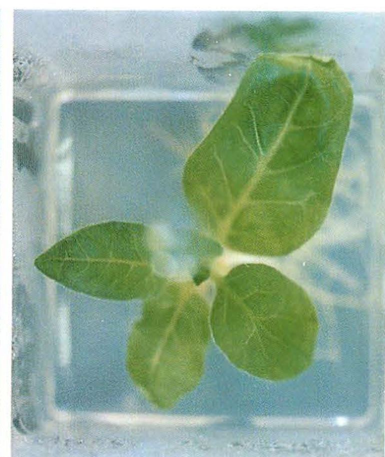
NATIONAL INSTITUTE FOR BASIC BIOLOGY

岡崎国立共同研究機構

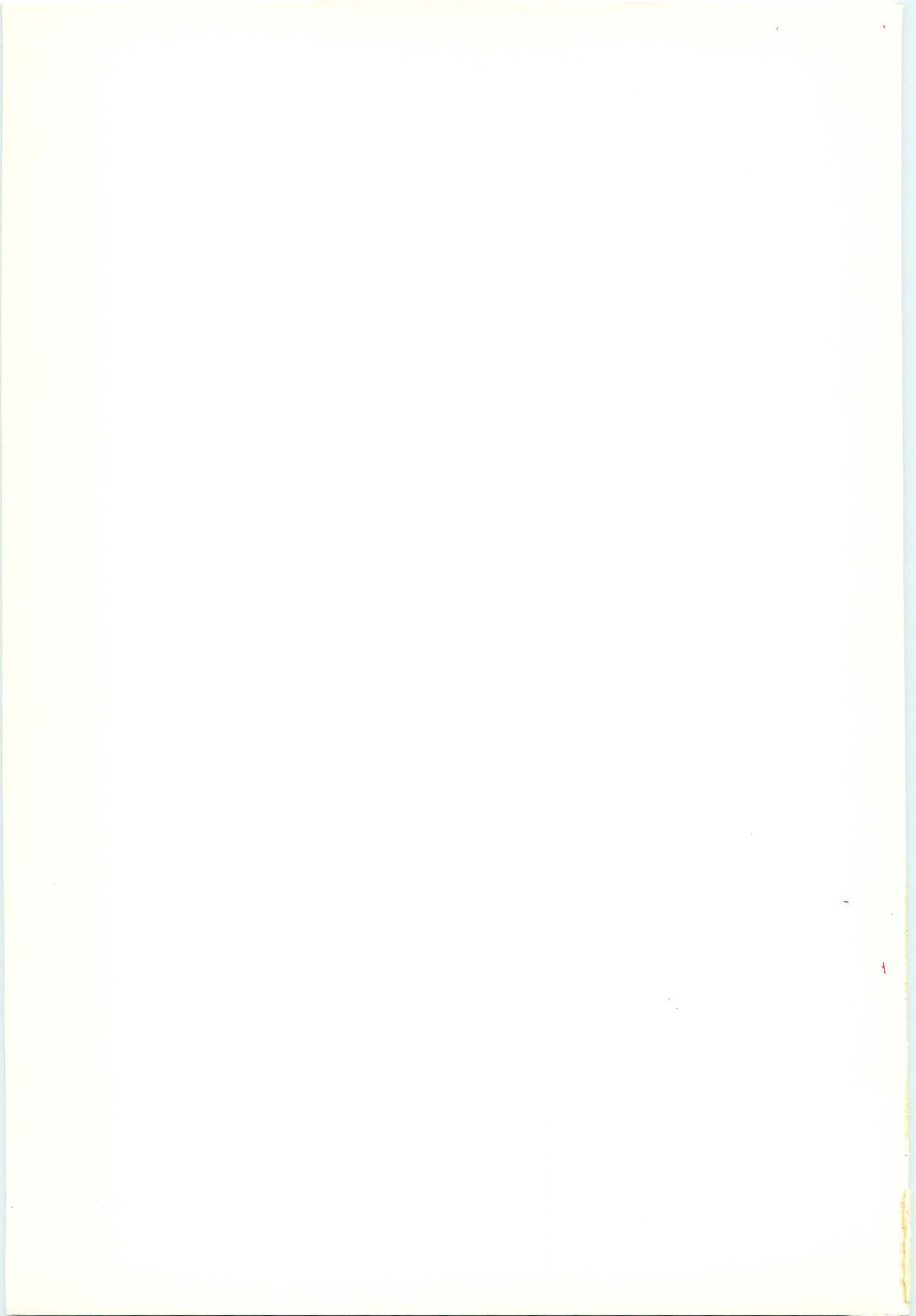
基 礎 生 物 学 研 究 所



Transgenic tobacco
with
(a) vector plasmids (b) squash cDNA (c) *Arabidopsis* cDNA

ANNUAL REPORT

1991



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.

Two new professors joined the NIBB during the last year. Prof. Masaharu Noda (former Associate Professor of Kobe University) filled the position of Professor of Molecular Neurobiology to promote molecular biological studies on the development of the vertebrate central nervous system. Prof. Hitoshi Sakano of the University of California at Berkeley was appointed to be in charge of Division of Cell Fusion (adjunct) to explore the possibility of the somatic DNA rearrangement in the development of the central nervous system.

The NIBB is an inter-university research institute and plays many roles as a national 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 26th and 27th Conferences in 1991, entitled "Plant organelle proteins; biosynthesis, targeting and assembly" (organized by Prof. Nishimura) and "Dynamic aspects of the cell cycle" (organized by Prof. Anraku).

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 1991.

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

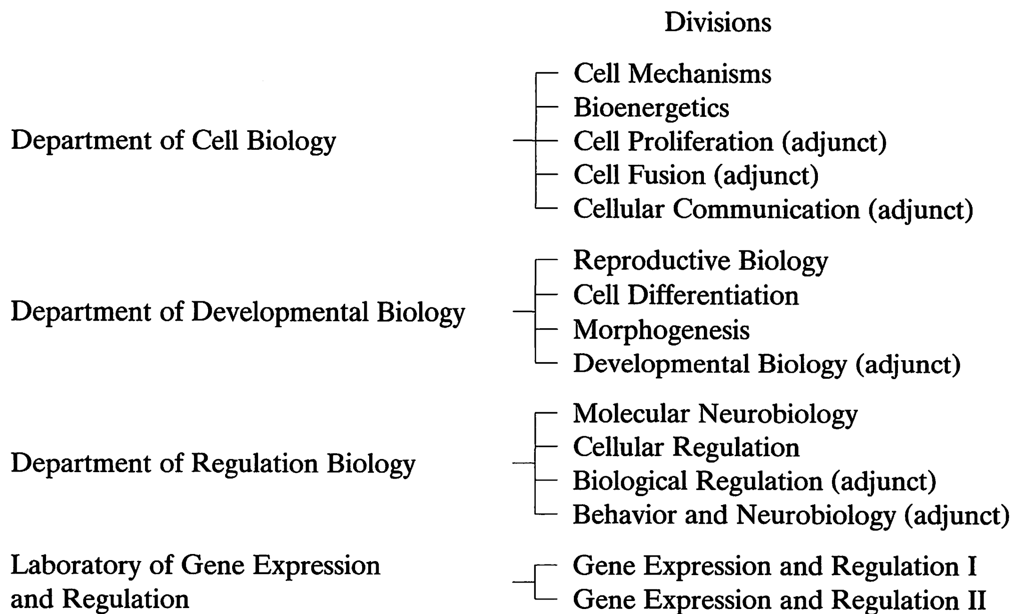
The Director-General oversees the operation of the Institute assisted by two advisory bodies, the Advisory Council and Steering Council. The Advisory Council is made up of distinguished scholars representing various fields of science and culture, and advises the Director-General on the basic policy of 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 15 divisions.



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

Morio, T., Ozaki, T., Takeuchi, I., and Tasaka, M. (1991). Transcriptional regulation of cell-type-enriched genes during development in *Dictyostelium discoideum* analyzed by nuclear run-on assays. *Develop. Growth & Differ.*, **33**, 293–298.

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DEPARTMENT OF CELL BIOLOGY

Chairman: Goro Eguchi

The Department is composed of two research divisions and three adjunct research divisions. In this department members have been conducting 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
JSPS Post-doctoral Fellow: Luigi De Bellis
Graduate Students: Ryuji Tsugeki
Katunari Tezuka[1)]
Kaori Inoue[2)]
Technical Staff: Maki Kondo

([1)]from Nagoya City University)
([2)]from Kobe University)

Eukaryotic cells contain several distinct organelles that play vital roles in cellular physiology. During proliferation and differentiation of the cells, the organelles often undergo dynamic changes. The biogenesis of new organelles may occur, existing organelles may undergo a transformation of function, which other organelles may degenerate. Because the dynamic transformation of organellar function (differentiation of organelles) is responsible for differentiation events in eukaryotic cells, the following two research projects are currently being studied in this division.

1. Functional transformation of microbodies during greening of the seedlings.

In many seeds such as 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 energy source for the growth of young seedlings. During the period when cotyledons emerge from soil, the tissues become green and photosynthetically active upon exposure to light. Photosynthesis primarily supplies the energy needed for the growth of the seedlings after greening.

During the greening process, dramatic metabolic changes which underlie 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. Since cell division does not occur in the cotyledons of these fatty seedlings following germination, all of these developmental events take place in a fixed number of preexisting cells. Therefore, greening cotyledon is an excellent model system for studies to elucidate the regulatory mechanism underlying light-induced organelle differentiation.

As a step to understand the regulatory mechanisms that operate during microbody transition in pumpkin cotyledons (*Cucurbita* sp., Amakuri Nankin), nine microbody enzymes, i.e., three glyoxysomal enzymes, three leaf peroxisomal enzymes and three enzymes present in both microbodies have been purified and characterized. Immunocytochemical analyses using their specific antibodies demonstrated that glyoxysomes are directly transformed to leaf peroxisomes during microbody transition. cDNA clones for the glyoxysomal enzyme, malate synthase, and the leaf peroxisomal enzyme, glycolate oxidase, have been screened and characterized. By the analysis using these cDNA clones, decrease in glyoxysomal enzymes during microbody transition was shown to be caused not only by decrease in level of the mRNA but also by induction of specific degradation system for glyoxysomal enzymes in microbodies.

Reversible transformation of microbodies from leaf peroxisomes to gly-

oxysomes was found to occur in the cotyledons during the senescence. The development of glyoxysomal enzymes was also observed in petals of pumpkin flower during the senescence, indicating that glyoxysomal enzymes may play a role in senescence process.

