70 and HSP64, were recognized in *Synechocystis* PCC6803 by antibodies raised against authentic DnaK and GroEL from *Escherichia coli.* HSP64 from *Synechocystis* PCC6803 was purified to homogeneity. Two open-reading frames corresponding to *groEL* and *groES* genes were found in the genome of *Synechocystis* PCC6803. Southern blot analysis indicates that only one *groESL* operon is present in the genomic DNA of *Synechocystis* PCC6803, whereas the existence of at least two copies of *groEL*-analogous genes was anticipated. The level of the transcript of *groESL* increased 100-fold within 15 min upon heat stress.

VI. Glycinebetaine stabilizes the oxygen-evolving complex of higher plants and cyanobacteria

The photosynthetic oxygen-evolving activity of the photosystem 2 complex, prepared from spinach, was labile when the complex was exposed to high-salt conditions under which the extrinsic proteins were dissociated from the complex. Glycinebetaine, which is accumulated in the chloroplast of halophilic higher plants, prevented the dissociation of the 18-kDa and the 23-kDa extrinsic proteins from the photosystem 2 complex in the presence of 1 M NaCl. It also prevented the dissociation of the 33-kDa extrinsic protein from the complex in the presence of 1 M $MgCl_2$ or 1 M $CaCl_2$.

We examined the heat stability of various electron-transport and phosphorylation reactions of photosynthesis in thylakoid membranes isolated from *Synechocystis* PCC6803. The order of sensitivity to heat was as follows; evolution of oxygen > Photosystem 2 = synthesis of ATP > cytochrome b_6/f > Photosystem 1. While glycinebetaine was effective in protecting the evolution of oxygen against heat, it did not protect any of the other reactions.

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H. Wada, Z. Gombos, T. Sakamoto, and N. Murata (1992) Genetic manipulation of the extent of desaturation of fatty acids in membrane lipids in the cyanobacterium *Synechocystis* PCC6803. *Plant Cell Physiol.* **33**, 535–540.

Books

N. Murata, Ed. (1992) Research in Photosynthesis Vol. I–IV, Kluwer Academic Publishers, The Netherlands.

DIVISION OF BIOLOGICAL REGULATION AND PHOTOBIOLOGY (ADJUNCT)

Professor: Kimiyuki Satoh
Associate Professor: Hirokazu Kobayashi
Research Associate: Satoru Tokutomi
Post-doctoral Fellow: Noritoshi Inagaki
*Graduate Student: Tatsuya Tomo**
*(*from Okayama University)*

Efficient energy transformation in the primary process 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 started from September 1992, in this newly established division, is aiming to elucidate organization of the reaction center of oxygenic photosynthesis, the photosystem II reaction center, in two aspects. In the first approach, the molecular structure of the reaction center will be analyzed by various methods in order to provide a solid basis for the second approach. In the second approach, the effort will be focused on elucidation of the molecular mechanism of light-regulated metabolic turnover of a subunit of the reaction center, the D1 protein. The process involves apparently unique, but in essence very general problems in molecular and cell biology; *i.e.*, stability and photodegradation of proteins *in vivo*, light-regulation of gene expression at the translational level, post-translational cleavage of the C-terminal extension of proteins and incorporation of cofactors and subunits into multi-component pigment-protein complexes.

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(related to previous projects)

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DIVISION OF BEHAVIOR AND NEUROBIOLOGY (ADJUNCT)

Professor: Masatoshi Takeichi
Research Associate: to be appointed

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 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, strongly suggesting its involvement in the neuro-muscular recognition. We are currently conducting the following research projects.

1) *Molecular genetic analysis of the role of connectin and Toll.* We are analysing the mutant animals which lack connectin or Toll to see if there are any defects in the neuro-muscular connectivity. We are also generating transgenic lines which ectopically express connectin on muscle fibers that normally would not express the molecule.

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

3) *Identification of more genes implicated in neuro-muscular recognition.* We are conducting molecular and genetic analysis of more enhancer trap lines that are expressed in specific subsets of muscles and/or motoneurons to examine their possible roles in neuro-muscular connectivity.

LABOLATORY OF GENE EXPRESSION AND REGULATION

Head: Yoshihiko Fujita

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

Professor: Yoshiro Shimura
Associate Professor: Kiyotaka Okada
Research Associate: Hideaki Shiraishi
Post-doctoral Fellows: Nobuyoshi Mochizuki
Kazuhito Akama
Graduate Students: Koji Sakamoto
Azusa Yano
Yoichi Ono
Toshiro Ito (from Kyoto University)
Technical Staff: Hideko Nonaka
Akiko Kawai

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. Our efforts have been focused on flower development and morphogenesis and also on root responses toward gravity, light or touching stimuli. 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 150 loci and more than 250 RFLP 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 stimulus-response interactions in root.

Mutants with abnormal floral morphology could be divided into the following types 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 with floral organs at asymmetric 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).

One of the homeotic genes, *AGAMOUS,* which is involved in the development of stamens and carpels is known to encode a putative DNA-binding protein which share a homologous region with the DNA-binding domains of transcription factors, yeast MCM1 and human SRF. Using the AGAMOUS protein overproduced in *E. coli,* we have shown that it binds to specific DNA sequences *in vitro.* The consensus sequence for the binding of the AGAMOUS protein was similar to that of yeast MCM1 (Shiraishi *et al.* (1993) *Plant J.*, in press). Attempts were also made to isolate cDNAs which are specifically expressed in floral organs. Two cDNA clones which are specifically expressed in stamen and pistil were

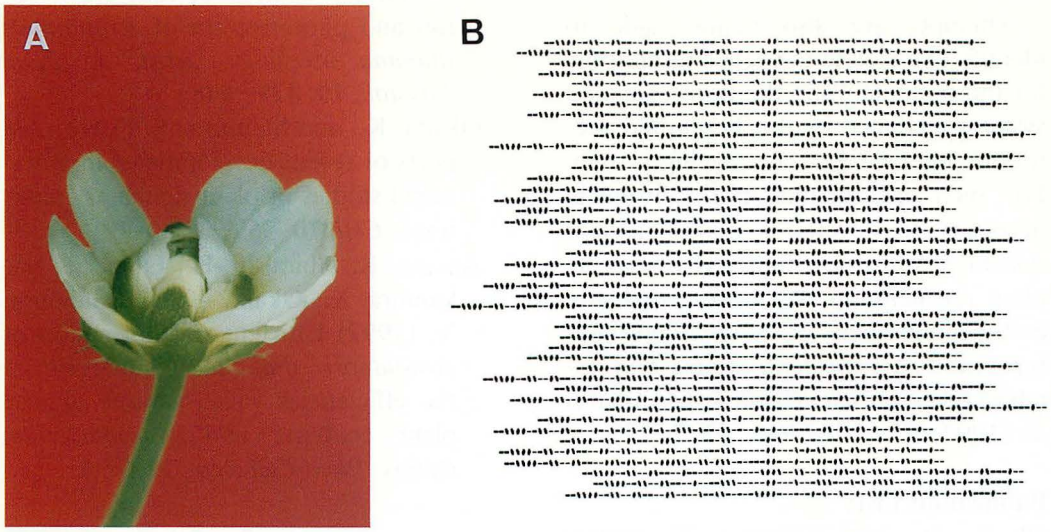

Fig. 1. (A) Flower of the *AGAMOUS* mutant. (B) Alignment of the nucleotide sequences of the oligonucleotides which are recognized by the AGAMOUS protein. DNA molecules recognized by the AGAMOUS protein were selected from a mixture of synthetic oligonucleotides containing random sequences in the central region. Nucleotide sequences of the selected oligonucleotides are shown with the following characters. A, −; T, =; G, #; C, %. A sequence motif which is recognized by the protein is visible in the central region of each oligonucleotide.

isolated, and their expression patterns were analyzed by *in situ* hybridization (Ito *et al.*, in preparation).

Roots alter their growth direction when they encounter obstacles (obstacle-escaping response), when their relative orientation against gravity is changed (gravitropic response), or when they are illuminated from aside (phototropic 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.

Polypeptides induced by gravitropic stimuli were analyzed using the 2-dimensional gel electrophoresis. Amounts of at least 11 spots were shown to be increased by the stimuli (Sakamoto *et al.* (1993) *Plant Cell Physiol.*, **34**,

297–304.).

Attempts are also being made to identify and isolate the genes responsible for the mutants using the transformation systems mediated by the Ti-plasmid vectors or by a direct gene-tagging system. For such experiments, it is absolutely necessary to develop a good, efficient system of transformation and 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).