Concerning protein transport into microbodies, we have established an *in vitro* translocation system using isolated microbodies and products of transcription-translation of cDNA for microbody enzymes. Since the enzyme compositions and functions of glyoxysomes and leaf peroxisomes differ from each other, it is of interest that these microbodies possess different machineries for protein import or not. By the analysis of *in vitro* translocation system, it was shown that glyoxysomal enzyme, malate synthase can be translocated in leaf peroxisomes and leaf peroxisomal enzyme, glycolate oxidase is able to be imported into glyoxysomes. These results indicate that transport machinery of glyoxysomes is similar to that of leaf peroxisomes. cDNA for plant microbody enzyme, malate synthase was expressed in COS 1 cells, a monkey kidney cell line. The expressed malate synthase was shown immunocytochemically to be accumulated in microbodies of COS 1 cells, suggesting that transport machineries to microbodies are common in plant and animal cells and are highly conserved through evolution.

2. Biogenesis and transformation of protein bodies.

i) Biogenesis of protein bodies during seed maturation.

Protein bodies in pumpkin and castor bean seeds are separated into three parts, namely crystalloid, matrix and membrane. Crystalloid is composed of crystalline form of a major storage protein, 11S globulin. We have chosen three proteins located in each part of protein bodies and characterized their biosynthesis and intracellular transport. Although the three proteins are localized in the different suborganellar parts of protein bodies, they are synthesized on endoplasmic reticulum (ER) as larger precursors and transported via transport vesicles to vacuoles in a similar manner, suggesting that a unique machinery plays a role in intracellular targeting of these vacuolar proteins.

Proprotein precursors of vacuolar components are transported from the ER into vacuoles, where thay are proteolytically processed into their mature forms. Recently the processing enzyme was purified from castor bean seeds. Our results show that the purified enzyme can process three different proproteins isolated from either ER or transport vesicles in cotyledon cells to produce the mature forms of these proteins which are found at different suborganellar locations in the vacuole. Thus a single vacuolar processing enzyme is capable of converting several proprotein precursors into their respective mature forms.

We have succeeded to isolate the transport vesicles from maturing pumpkin cotyledons (Fig. 1). They contains a large amount of proprotein precursors. Further characterization of the isolated transport vesicles will be necessary to clarify the detailed mechanisms for intracellular transport of protein body proteins and specific recognitions between ER, transport vesicles and vacuoles at molecular level.

ii) Reversible transformation between vacuoles and protein bodies during seed maturation and germination.

Protein bodies are single membrane-bound organelles which accumulate seed

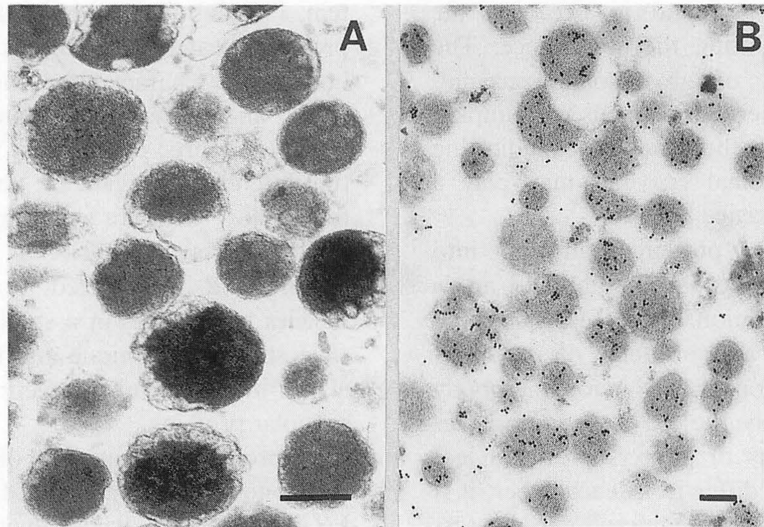

Fig. 1. Electron micrograph of dense vesicles isolated from maturing pumpkin cotyledons (A) and immunoelectron micrograph of the vesicles using an antiserum against 2S protein and protein A-gold (B). Bars are 200 nm.

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 seed maturation, while vacuoles result from the fusion of protein bodies during seed germination. Therefore, protein body is a specialized form of vacuoles in dry seed. It must be emphasized that dramatic transformation of protein bodies from a storage to a lytic compartment occurs during the maturation and germination of seeds. In order to clarify the regulatory mechanisms underlying the processes of the budding and the membrane fusion, we have especially focused on one membrane protein (MP32) of protein body which was rapidly degraded just after seed imbibition. Recently cDNA clone for MP32 was isolated and the primary structure of its precursor was deduced from the nucleotide sequence, indicating that the precursor protein included another membrane protein (MP27) and MP32 on the polypeptide chain. We have isolated protein bodies and vacuoles from various developmental stages of maturing and germinating pumpkin seeds. Using isolated protein bodies, we have recently established an *in vitro* system to reconstitute the fusion process. Experiments employing this *in vitro* system are currently underway to elucidate the molecular mechanisms of membrane fusion during seed germination.

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DIVISION OF BIOENERGETICS

Professor: Yoshihiko Fujita
Associate Professor: Shigeru Itoh
Research Associate: Mamoru Mimuro
Kaori Ohki
Katsunori Aizawa
*NIBB Visiting Scientist: Siba Plasad Adhikary**
Fellows: Tohru Ikeya
Jayanti Kumari Sahu
Technical Staffs: Akio Murakami
Masayo Iwaki

*(*Department of Botany, Utkal University, Orissa, India)*

Light-energy conversion in photosynthesis has been investigated at cell biological and biophysical levels. Cell biological study has been focused on the mechanism for regulation of stoichiometry between the two photochemical reactions in thylakoids, PSI and PSII complexes, in response to the photosynthetic conditions. Nitrogen-fixation in cyanophytes directly coupled with oxygenic photosynthesis has been another subject in the study of this level. Biophysical study has been focused on mechanisms of the excitation-energy transfer in light-harvesting pigment-protein complexes and of the electron transfer in the photosynthetic reaction centers.

(1) *Regulation of stoichiometric composition of thylakoids*

We have found that the stoichiometry between PSI and PSII in thylakoid system is not fixed but is changed in response to the photosynthetic conditions. This regulatory change adjusts the balance between the two light reactions and maintains a high efficiency of photosynthesis under each condition (cf. Fig. 1 and Fujita 1990). Our previous study for cyanophytes has revealed that this regulatory change is achieved by control of PSI formation during thylakoid development, and the signal for the control is closely related to the redox state of Cyt b_6-f in the thylakoid ETS. Analysis of the electron transport within Cyt b_6-f in relation to the control of PSI formation was indicated that (1) HQNO-sensitive Cyt-b_6 oxidation is involved in the signal system and that (2) the signal from ETS causes stimulation of PSI formation. Analysis of PSI formation in both processes for apoprotein and Chl *a* synthesis has also indicated that (1) the controlled changes in apoprotein synthesis occurs at translational level and (2) Chl *a* synthesis is also regulated at the step after Pchlide synthesis. Results suggest a possibility that the signal from ETS stimulates Chl *a* synthesis and resultant larger supply of Chl *a* accelerates synthesis and assembly of PSI complex.

The abundance of Cyt oxidase, another terminal of thylakoid ETS, is also changed in parallel with that of PSI suggesting that the abundance is regulated by a similar mechanism to that for PSI. However, some of Cyt oxidase has been reported to be located not in thylakoids but in cytoplasmic membranes in some particular strain, so that changes in abundance of Cyt oxidase may be induced by a different signal mechanism from the control of PSI formation. We re-examined the location of Cyt oxidase in the same strain as used for the above observation by a cytochemical electron microscopy. Results have shown that Cyt oxidase activity is found only in thylakoids, suggesting that the abundance of Cyt oxidase is also regulated in response to the thylakoid signal system.

(2) *Day/night oscillation of nitrogenase activity in the cyanophyte Trichodesmium*

The activity for nitrogen-fixation in cyanophytes oscillates under day/night regime; in most cases, high activity at night and low or insignificant in day

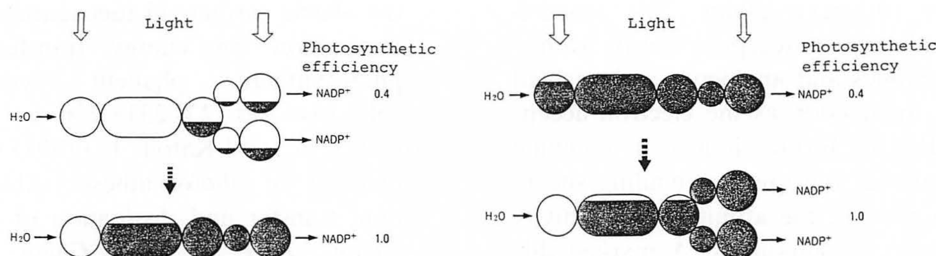

Fig. 1. Schematic presentation of regulation of PSI/PSII stoichiometry and electron transport state. Shaded area in each circle indicates level of reduced state of each component. Reduced PSII and oxidized PSI are photochemically inactive, respectively, and large occurrence of such states causes low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

time. This oscillation has been interpreted as a time separation of the O_2-sensitive nitrogen-fixation from O_2 evolving photosynthesis. However, *Trichodesmium* sp. shows a reverse pattern due to a direct coupling of nitrogen-fixation with oxygenic photosynthesis; high in day time, and insignificant at night. To know the mechanism for protection of nitrogenase from O_2 evolved by photosynthesis, changes in expression and modification of nitrogenase proteins were examined in relation to changes in the activity. Results have indicated that the oscillation is due to activation and inactivation of the Fe-protein of nitrogenase; the Fe-protein is modified to the inactive form in the dark, but light causes the activating modification. Results suggest that a light-dependent activation of the Fe-protein can compensate the inactivation by O_2 and maintain a high activity of nitrogenase under simultaneous occurrence of photosynthesis in this organism.

(3) *Excitation energy transfer in light-harvesting pigment-protein complexes*

The study in 1991 was focused on the energy transfer mechanism in fucoxanthin-Chl *a*/*c* protein complex, an intact light-harvesting assembly newly isolated from the brown alga *Dictyota dichotoma* (FCPA), and in chlorosomes in the green bacterium *Chloroflexus aurantiacus.* Spectroscopic analysis of FCPA has revealed that (1) fucoxanthin is present in the protein complex with a specific molecular geometry, which enables the pigment to transfer efficiently its excitation energy to Chl *a.* This study also provided the first success in detection of the fluorescence from fucoxanthin and other keto carotenoids. Analysis of the fluorescence from keto carotenoids has indicated that keto group of carotenoids induces S1 fluorescence with a long lifetime, both of which are responsible for the efficient energy transfer to Chl *a.* The study for chlorosomes has indicated a heterogeneity of molecular state of Bchl *c* in chlorosomes and also occurrence of plural pathways of the energy transfer in chlorosomes. A model for molecular arrangement of two types of Bchl *c* oligomers in chlorosomes has been proposed.

(4) *Electron transfer in photosynthetic reaction center complexes*

Mechanism of energy conversion in photosynthetic reaction center complexes has been investigated by replacing native quinone with artificial ones. Special attention has been paid for the action of phylloquinone in PSI reaction

center of green plants. The reaction center complex accepted a wide variety of quinones and analogues and enabled them to function as the electron acceptor. Results further indicated a unique structure of the quinone-binding site in the aspects of the affinity and reactivity of artificial compounds. A marked difference was observed for the electron transfer in the reaction center complex from that in solution. Similar studies are also undergoing with reaction centers of PSII and of green-sulfur photosynthetic bacteria.

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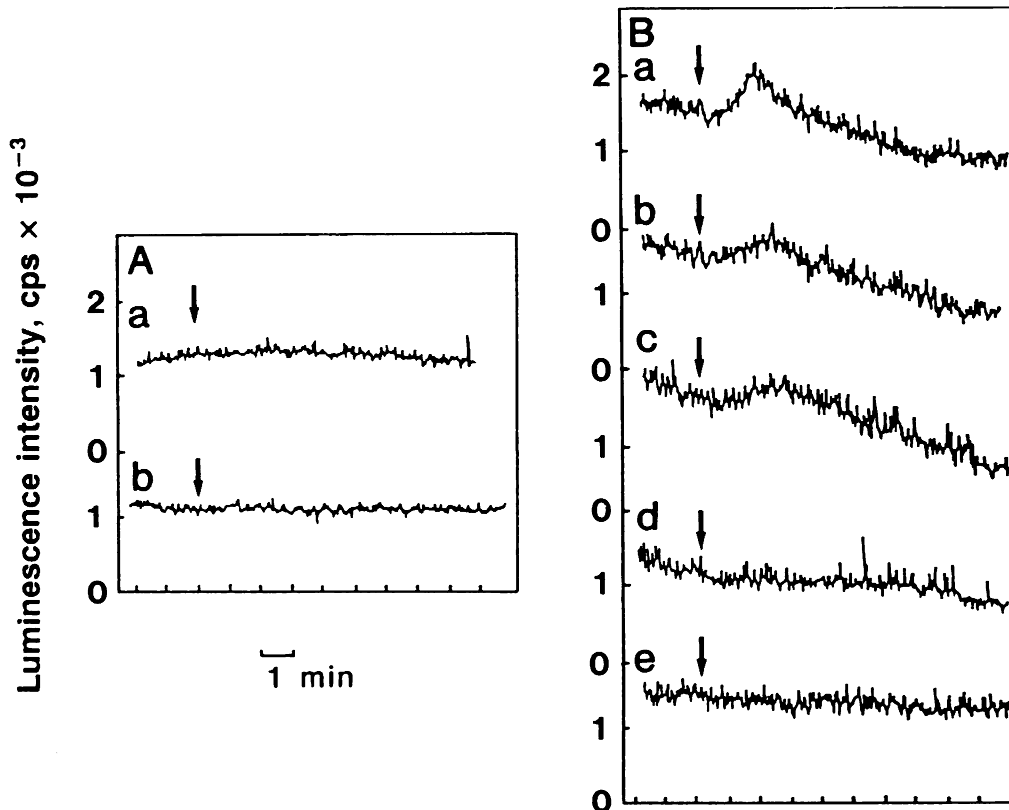
DIVISION OF CELL PROLIFERATION (ADJUNCT)

Professor: Yasuhiro Anraku
Associate Professor: Souichi Nakamura
Research Associate: Hidetoshi Iida
NIBB Post-doctoral Fellow: Hiro Nakamura
JSPS Post-doctoral Fellow: Junko Nakajima-Shimada
Graduate Student: Tomoko Ono (from Nara Women's University)
Visiting Scientist: Makiko S. Okumura

Ca^{2+} is a growth-regulating substance in the cell cycle of eukaryotic cells. For example, when the availability of Ca^{2+} becomes limited in the presence of sufficient amount of growth factors, mouse fibroblastic cells are arrested in the G_1 phase of the cell cycle. Most of growth factors elicit transient increase in the cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$). There is also a rise in $[Ca^{2+}]_i$ during mitosis. In recent years, several Ca^{2+} channels have been identified and methods for the monitoring of $[Ca^{2+}]_i$ have been developed, but molecular mechanisms of how Ca^{2+} is mobilized and how the mobilized Ca^{2+} acts to regulate the cell cycle are still obscure. In this Division, recent research has focused on 1) the role of Ca^{2+} in the cell cycle and mating process of the yeast *Saccharomyces cerevisiae*, 2) the establishment of measuring systems for $[Ca^{2+}]_i$ in yeast cells and 3) the mechanism of regulating Ca^{2+} homeostasis, with the hope that combination of cell biological and genetic approaches will uncover the molecular mechanisms of Ca^{2+} signaling.

Establishment and Application of a $[Ca^{2+}]_i$ Monitoring System by Using the Apoaequorin cDNA

The use of fura-2 in conjunction with digital image processing enabled us to quantify $[Ca^{2+}]_i$ in a single yeast cell, as described in the last issue of the NIBB Annual Report (also see Iida *et al.* (1990) J. Biol. Chem., **265**, 13391–13399). The fura-2 method has advantages to measure $[Ca^{2+}]_i$ in single cells and spatial distribution of $[Ca^{2+}]_i$ in the cytosol and organelles, but has some drawbacks: 1) fura-2 is sequestered eventually into an organellar compartment, the vacuole, and 2) fura-2 requires excitation at 340 and 380 nm. These two problems make it difficult to monitor $[Ca^{2+}]_i$ for a long period. To circumvent these problems, we have chosen a second Ca^{2+}-specific probe, aequorin. It is a Ca^{2+}-binding protein that emits light upon reacting with Ca^{2+} and consists of three components: apoaequorin (apoprotein), coelenterazine (chromophore) and molecular oxygen. The cDNA for apoaequorin of jellyfish *Aequorea victoria* has been cloned and sequenced. Apoaequorin is composed of 189 amino acid residues with three EF-hand structures. Although aequorin has been used as a probe, the use of this protein has been limited by the difficulty of introducing sufficient amount of it into small cells, including yeast. To overcome this difficulty, we have designed the present study to express the apoaequorin cDNA in yeast cells under the control of *GAL1* or the *GAP* promoter and tested whether apoaequorin can be accumulated in high enough concentration in the cells to detect a Ca^{2+} signal. The results showed that the cells accumulated sufficient amounts of recombinant apoaequorin and that the protein was active and not toxic to the cells. For an *in vivo* method for monitoring of changes in $[Ca^{2+}]_i$, introduction of coelenterazine into intact yeast cells and regeneration of aequorin in the cells are essential steps. We found that aequorin can be effectively regenerated by simply incubating



Changes in $[Ca^{2+}]_i$ in response to glucose in glucose-starved G_0/G_1 cells, as revealed by luminescence intensity of aequorin regenerated in intact yeast cells. Twenty-four hours after the start of glucose starvation, cells expressing the recombinant apoaequorin were harvested, resuspended in Mes/Tris buffer, incubated with coelenterazine, washed, resuspended in appropriate buffers and monitored for changes in $[Ca^{2+}]_i$. (A) Cells were resuspended in Mes/Tris buffer containing no $CaCl_2$ and then supplemented (arrow) with glucose (trace **a**) or 2-deoxyglucose (trace **b**). (B) Cells were resuspended in Mes/Tris buffer containing 10 mM $CaCl_2$ and then supplemented (arrow) with glucose (trace **a**), fructose (trace **b**), mannose (trace **c**), 2-deoxyglucose (trace **d**), or 6-deoxyglucose (trace **e**). This figure shows that a rise in $[Ca^{2+}]_i$ is dependent on extracellular Ca^{2+}, and suggests that the rise is energy-dependent.

intact yeast cells with coelenterazine. Coelenterazine-incorporated cells responded to extracellular stimuli. A mating pheromone, α-factor, generated extracellular Ca^{2+}-dependent luminescence specifically in cells of *a* mating type with maximal intensity occurring 45—50 min after addition of α-factor. Glucose added to glucose-starved G_0/G_1 cells stimulated an extracellular Ca^{2+}-dependent luminescence with maximal intensity occurring 2 min after the addition (Fig. A and B-a). This stimulation corresponded to a change in $[Ca^{2+}]_i$ from the basal level of 180 ± 10 nM to 340 ± 40 nM (n=4). These results demonstrate the usefulness of the aequorin method in monitoring $[Ca^{2+}]_i$ response to extracellular stimuli in yeast cells. Using this method, we have investigated mechanisms of Ca^{2+} mobilization in the initiation of the cell cycle of glucose-starved G_0/G_1 cells triggered by glucose. The results showed that metabolizable hexoses

such as glucose, fructose and mannose, but not non-metabolizable hexoses such as 2-deoxyglucose and 6-deoxyglucose, elicited the $[Ca^{2+}]_i$ rise in glucose-starved cells. This result implies the possibility that ATP may be involved in the $[Ca^{2+}]_i$ response. To test this possibility, we added 10 μM KCN, a potent inhibitor of electron transport in mitochondria, with glucose. The $[Ca^{2+}]_i$ rise was dramatically reduced by KCN. This result is consistent with the notion that ATP is involved in the $[Ca^{2+}]_i$ response. To clarify this point, several experiments are in progress.

Ca^{2+} Homeostasis Maintained by the Vacuolar H^+-ATPase and Vacuolar Ca^{2+}/H^+ Antiporter

In vitro experiments have shown that the vacuolar membrane vesicles of the yeast have a H^+-ATPase capable of producing an electrochemical potential difference of protons ($\Delta\mu H^+$). The H^+-ATPase thus enables the vacuole to incorporate protons from the cytosol. The vesicles also have a Ca^{2+}/H^+ antiporter that enables the vacuole to take up Ca^{2+} by extruding protons from the vacuole. Interestingly, the Ca^{2+} uptake is inhibited by inhibitors of the vacuolar H^+-ATPase, such as Cu^{2+}, Zn^{2+} and *N, N'*-dicyclohexylcarbodiimide. This observation raises the possibility that the Ca^{2+} uptake carried out by the Ca^{2+}/H^+ antiporter is coupled with the activity of the H^+-ATPase *in vivo*. If this is the case, the vacuole of mutant cells defective in the vacuolar H^+-ATPase may fail to incorporate Ca^{2+} and the Ca^{2+} concentration in the cytosol may become higher in the mutant than in wild-type cells. To test this possibility, we measured $[Ca^{2+}]_i$ in cells of the *cls7, cls8, cls9, cls10* and *cls11* mutants, all of which are defective in H^+-ATPase activity, by using the fura-2 method. The result showed that the average $[Ca^{2+}]_i$ in wild-type cells was 150 ± 80 nM whereas the mean value in the five *cls* mutant cells was 900 ± 100 nM. This observation clearly indicates that Ca^{2+} homeostasis in the cytosol is maintained by the coupling of Ca^{2+}/H^+ antiport with H^+-ATPase on the vacuolar membrane.