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DIVISION OF GENE EXPRESSION AND REGULATION II

Professor: Takashi Horiuchi
Research Associate: Masumi Hidaka
JSPS Post-doctral Fellow: Takehiko Kobayashi
Technical Staff: Yoko Fujimura

We are examining mechanisms related to chromosomal replication, recombination and repair. Homologous recombination, replication, especially initiation and termination processes, and coupled events have been given special attention.

I. DNA replication terminus (*Ter*) site in *E. coli* is an entrance for recombination enzyme (RecBCD)

From the *E. coli* chromosome, we isolated *Eco*RI DNA fragments with higher homologous recombinational hotspot (Hot) activity, as follows: whole *E. coli* chromosomal DNA was digested with *Eco*RI enzyme, ligated with the 7 kb Km^r fragment and introduced into the RNase H-defective (*rnh*$^-$) host, then a number of Km^r transformants were collected. From these transformants, plasmid-like ccc (covalently closed circular) DNA was extracted by the alkaline miniprep method. Some clones contained the plasmid-like ccc DNA in higher amounts than in other clones. These DNA fragments were classified into 8 groups, termed HotA to H DNA. The Hot DNA was formed through excision from the host chromosome into which Hot DNA was once integrated rather than through autonomous replication, because Hot DNA was unable to be transformed into a mutant in which the Hot corresponding region on the chromosome was deleted. Thus, the amount of ccc Hot DNA recovered from the transformant indicates the recombinational Hot activity. We, therefore, called this "Hot assay". Hot DNA shared the following properties; (1) Hot activity was extraordinarily enhanced in the *rnh*$^-$ strain, (2) all Hot DNA, so far tested has Chi activity. As shown in Fig. 1, seven groups were clustered into a narrow DNA replication terminus region (about 280 kb). In particular, two Hot activities, HotA and C, were located on *Eco*RI fragments just next to the two *Eco*RI fragments carrying the DNA terminus sites, *TerB* and *C*, respectively. To examine the relationship between Hot and Ter activity, we carried out a Hot assay using the Ter-binding protein less (*tau*$^-$) mutant. The Hot activities of A, B and C groups disappeared when the *tau*$^-$ strain was used as a host, hence the Hot activities are termination event-dependent. In addition, at least HotA activity proved to require a Chi sequence present on the HotA fragment, because mutational destruction of the Chi sequence resulted in disappearance of the Hot activity. From these results, locations of the Hot and the Chi sites and their orientations, we proposed the following models: RecBCD, a Chi responsible enzyme, has good access to the DNA replication fork blocked at the *Ter* site, the molecule of which is known to be accumulated extensively under *rnh*$^-$ conditions. It enters duplex DNA, travels on a newly synthesized (branched) portion of duplex DNA and triggers recombination of the nearby region when it meets a Chi sequence. Other Hot DNA groups, Hot activity of which is termination event-independent, might be activated by other events induced under *rnh*$^-$ conditions. In *rnh*$^-$ cells, the two most active replication origins are located at the terminus region. Thus, termination independent Hot activities might require the initiation event occurring under *rnh*$^-$ conditions. In the T4 phage, there are hotspots