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DIVISION OF CELL FUSION (ADJUNCT)

Professor: Hitoshi Sakano
Research Associate: Masahiro Ishiura
Fumikiyo Nagawa

We are interested in the somatic DNA rearrangement in the immune and central nervous systems in the mouse. In the immune system, V-(D)-J joining generates a vast diversity of antigen receptor genes by combining multiple gene segments, as well as by modifying the junctional sequences. Somatic DNA rearrangement can also serve as an effective mechanism for the activation of a particular member of a multigene family. In the antigen receptor gene, this is achieved by bringing two separate regulatory DNA elements, the promoter and enhancer, into close proximity. This type of gene activation mechanism may be utilized to determine the irreversible fate of cells during development. It is tempting to ask whether such gene rearrangement is restricted to the immune system, or whether similar somatic events are exploited by other organ systems, e.g., the central nervous system.

Similarities between the immune system and the central nervous system have long been discussed. Both systems have to be capable of recognizing and remembering many different external signals. It is known that some lymphocyte surface proteins, e.g., CD4, are expressed in nerve cells. Furthermore, it has been shown that RAG-1, a gene involved in site-specific recombination in the immune system, is also transcribed in the brain. In order to study the developmental control of the V-(D)-J type of joining, we have constructed a recombination reporter gene and generated transgenic animals. The substrate contained two sets of V-(D)-J joining signals and the bacterial β-galactosidase gene, *lacZ*, in an opposite transcriptional orientation downstream from the promoter/enhancer complex (Matsuoka *et al.*, *Science* **254**: 81–86, 1991). While analyzing transgenic mice, we noticed that not only lymphatic tissues, but certain areas of the brain were stained blue with X-gal. This observation indicates that DNA recombination activates the transgenic reporter gene during the development of nerve cells. Regions showing β-gal activity appear around birth, and continue to emerge postnatally in newborn mice. While the distribution of the β-gal activity in the central nervous system is widespread, it is neither diffuse nor random.

Although the mode of gene rearrangement remains to be studied, it appears that recombination occurs in certain domains of the brain at specific stages of development. The question then arises, why do brain cells have to undergo gene rearrangement? Recombination in brain cells could be acting as switching system for gene expression by DNA inversion or by juxtaposing promoters and coding regions with enhancers by a deletion mechanism. The next task is obviously to identify the rearranging genes in nerve cells. For deletion type recombination, isolation of circular DNA has been instrumental in the study of Ig and TCR gene rearrangement in lymphocytes (Matsuoka et al., *Cell,* **62**: 135–142, 1990). If the rearrangement in the brain occurs by a DNA deletion mechanism, characterization of brain circular DNA will allow us to identify the rearranging genes in the central nervous system.

Division of Cellular Communication (Adjunct)

Professor: Yoshiki Hotta
Research Associate: Hitoshi Okamoto
Institute Research Fellow: Keiko Nakao
Graduate Stuedent: Keita Koizumi
(from Univ. of Tokyo)

Our division is currently involved in the study of molecular mechanisms of cellular communications and signaling, especially of the development and differentiation processes of the multicellular organisms. As model organisms, fruit fly (Drosophila melanogaster) and fish (Japanese Medaka and zebrafish) are chosen, to study the sensory receptors, nervous systems and muscles at the genetic, molecular and cellular levels.

Neurogenetics of Drosophila

For the study of Drosophila, many visual transduction mutants have been isolated by their abnormal visual behavior. The molecular defects of many vision mutants have been shown to be involved in the phosphatidyl-inositol metabolisms in the eye. The results suggest that the visual signal transduction system is linked to the phospholipid turnover, most likely that of inositol 1,4,5-triphosphate (InsP3). By molecular cloning of the genes, norpA (no receptor potential A) and rdgA (receptor degeneration A) have been identified as structural genes for eye-specific phosphatidyl inositol phosphoplipase C and diacylglycerol kinase, respectively.

Other genes of interest are the embryonic lethals of Drosophila which are normal in embryonic polarities and segmentations, but are causing defects in the early differentiation of mesoderm, neuroblasts and glia. One particularly interesting gene, 1(2)KN13 is shown to lack muscle cells both in vivo and in vitro (single embryo primary culture) due to the defects in myoblast fusion processes in spite of the presence of morphologically normal "muscle pioneer cells".

Molecular Embryology of Fish

After the recent explosive advance in neurobiology and molecular developmental biology of Drosophila, we are at the stage of extending our knowledge and molecular technology to vertebrate systems. It is especially important to study how the neurons in the vertebrate brain acquire their cellular identities and how they send their axons to proper targets. Embryos of small fresh water fish, such as zebrafish or Japanese Medaka fish are useful materials for such study. It is because the embryos stay transparent throughout their embryonic development, and many of their early neurons are identifiable. Many research groups, including ours, have started exploiting this advantage to carry out cell manipulations, such as dye-labeling, cell transplantation and cell ablation by laser beam.

Since April 1991, we have set up the facility for the fish study. We are currently modifying zebrafish genome by using gene transfer technique to make transgenic fish, whose embryos are designed to be amenable to various modern analyses like genetic mosaic method and enhancer-trap technique.

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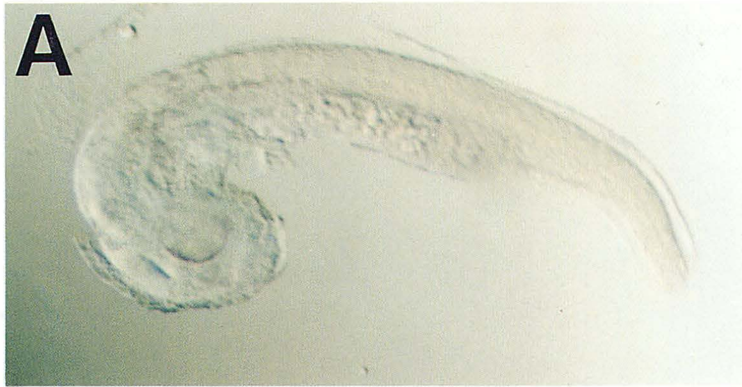

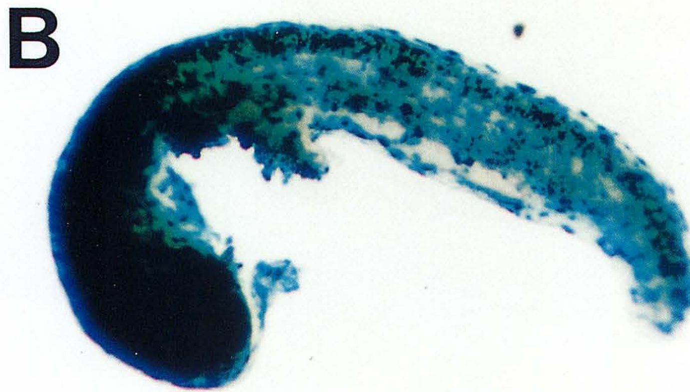

(A) Side view of a 24 hr zebrafish embryo. The yolk sac has been removed. (B) A 24 hr zebrafish embryo transformed with the E. coli lacZ gene under the control of the mouse hsp68 promoter. After heat shock and fixation, tissues of a whole mount embryo have been stained blue for β-galactosidase activity with X-gal.

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DEPARTMENT OF DEVELOPMENTAL BIOLOGY

Chairman: Yoshiaki Suzuki

The Department is composed of 3 regular divisions and 1 adjunct division and conducts molecular analysis on various aspects of developmental phenomena; differentiation and maturation of the gonad cells, molecular basis of the body plan, and gene regulation in the differentiated tissues, changes of the differentiated states, and different phases of the cell cycle.

LABORATORY OF REPRODUCTIVE BIOLOGY

Professor: Yoshitaka Nagahama
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Masakane Yamashita
Minoru Tanaka
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Visiting Scientists: Yan Shao-Yi
*Sergei G. Vassetzky**
*Devendra N. Saksena**
Jennifer Specker
*Kazu Haino-Fukushima***
*Michiya Matsuyama****
Technical Staffs: Hiroko Kajiura
Sachiko Fukada

*(*supported by JSPS)*
*(**from Tokyo Metropolitan University)*
*(***from Mie University)*

The germ cell is the most important cell in the body of any species, since it transmits genetic information from one generation to the next. The male gamete is specialized to deliver its nuclear package to the egg, but the egg contains not only a haploid nucleus but also everything else that constitutes the zygote. Thus, the origin, differentiation, growth and maturation of these essential cells are of fundamental interest and importance to developmental biology. The sequence of events leading to the production of fertile gametes takes place while germ cells reside within the gonads, and occurs in close association with gonadal somatic cells, by which various endocrine products (particularly steroid hormones) are secreted. 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.

1) 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.

To investigate the molecular basis for this switch, our current efforts center on the cloning and sequencing of the genes encodings five steroidogenic enzymes responsible for estradiol-17β and 17α, 20β-DP biosynthesis: cholesterol side-chain cleavage cytochrome P-450 (P-450scc), 3β-hydroxysteroid dehydrogenase (3β-HSD), 17α-hydroxylase/

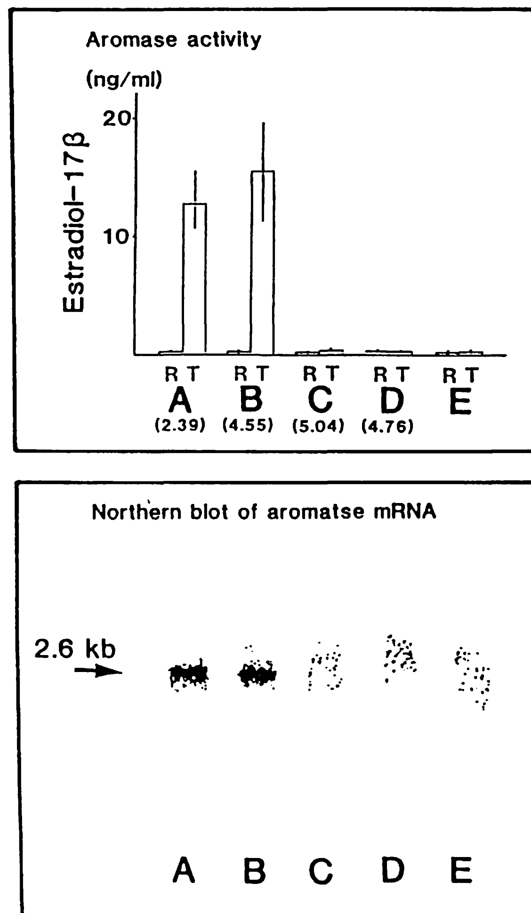

Fig. 1. Upper panel, changes in estradiol-17β production by isolated follicular preparations of trout during early (A) and late (B) vitellogenesis and oocyte maturation (migratory nucleus (C) and matured oocyte (D)) and by postovulatory follicles (E). Follicular preparations were incubated in Ringer alone (R) or in the presence of 100 ng testosterone/ml. Levels of estradiol-17β in the culture medium following incubation were measured by RIA. The diameters of the follicles used are shown in parentheses (mm). Lower panel, Dectection of 2.6 Kb RNA transcripts that hybridized to the trout aromatase probe from various stages of trout ovaries. Poly(A)$^+$RNAs were extracted from the same ovaries as shown in the upper panel.

$C_{17,20}$-lyase cytochrome P-450 (P-450c17), P-450 aromatase (P-450arom) and 20β-hydroxysteroid dehydrogenase (20β-HSD). We have already isolated cDNA clones for these five enzymes; the complete nucleotide and deduced amino acid sequences of P-450c17, P-450arom and 20β-HSD cDNAs have been determined. A cDNA clone encoding P-450c17 was isolated from a rainbow trout ovarian follicle cDNA library. The cDNA contained an open reading frame of 1542 nucleotides encoding a protein of 514 amino acid residues. The trout P-450c17 expressed in nonsteroidogenic mammalian COS-1 cells showed both 17α-hydroxylase and $C_{17,20}$-lyase activities. The cDNA only hybridized to a single species of mRNA (2.4 Kb) isolated from rainbow trout ovaries; the

2.4 Kb transcripts were abundant in trout ovaries during the later stages of oogenesis. A cDNA clone encoding rainbow trout P-450arom was also sequenced and found to contain an open-reading frame of 1566 nucleotides predicted to encode a protein of 522 amino acid residues. Northern blot analysis revealed 2.6 Kb RNA transcripts which were present in the trout ovary during vitellogenesis and hybridized to the cDNA insert. The RNA transcripts were present only during stages of estradiol-17β production by the ovarian follicles, suggesting that estradiol-17β production is regulated, in part, by the amount of P-450arom mRNA present (Fig. 1). Using these P-450c17 and P-450arom cDNA clones, we are now attempting to clone genomic DNAs for these two enzymes.

Using synthetic oligonucleotides deduced from the partially determined amino acid sequence of purified pig 20β-HSD, we have, for the first time in any species, isolated and sequenced a cDNA encoding 20β-HSD from a pig testis cDNA library; this work has been carried out in collaboration with Drs. S. Nakajin and S. Ohno (Hoshi University). The cDNA contains an open-reading frame of nucleotides predicted to encode a protein of 289 amino acid residues. Surprisingly it has 85% amino acid homology with human carbonyl reductase. The steady state levels of the mRNA transcripts increased to a maximum in testes from 10-day old pigs but rapidly declined thereafter to the same levels found in testes of mature animals. The pig 20β-HSD will be used as a probe to clone rainbow trout 20β-HSD.

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 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. We purified MPF from mature oocytes of carp (*Cyprinus carpio*). Carp MPF consists of two components; one is homolog of the $cdc2^{+}$ gene product of fission yeast (*Schizosaccharomyces pombe*), referred to as $p34^{cdc2}$, and the other is cyclin B. Using monoclonal antibodies against the PSTAIR sequence of $p34^{cdc2}$ and *E. coli*-produced goldfish cyclin B, we examined the levels of $p34^{cdc2}$ and cyclin B during goldfish oocyte maturation induced *in vitro* by 17α, 20β-DP. Protein $p34^{cdc2}$ was found in immature oocyte extracts and did not remarkably change during oocyte maturation. Cyclin B was absent in immature oocyte extracts and appeared when oocytes underwent germinal vesicle breakdown. Cyclin B that appeared during oocyte maturation was labeled with [^{35}S]methionine, indicating its *de novo* synthesis. The introduction of *E. coli*-produced cyclin B into immature oocyte extracts induced $p34^{cdc2}$ activation, which was associated with threonine phosphorylation of $p34^{cdc2}$ and serine phosphorylation of cyclin B, as found in oocytes matured by 17α, 20β-DP. Cyclin B-induced $p34^{cdc2}$ activation was not induced by inhibiting threonine phosphorylation of $p34^{cdc2}$ by protein kinase inhibitors. These results suggest

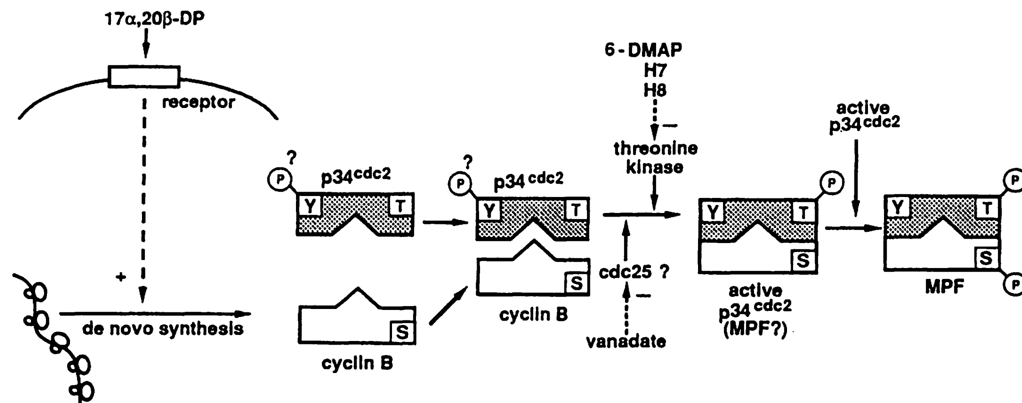

Fig. 2. A hypothesis for the formation and activation of MPF during fish oocyte maturation (see text for details).

that 17α, 20β-DP induces oocytes to synthesize cyclin B, which in turn activates preexisting $p34^{cdc2}$ through threonine phosphorylation of $p34^{cdc2}$ (Fig. 2).

In collaboration with Dr. T. Choi and others (University of Tokyo), we examined the changes in the phosphorylation state of $p34^{cdc2}$ and its histone H1 kinase activity in mouse oocytes and embryos. The results suggest that changes in phosphorylation states of $p34^{cdc2}$ triggered its activation at the first metaphase, but not inactivation and reactivation at the first and second metaphase, respectively. However, in the mitotic cell cycle, the activation of $p34^{cdc2}$ protein kinase may be triggered by its dephosphorylation.

2) Endocrine regulation of male germ cell development and maturation

We have identified two steroidal mediators of male germ cell development in salmonid fishes (11-ketotestosterone for spermatogenesis and 17α, 20β-DP for sperm maturation). A steroidogenic switch, from 11-ketotestosterone to 17α, 20β-DP, occurs in salmonid testes around the onset of final maturation. *In vitro* incubation studies using different testicular preparations have revealed that the site of 17α, 20β-DP production is in the sperm, but its production depends on the provision of precursor steroids by somatic cells. The site of 11-ketotestosterone production is in the testicular somatic cells.

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

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. More recently, in collaboration with Dr. T. Kobayashi (Niigata University), we identified 17α, 20α-dihydroxy-4-pregnen-3-one as the spermiation-inducing hormone in a frog (*Rana nigromaculata*). It is well established that gonadotropins and androgens are the two major hormones involved in the regulation of vertebrate spermatogenesis. Thus, our findings demonstrate, for the first time, the distinct role of progestational steroids in vertebrate spermatogenesis. Progestational steroids may represent endocrine effects that have been heretofore unrecognized among the more classical hormonal regulators of male germ cell formation in vertebrates.

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

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Division of Cell Differentiation

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Members of the Division have been involved in two well associated projects. One, which was initiated in 1968, is to understand how a special tissue like the silk gland of *Bombyx mori* is differentiated along the developmental programs and results in transcribing a specific set of genes like the silk fibroin, fibroin L-chain, P_{25}, sericin-1, and sericin-2 genes. The other initiated at the time when the Division was established in 1978 is concerned with how the body plan of the silkworm is controlled and how the homeotic genes regulate a set of target (structural) genes in specifying the segment identities.

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

Development of the silk gland is initiated in the labial segment at the embryonic stage 18 or 19, 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, Bm in,* and others are expressed in the larval silk gland. The *Bm Antp* was mapped on the *Nc* mutation locus which had been mapped about 1.4 cM apart from the *E* loci, the *Bombyx* counterpart of the BX-C. The developmental morphology of the wild type and the *Nc/Nc* embryos has been studied, and abnormal features of the *Nc/Nc* embryos in the gnathocephalon and thorax regions have been recognized (see the following section). Among the features, a severe disturbance of the silk gland development in the *Nc/Nc* embryos (Fig. 1) has attracted our attention. This observation triggers our interest to find target genes of the *Bm Antp* in the silk gland development.

Since the *Bm en* and *Bn in* are expressed in the middle portion of the silk gland, we are interested to know which portion of the silk gland expresses a *Bombyx* homolog of *Wnt-1. Bm Wnt-1* cDNA has been obtained from an embryonic cDNA library and identified by sequence comparison with the *Wnt-1.*

Since PCR studies indicated that Bombyx homologs of *cad, lab, Dfd, pb,* and others are expressed in the larval posterior silk gland, cDNA cloning has been carried out, and *Bm cad* cDNA has been obtained from an embryonic library and identified by sequencing.

We have been studying the molecular mechanisms that control the differential transcription of the fibroin and sericin-1 genes in the silk gland. Several factors (SGF-1 to -4, FBF-A1, and OBF-1 to -5) are proposed to bind and control the silk genes.