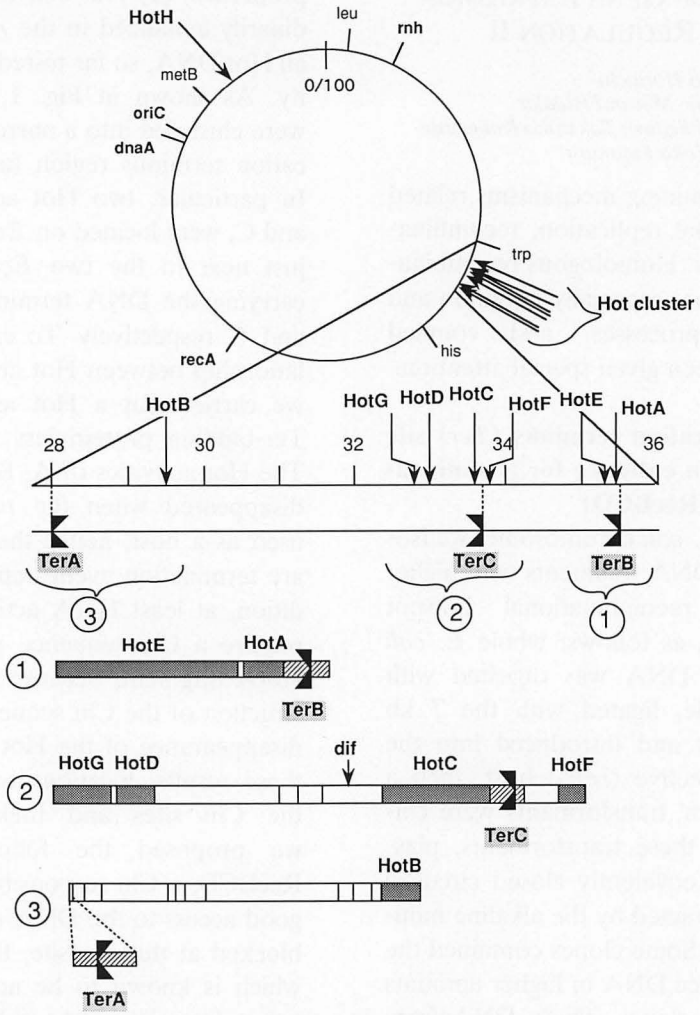

Figure 1. Location of Hot DNA fragments of the *E. coli* chromosome.
The upper part of the Figure shows locations of Hot DNA fragments on the *E. coli* circular map and lower one represents the three expanded *Eco*RI restriction maps, each of which contains a *Ter* site indicated by (◤) and on which 7 kinds of Hot DNA are clustered. Relevant markers are also shown.

closely linked to replication origins. Furthermore, *HOT1*, a recombination hotspot in yeast, might express activity through a mechanism similar to that for *E. coli* Hot DNA, as described below.

II. Molecular analysis of *HOT1*, a recombinational hotspot in *Saccharomyces cerevisiae*

HOT1 is an active recombinational hotspot present at the transcriptional enhancer-initiator region of repeated rRNA genes in *S. cerevisiae*. Data obtained from analysis of the *HOT1* phenomenon by other workers suggest that several properties of the *HOT1* are similar to those of the *E. coli* Hot-Chi system described above. (1) Both systems enhance homologous recombination (see Fig. 2), (2) both require two distinct

Figure 2. Colony color sectoring assay for yeast *HOT1* (developed by Lin and Keil, 1991).
Left; colonies of the strain containing no *HOT1* element produce very few white sectors. Right; colonies of the strain carrying the *HOT1* elements, E and I, produce numerous white sectors. The elements enhance excisive recombination between repeated *leu2* genes, a *ADE5, 7* gene located between which is eliminated frequently and white sectors are produced. Using this assay we are examining whether the *sog* site (E element) in yeast is functionally exchangeable with the *E. coli* analogous site, *Ter.*

elements for activities; In Yeast, E (enhancer) and I (initiator), in *E. coli, Ter* (terminus site) and Chi are essential. (3) One of the two elements in both cases, has DNA replication fork blocking activity. In *E. coli,* the *Ter* site has this activity. Similarly, in yeast, Fangman's and our groups found that the E element has fork blocking (we named "sog") activity. Thus, the replication fork stopped at the E element to which yeast recombination enzyme(s) such as RecBCD enzyme might access and stimulate recombination. To test this hypothesis, the following experiments are ongoing. (1) In crude extract of yeast, we are searching for protein(s) that specifically bind to the *sog* (E element) site, using gel retardation assay. (2) We are investigating whether the *sog* site (E element) in yeast is functionally exchangeable with the *E. coli* analogous site "*Ter*" (Fig. 2).

III. DNA fragments that delay DNA replication fork movement on the plasmid carrying them

During the course of experiments to identify DNA fragment carrying terminus (*Ter*) activity, we found two DNA plasmid clones, on which progress of the DNA replication fork is significantly and uniformly delayed, but not stopped at a definite site such as *Ter.* In prokaryotes, the rate of replication fork is rapid (about 1 kb/sec). Thus, the replication intermediate of a plasmid in *E. coli* cells, which appears as an arc in this 2D gel, cannot be recovered to any great extent. Southern hybridization using radiolabeled DNA as a probe is essential for detection. However, in the case of this particular plasmid, because of the delay in replication, the intermediate molecules greatly accumulated and EtBr staining facilitated visibility.

Both DNA fragments were derived from the terminus region of the *E. coli* chromosome and had activities that delayed replication. They shared the following properties.

(1) The activity has polarity. Rate of DNA replication in a clone was delayed, while that of an orientation isomeric clone (with the same fragment but in an

opposite orientation) was normal. (2) Cells carrying either plasmid were elongated. We are examining mechanisms through which the two DNA fragments delay replication of the plasmid carrying them.