The SGF-2 is specific to the posterior silk gland, is presumed to be a key transcription factor binding to the upstream C, D, and E regions (enhancer I) of the fibroin gene, and is also presumed to be

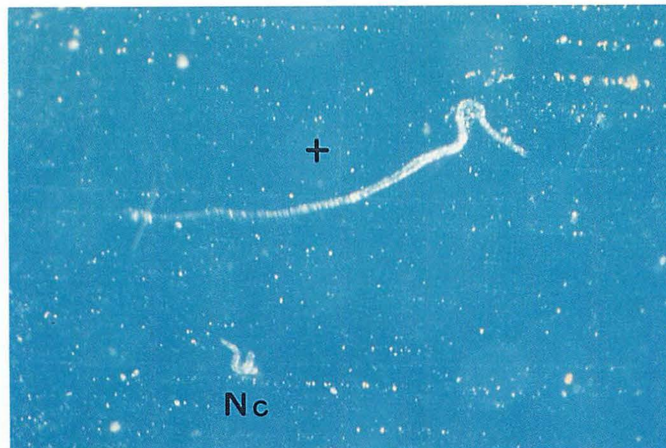

Fig. 1. Silk glands from the wild type (+) and the *Nc/Nc* (Nc) embryos at the stage 24.

composed of the FF1 and FF2. The FF1 has a DNA binding ability and is specific to the posterior silk gland while non-functional FF1 molecules are present ubiquitously. The FF2 is present ubiquitously, and has no DNA binding ability but stabilizes the FF1 binding.

Fibroin gene enhancer II (+156/+454) in the intron is composed of 6 octamer-like elements. Footprint experiments and gel shift assays indicated that these elements are targets of cell type specific DNA binding proteins (OBF-1 to -5). The OBF-1 is present in the posterior silk gland and binds to the elements at around +220 and +290. The OBF-2 and -3 are present in the middle silk gland and bind to the elements at around +290 and +420, respectively. The OBF-4 is present in both the posterior and the middle silk glands and binds to the element at +370. The OBF-5 is present in a cultured cell line derived from embryos but not in the silk gland, and binds to the element at +250. An expression cDNA library from the posterior silk gland was screened with oligonucleotide probes covering the elements at +220 and +290, and a candidate clone for the OBF-1 was obtained. The cDNA encodes a zinc-finger type protein, ZP-P1, having 3 finger domains. The ZP-P1 mRNA was shown to be expressed preferentially in the posterior silk gland.

Previously we have shown that a POU-domain containing protein (POU-M1, a *Bombyx* homolog of *Cf1-a*) is probably identical with the SGF-3 which is proposed to be a key transcription factor binding to the SC element of the sericin-1 gene. For the definite identification we produced antibodies against oligopeptides in the POU-M1 protein sequence. Two antibodies, one against the N-terminal peptide and the other against the C-terminal peptide, were found interacting with the POU-M1 protein as well as the SGF-3. Both antibodies gave a specific hypershift of the POU-M1/SC probe complex or the SGF-3/SC probe complex in gel shift assays demonstrating that the POU-M1 is identical with the SGF-3.

The SGF-3 gene has been cloned and proteins binding to its presumed pro-

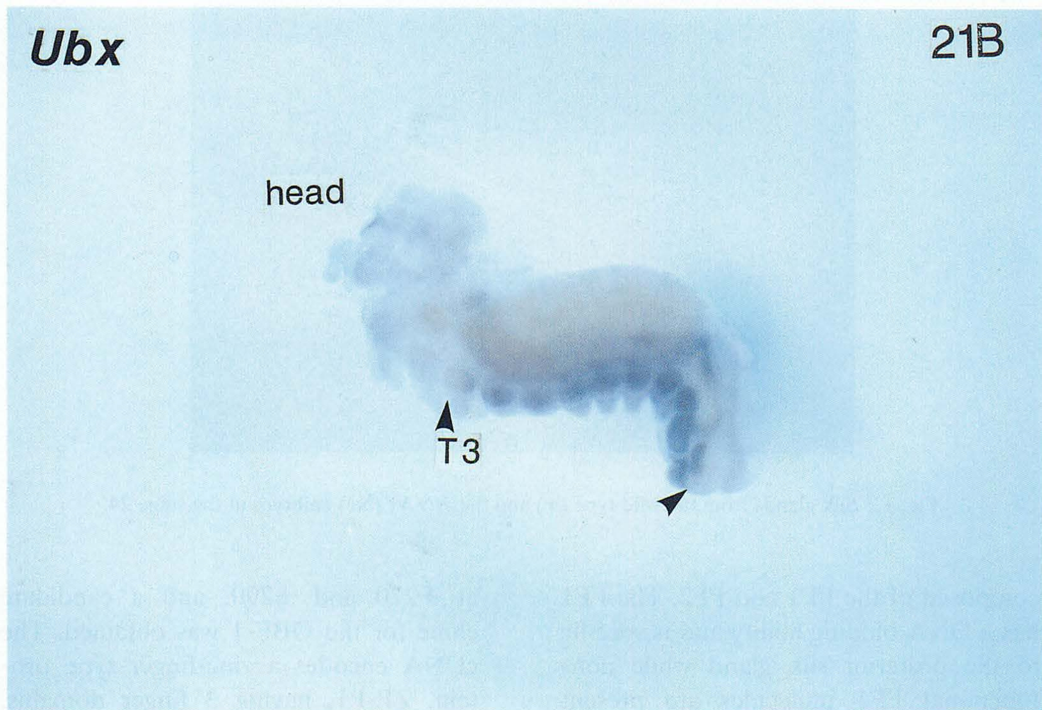

Fig. 2. *In situ* hybridization with *Bm Ubx* probe at the stage 21B.

moter region have been analyzed by footprints and gel shift assays. Cell-free transcription experiments of a series of deletion mutants of the SGF-3 gene have indicated the presence of negative element(s) as well as positive element(s).

Through the characterization of these factors and their genes we hope to decipher the control mechanisms at the upper hierarchy as well as at the level of the fibroin and sericin-1 genes transcription.

Genes involved in the *Bombyx* body plan

When the *Bombyx* body plan is compared with the *Drosophila*, some features are common but unique features that are common to many insets, are also evident, like ways of blastokinesis and dorsal closure, development of the silk gland in the labial segment, and formation of the legs in the thorax and abdomen in the embryonic stages. To study these problems we have been analyzing the natures of the genes that are presumed to play important roles in *Bombyx* development. In the past we cloned and identified *Bombyx* homologs of *Antp*, *Scr*, *Ubx*, *abd-A*, *Abd-B*, *en*, *in*, and *Cfl-a*. In 1991 *Bombyx* homologs of *cad* and *Wnt-1* have been added to the list. Using these clones as probes we have analyzed the natures of some mutants and the expressions of these genes in the wild type embryos.

By Northern blotting and *in situ* hybridization the expressions of *Bm Ubx*, *Bm abd-A*, and *Bm Abd-B* have been analyzed. At and after 1 day of embryonic development 5.4 kb transcript of the *Bm Ubx* became detectable, which

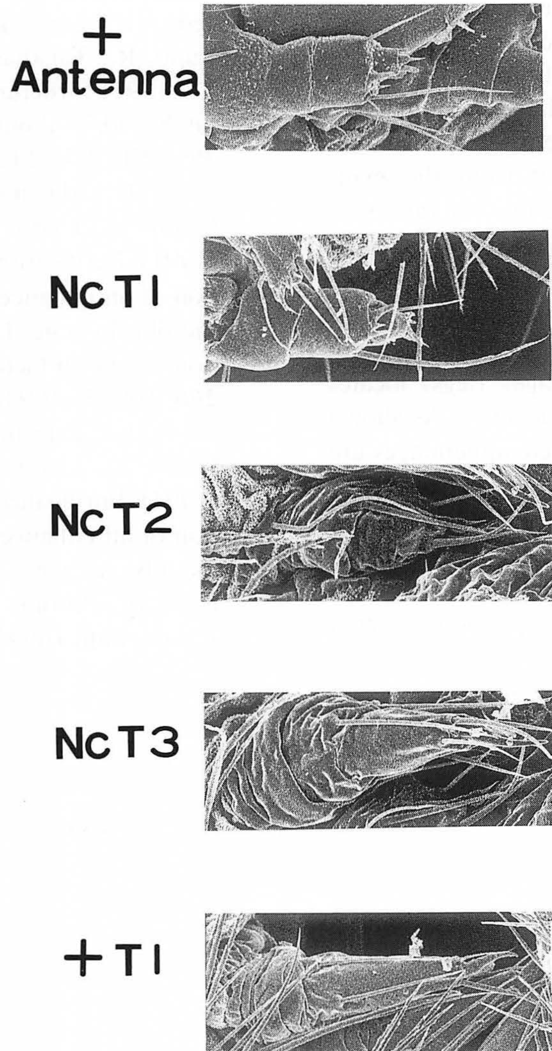

Fig. 3. Morphological appearance of the appendages of the antenna, the prothorax (T1), the mesothorax (T2), and the metathorax (T3) in the wild type (+) and the *Nc/Nc* (Nc) embryos.

was followed by a strong expression of 5.6 kb transcript peaking at 3 days. Another component of 1.2 kb was expressed at and after 6 days. Also peaking at 3 days 5.3 kb transcript from the *Bm abd-A* and 3.2 kb transcript from the *Bm Abd-B* were expressed. An example of *in situ* hybrifization for *Bm Ubx* expression at 3 days of development is shown in Fig. 2. This stage corresponds to about the stage 21 when the katatrepsis and the dorsal closure are actively taking place. As shown in Fig. 2 a strong expression is localized from the metathorax to the telson at the ventral side. Expressions of the *Bm abd-A* and the *Bm-Abd-B* were also detected strongly at the stage 21; the *Bm abd-A* from the A1 to the A6 at the ventral side, and the *Bm Abd-B* in the whole

area of the A4 to the telson.

Morphological appearance of the development of the wild type and the *Nc/Nc* mutant embryos has been analyzed. Abnormal development of the mutant embryos was detected from the mandibular segment to the mesothoracic segment. Especially, the prothorax and the mesothorax are fused, and the pair of prothoracic appendages (legs) comes closer to the mid-line and the pair of mesothoracic appendages (legs) locates widely apart from each other. As shown in Fig. 3 the prothoracic appendages are transformed into antenna-like structures, and the mesothoracic appendages partly lose their thoracic leg characteristics. Other abnormality in the labial segment was described in the previous section (see Fig. 1).