Publication List:

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Division of Speciation Mechanisms

Professor: To be appointed

TECHNOLOGY DEPARTMENT

Head: Hiroyuki Hattori

Common Facility Group

Research Support Facilities
Mamoru Kubota
Chieko Nanba
Toshiki Ohkawa
Kaoru Sawada
Tomoki Miwa
Mariko Saitoh
Kimiko Yamamiya
Takeshi Mizutani

Radioisotope Facility
Kazuhiko Furukawa(Unit Chief)
Yoshimi Matsuda
Nobuya Sugimoto

Center for Analytical Instruments
Hisashi Kojima
Yukiko Kabeya
Yumiko Makino

Glassware Washing Facility
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Akio Murakami (Unit Chief)
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Regulation Biology Group
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Sonoko Ohsawa
Miki Ida
Tomoko Mori

Gene Expression and Regulation Group
Yoko Fujimura
Hideko Nonaka
Koji Hayashi
Akiko Kawai

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 technicalarea. Each technical staff is proceeded to the fixed division usually and they support the various research with their special biological, biochemical and biophysical techniques.

RESEARCH SUPPORT FACILITY

Head of Facility: Norio Murata
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
Kimiko Yamamiya
Takeshi Mizutani

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 level. This facility is routinely used for DNA recombination experiments.

3. *Computer Laboratory*

To meet various computational needs in the Institute, various kinds of computers are provided: VAX/VMS machines (VAX11/780 and micro VAX II), UNIX workstations (SPARC stations IPC, IPX, and 10 and DEC station 2100), and personal computers (PC9801, IBM compatible, and Macintosh machines). All of them are linked to the FDDI loop LAN of the Okazaki National Research Institutes (ORION) via ethernet. All laboratories in the Institute have at least one microcomputer

connected to the ethernet and thus they can access to this facility or to any site connected by internet. In addition, a NetWare server machine (Quarter L) is used to integrate PCs and it enables reference searches and sharing of Post Script printers from any laboratory. Various databases and software for molecular biological researches are also provided.

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 are equipped for experimental cultures of plant and microbial cells.

7. *Molecular Biological Analysis Laboratory*

In order to facilitate molecular biological analyses, high performance equipments such as DNA sequencer (ABI 370A), peptide synthesizers (ABI 430A and 431A), nucleotide synthesizer (ABI 381A), imaging analyzers (Fuji BAS 2000–3060 and –3080; PDI Discovery Series) 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-videomicrographs of the motile behavior of the cells at the cellular and subcellular levels. Photoreceptive and signal transduction 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 myosin in non-muscle cells might be better understood if wild-type cells 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. An expert system to predict various protein localization sites from amino acid sequence only has been constructed, and used to analyze unknown ORFs determined by an European yeast genome project, in collaboration with A. Goffau et al (Univ. Catholique Louvain, Belgium). Now, main efforts are concentrated on finding some rules for splice-site selection from mRNA precursor sequences in collaboration with H. Sakamoto (Kobe Univ.). For this purpose, an aberrant splicing database was constructed. Some hypothetical rules extracted from the database are now being tested for their predictability by incorporation into a computer program.

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

Nakai, K., and Kanehisa, M. (1992) A knowledge base for predicting protein localization sites in eukaryotic cells. *Genomics* **14**, 897–911.

Takahashi, T., Kubota, M., Watanabe, M., Yoshihara, K., Derguini, F., and Nakanishi, K. (1992) Diversion of the sign of phototaxis in a *Chlamydomonas reinhardtii* mutant incorporated with retinal and its analogs. *FEBS Lett.* **314**, 275–279.

II. Cooperative Research Program for the Okazaki Large Spectrograph

Johnson, C.H., and Kondo, T. (1992) Light pulses induce "singular" behavior and shorten the period of the circadian phototaxis rhythm in the CW 15 strain of *Chlamydomonas. J. Biol. Rhythms*, **7**, 313–327.

Kawai, H. (1992) Green flagellar autofluorescence in brown algal swarmers and their phototactic responses. *Bot. Mag. Tokyo* **105**, 171–184.

Srivastava, P.K., Mori, Y., and Hanazaki, I. (1992) Photo-inhibition of chemical oscillation in the $Ru(bpy)_3^{2+}$-catalyzed Belousov-Zhabotinskii reaction. *Chem. Phys. Lett.* **190**, 279–284.