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DIVISION OF MORPHOGENESIS

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The lost tissues in the eye of the newt are completely regenerated through the transdifferentiation process in which once-specialized cells lose their phenotypes and redifferentiate into other cell types. The main source of the regeneration in the urodele eye is the pigmented epithelium. The in vitro experiments have revealed that the lens transdifferentiation is observed in the cultures of the pigmented epithelial cells (PECs) not only from urodeles but also from other vertebrates including birds and human. So the transdifferentiation of the PEC is a conserved phenomenon among vertebrates, whether or not the lost lens is regenerated in the animal. We are studying in order to answer the questions why the PECs can transdifferentiate into the lens cells and why the pigmented epithelia of the species other than the newt never regenerate the lost lens in vivo. Itoh and Eguchi previously established a unique culture system for the lens transdifferentiation using the PECs from the chicken embryo, in which the differentiated states of the cells are well regulated by the modification of the medium. Thus, molecular biological approaches have become possible using cultured chicken PECs. The phenomena related to the lens regeneration in vivo are also studied using newts. In addition, we are trying to establish a transgenic system of birds in which the functions of individual genes can be examined directly. The investigations and the results achieved in 1991 by the members of this division are as follows.

1. The extracellular factors modulating the lens transdifferentiation of the PECs in vitro.

Itoh and Eguchi have revealed that the lens transdifferentiation from the chick embryo PECs is greatly enhanced by phenylthiourea and testicular hyaluronidase in the medium. Recently we revealed that the main activity of the enhancement in the crude hyaluronidase preparation is not due to the hyaluronidase itself but to a trace of basic fibroblast growth factors (bFGF) contaminating the preparation. In fact the pure bFGF enhanced the lens transdifferentiation of the PECs in the presence of phenylthiourea. Other groups reported that the bFGF also enhanced the transdifferentiation of the pigmented epithelium into the neural retina. Now we are investigating the molecular changes in the PECs in response to the stimulus by the bFGF.

Another growth factor which affects the differentiated states of the cultured PECs is the transforming growth factor-β (TGF-β). As will be described in the next section, a gene coding for the TGF-β binding protein is transcriptionally regulated in the process of the lens transdifferentiation, suggesting an important role of the TGF-β in the transdifferentiation. The effect of the TGF-β on the pigmented epithelial cells was examined in vitro. The PECs maintain the monolayer cell sheet and exhibit a contact-

inhibition of growth in a basic culture medium, but dramatically change their morphology in the presence of the TGF-β. They continue to grow even after the culture has attained the confluency, produce a large amount of extracellular matrix (ECM) components, and result into multi-cell layers. We revealed that the TGF-β have a strong effect in changing the stable PECs into a quite different state of cells. However, the relationship between thus attained cells and the cells obtained through dedifferentiation by the effect of dFGF is yet to be clarified. Whether the TGF-β plays a crucial role in the regeneration process in vivo should be an important question.

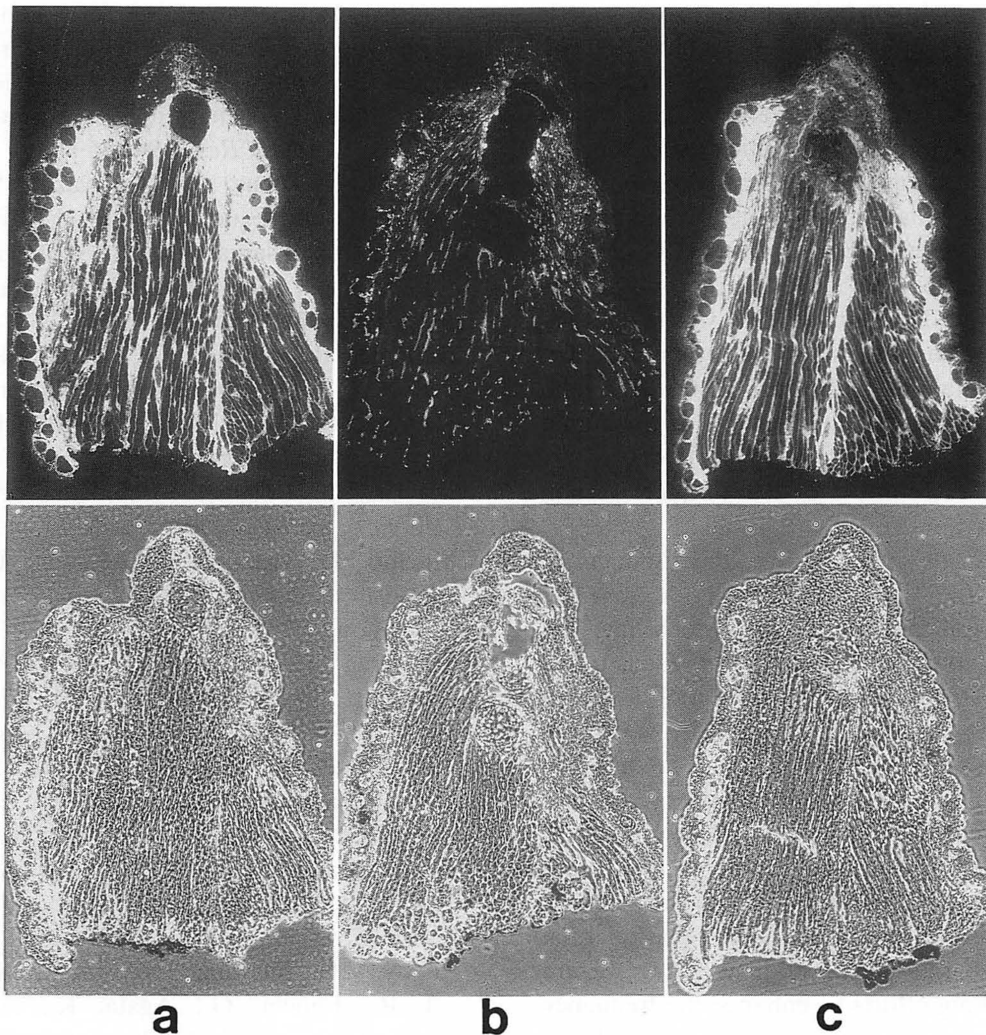
The ECM components also have important roles to control the differentiated states of the PECs. It is known that both the bFGF and the TGF-β function interacting with the components of the ECMs in many experimental systems. Immunocytochemical studies have revealed that the major components of the ECM in the PEC culture are once degraded dramatically and reorganized into a different structure when the TGF-β is added to the medium. The specific adhesion of the PECs to some ECM components was demonstrated in a serum-free culture. (Kosaka et al. 1992, Exp. Eye Res., in press).

2. Gene regulation in the process of the lens transdifferentiation.

To investigate the regulatory mechanisms of the gene expression during the lens transdifferentiation, various cDNA clones encoding the lens-specific and the PEC-specific proteins have been isolated and characterized. Fr example, the cDNA clones coding for α- and β-crystallins are used to characterize the cells changing to the lens cells. The cDNA clones coding for a melanosomal matrix protein and a tyrosinase were characterized to specify the PEC phenotype.

Two cDNA clones were already identified by a differential screening in which the mRNA levels are compared between the PEC and the dedifferentiated PEC. One cDNA clone termed pP64 encodes a polypeptide closely related to the TGF-β binding protein identified in mammals. Another is termed pP344 and codes for a polypeptide which has the common sequence among the members of the serine-protease inhibitor family. The gene of the former clone is transcribed to the two species of the messengers whose sizes are 6.0kb and 5.0kb in the PEC, but in the dedifferentiated state only the 6.0kb one is transcribed. The translated product from the 5.0kb messenger lacks the N-terminal region of the 6.0kb one. Phenotypic changes of the PECs by the TGF-β as mentioned above leads us to an assumption that the products of the pP64 gene control the differentiated states of the PECs by modulating the action of the TGF-β. We should reveal whether the two species of the pP64 products have different effects on the action of the TGF-β. The product of the latter gene, pP344, has an activity of the trypsin-inhibitor, and expected to inhibit some protease in tissues. The pP344 gene is expressed only by the fully-differentiated PEC. So the expression of the pP344 gene may stabilize the differentiated state of the PEC by protecting the cells from the action of proteases.

We revealed that the chicken connexin 43 is the major component of the gap junction in the PEC. Now the cDNA clone and the specific antibodies are available to investigate the molecular change in the cell-cell communication system during the lens transdifferentiation.



Transient disappearance of the 2NI36 antigens during limb regeneration. Longitudinal sections of the stumps of the amputated forelimbs of the newt 3(a), 8(b) and 14 days(c) after the amputation. Upper row shows indirect immunofluorescent stains and lower shows the corresponding phase-contrast images.

3. An antigen related to the process of the lens regeneration in newt

We have already reported that an antigen recognized by a monoclonal antibody, 2NI36 disappear in the dorsal region of the newt iris before the dedifferentiation of the region becomes obvious after the lentectomy, and that a piece of the ventral iris which never regenerate a lens did regenerate a new lens when it was treated with the monoclonal antibody. The spatial and temporal distribution of the antigen were investigated and the antigen molecule was biochemically characterized. The 2NI36 antigen is widely distributed in various organs of the adult newt, especially in the tissues derived from the mesoderm (Imokawa et al., 1992, Int. J. Dev. Biol., in press). But the antigen was never observed in

the embryo before the organogenesis was completed (Imokawa and Eguchi, 1992, Int. J. Dev. Biol., in press). The antigen molecules were purified by the affinity-chromatographies using the lectin and the antibody, and a group of glycoproteins were identified as the antigen molecules. We assume that the antigen molecues may serve for the stabilization of the differentiated cell states. This assumption was supported by the fact that the staining with the 2NI36 in the limb also becomes undetectable at the stump after an amputation (Figure).

4. Development of a transgenic system in avian.

As described above, the products of the pP64 gene and the pP344 gene are the candidate molecules which have the key roles in the process of the lens transdifferentiation. The function of the genes must be examined in vivo by manipulation of the genomes of the animal. We have already developed an experimental system to introduce an exogenous DNA into a fertilized chicken egg and also succeeded in the production of a chimera by injecting the dissociated blastodermal cells into the blastoderm. Now we are making efforts to enhance the frequency of the integration of the introduced DFNAs into the genome. The DNA injection into a fertilized egg and the production of a blastoderm chimera are succeeded also in the quail which have some advantages in experimental procedures.

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DIVISION OF DEVELOPMENTAL BIOLOGY (ADJUNCT)

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Maki Minami (from Kyoto University)
Takahiro Morio (from Kyoto University)

This division is devoted to the study of the regulatory mechanisms of transcription of genes involved in cell proliferation and differentiation in higher plants and a cellular slime mold. We have carried out the following two projects.

(1) Transcriptional regulation of cell cycle-dependent genes in plants by transcription factors.