Takahashi, T., Kubota, M., Watanabe, M., Yoshihara, K., Derguini, F., and Nakanishi, K. (1992) Diversion of the sign of phototaxis in a *Chlamydomonas reinhardtii* mutant incorporated with retinal and its analogs. *FEBS Lett.* **314**, 275–279.

Takeda, J., and Abe, S. (1992) Light-induced synthesis of anthocyanin in carrot cells in suspension. IV. Action spectrum. *Photochem. Photobiol.* **56**, 69–74.

RADIOISOTOPE FACILITY (managed by NIBB)

Head of Facility: Yoshihiko Fujita
Technical Staffs: Kazuhiko Furukawa
Yoshimi Matsuda
Nobuya Sugimoto

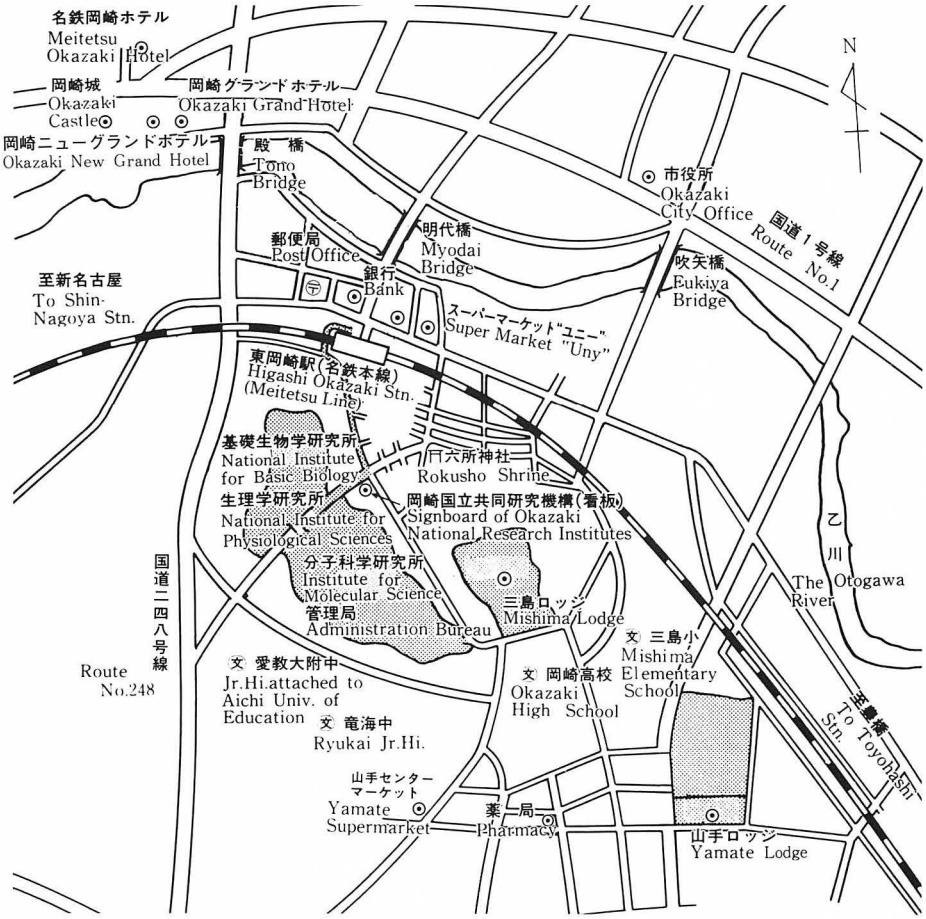
The facility is composed of a main center and two subcenters, one in the NIBB and the other in the NIPS. The facility is being used for molecular analyses of organisms. At the center, a variety of radioisotopes including ^{3}H, ^{14}C, ^{22}Na, ^{32}P, ^{35}S, ^{45}Ca, ^{125}I and various species of beta and gamma-ray emitting nuclides are handled. A laboratory facility 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
   - 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
   - Spectrofluorometer
     - HITACHI 850
     - HITACHI MPF-4
     - SIMADZU RF-5000
   - Spectrophotometer
     - HITACHI 330
   - Spectropolarimeter
     - JASCO J-40S
   - Microplate Reader
     - CORONA MTP-120
     - CORONA MTP-100F
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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