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 unknown. We have chosen wheat histone genes as a model system to elucidate the regulatory mechanisms of S phase-specific transcription in higher plants. Until now, three *cis*-acting positive elements, the hexameric (ACGTCA), octameric (CGCGGATC), and nonameric (CATCCAACG) motifs, have been identified in the proximal promoter region of the wheat histone H3 gene (TH012). *In vivo* transcription experiments with deletion and site-directed mutations of the H3 gene have suggested that some of these motifs may contribute to the S phase-specific transcription of this gene. These motifs are also shown to be conserved in the regulatory regions of other plant histone genes. We have identified three nuclear DNA-binding proteins, HBP-1a, HBP-1b and HBP-2, as candidates of *tarns*-acting factors. The former two proteins specifically bind to the hexameric motif and the latter one to the nonameric motif. The cDNA clones encoding HBP-1a, HBP-1b and HBP-2 have been isolated from a wheat cDNA library in λgt11 using South-western screening method. Sequence analyses of the cDNAs and DNA-binding experiments with bacterially expressed HBP-1a and HBP-1b have revealed that the both proteins have the characteristic properties of the bZIP-type transcription factors and HBP-2 has two zinc-finger structures. In wheat seedlings, there are parallel relationships among the levels of transcripts from HBP-1a, HBP-1b, and four histone subtype (H2A, H2B, H3 and H4) genes. We also isolated of other three cDNA clones which are similar to HBP-1a or HBP-1b cDNA. Analyses of the cDNA clones revealed that HBP-1a and HBP-1b are classified into the HBP-1a and HBP-1b protein subfamilies. It is demonstrated that the hexameric motifs are located in the promoter regions of genes other than histone genes, and that the hexamer-specific DNA binding proteins are present in some higher plants. We isolated a cDNA clone for the counterpart of HBP-1b from *Arabidopsis thaliana*.

To analyze the regulation of HBP-1a and HBP-1b genes, we have isolated their cognate genomic clones. Structural analyses of the clones and DNA-binding assays using baterially expressed HBP-1a and HBP-1b have suggested the possibility of the autoregulation in the expression of genes by HBP-1a and HBP-1b themselves.

To know how HBP-1a, HBP-1b and HBP-2 interact with a basic transcription machinery, we isolated a cDNA clone

encoding a TATA box-binding protein (TFIID) from the wheat seedling cDNA library. We hope this will help us to make a reconstitution system of *in vitro* transcription in higher plants.

(2) Regulation of cell-type specific gene expression during development of *Dictyostelium discoideum.*

After vegetative growth of *D. discoideum*, cells aggregate to form a multicellular cell mass and differentiate into two kinds of cells, prespore and prestalk cells. To elucidate the regulatory mechanisms of cell differentiation during development, we have isolated several genes which were specifically transcribed in prespore and prestalk cells, respectively. Recently, we have concentrated our effort to understand the regulatory mechanisms of expression of two prespore-specific genes (SP96 and Dp87). Promoter region of each gene has been shown to include the sufficient information for prespore-specific transcription and we have identified one of the *cis*-acting regulatory regions of both genes and a short homologous C-rich element 'ACACCCA' was found in these *cis*-acting regions. We have found a nuclear factor by gel retardation assays, which specifically bound to these *cis*-acting regions. The following results and developments have been achieved in 1991.

To obtain detail information for the Dp87 gene transcription, some parts of the promoter region of Dp87 gene were deleted or replaced by heterologous DNA fragments, jointed to the *E. coli* β-gal gene, then introduced into *D. discoideum* cells and the expression of the chimeric gene in transformed cells was examined by a histochemical staining of β-galactosidase. The results were summarized into three parts: a) The sequence between −664 and −432 contained an element which increased the transcriptional activity by 10-fold. b) In addition to the region between −447 and −356, which has been identified previously, other three regions from −351 to −231, from −231 to −159 and from −159 to −64 also included the *cis*-acting elements essential for the transcription. c) All these *cis*-acting regions except for the region between −432 and −356 were functional when they were jointed to the downstream sequence of Dp87 gene, and were not functional when they were jointed to the heterologous basal promoter of the actin 15 gene of *D. discoideum*. However, when the region from −432 to −356 was jointed to the actin basal promoter, it increased the transcriptional activity. These results indicate that the transcription of Dp87 gene is regulated by the combination of some independent *cis*-acting elements.

As the chimeric gene including the 5' upstream region up to −664bp and the β-gal was expressed only in prespore cells, histochemical detection of cells expressing β-galactosidase showed that prespore cells were randomly appeared at early aggregating stage on an agar plate. It was believed that the prespore cell differentiation was not induced under submerged condition. When transformed cells were incubated in the liquid condition with 1mM of cAMP, some cells were expressed the β-galactosidase which indicated to differentiate to prespore cells. The difference between our result and previous observations was due to the different markers; ours is the earliest marker and the other are late markers. Our result suggests that there are two steps of prespore cell differentiation, the first step is induced in the liquid condition but the next step is not. This new *in vitro* method is very useful to

study the cell-cell interaction or morphogen(s) which required for the prespore cell differentiation.

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DEPARTMENT OF REGULATION BIOLOGY

Chairman: Norio Murata

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

DIVISION OF MOLECULAR NEUROBIOLOGY

Professor: Masaharu Noda
Technical Staff: Sonoko Ohsawa

The division just started its new projects from September 1991. This division deals with developmental neurobiology of the vertebrate central nervous system. The project is aiming to elucidate the mechanisms that govern the development of topographic projections in the central nervous system. Main subjects are the molecular analysis of the neuronal migration in corticogenesis and the formation of neural connections in the retinotectal system.

DIVISION OF CELLULAR REGULATION

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The research effort of this division is directed toward the understanding of tolerance and adaptation of higher plants and microbial plants to high and low temperature as well as to high-salt stress. In particular, our research focuses on the molecular mechanism by which plants adapt or tolerate such stress conditions.

1. Gene-engineered alteration of the fatty-acid unsaturation modifies the chilling sensitivity of higher plants.

The chilling sensitivity of plants is correlated to the extent of fatty-acid unsaturation of phosphatidylglycerol in chloroplast membranes. Plants with high contents of unsaturated fatty acids in phosphatidylglycerol are resistant to chilling, whereas species with low contents of unsaturated fatty acids in phosphatidylglycerol are sensitive to chilling. 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 its role in the low-temperature sensitivity of higher plants, cDNAs encoding the precursor protein of this enzyme have been isolated from a **low-temperature-sensitive plant**, squash, and from a **low-temperature-resistant plant**, *Arabidopsis*. Tobacco plants were transformed with Ti plasmids that were constructed with the squash and *Arabidopsis* cDNAs for acyltransferases under control of the 35S CaM promoter and the NOS terminator. Analysis of the lipids of these transformants demonstrated that only phosphatidylglycerol was significantly altered in its fatty-acid unsaturation by transformation with the cDNA for glycerol-3-phosphate acyltransferase. When squash cDNA was introduced, the content of unsaturated fatty acids in the phosphatidylglycerol was decreased. In contrast, the introduction of the *Arabidopsis* cDNA caused a small but significant increase in these fatty acids.

Chilling sensitivity of transformed plants was assayed by two methods: Inactivation of photosynthesis of excised leaves and visual damage of whole plants at chilling temperature (Figure 1). With both methods, the wild-type tobacco plants showed some sensitivity to chilling temperature. Upon introduction of the squash cDNA for glycerol-3-phosphate acyltransferase, this sensitivity was markedly increased. In contrast, the

(a) Control (transgenic tobacco with vector plasmids) (b) Transgenic tobacco with squash cDNA (c) Transgenic tobacco with *Arabidopsis* cDNA

Fig. 1. The low temperature induced symptom of wild-type and transgenic tobacco plants. (a) Control (transformant with vector plasmids). (b) Transformant with the cDNA for glycerol-3-phosphate acyltransferase from squash. (c) Transformant with cDNA for glycerol-3-phosphate acyltransferase from *Arabidopsis.*

introduction of the *Arabidopsis* cDNA for glycerol-3-phosphate acyltransferase decreased the chilling sensitivity. These remarkable differences in the chilling sensitivity were well correlated with the extents of fatty-acid unsaturation of phosphatidylglycerol.

These results, therefore, demonstrate that it is possible to regulate the chilling sensitivity of higher plants solely by the gene-engineered alteration of fatty-acid unsaturation of phosphatidylglycerol with introduction of an appropriate acyltransferase. Other factors could also contribute to chilling sensitivity, but these studies provide the first direct evidence that the fatty-acid unsaturation of phosphatidylglycerol is a major contributor. Studies on photosynthetic characteristics of the transformants are in progress.

2. The fatty-acid unsaturation affects thermal properties of cyanobacterial photosynthesis.

Alteration of the fatty-acid unsaturation of membrane lipids by genetic manipulation could provide an important information in our understanding of the role of fatty-acid unsaturation in plant membranes. By combination of techniques of mutation and gene disruption we could produce three strains of the cyanobacterium, *Synechocystis* PCC-6803, having distinctly different levels of fatty-acid unsaturation of membrane lipids. The wild-type strain contains mono-, di- and triunsaturated fatty acids. The desaturation mutant (termed Fad6), defective in fatty-acid desaturation at the delta-6 position, contains mono- and diunsaturated fatty acids. The transformant of Fad6 (termed Fad6/desA::Km^r in which the gene (desA) for the desaturation at the delta-12 position of fatty acids has been disrupted, contains only a monounsaturated fatty acid.

The effect of fatty-acid unsaturation on thermal properties of photosynthetic activities was investigated with these

three strains. It was found that temperature profiles of the photosynthetic activity at room temperature and of the inactivation of photosynthesis at high temperature were not affected by the changes in fatty-acid unsaturation. In contrast, exposure to low temperature, in the light, produced distinctly different degrees of inactivation of photosynthesis among the strains. Fad6/desA::Km^r was much more damaged than the wild type and Fad6. This observation suggests that the presence of the second double bond in the fatty acids is essential for this cyanobacterium to tolerate the low temperature.

3. Glycinebetaine stabilizes the oxygen-evolving complex of higher plants and photosynthetic microbes.

The protective effect of glycinebetaine on the photosystem 2 activity was investigated. Glycinebetaine is a nontoxic osmolyte found in halophilic higher plants and microbial plants. Spinach photosystem 2 complex loses its oxygen-evolving activity during the incubation at room temperature for hours. The loss of the activity was greatly prevented by the presence of glycinebetaine at high concentration such as 1 M. It was found that glycinebetaine protects the Mn cluster, which serves an essential role in oxygen evolution.

We have also discovered that the presence of glycinebetaine in the medium allows the isolation of cyanobacterial thylakoid membranes that are stable and highly active in the electron-transport and phosphorylation. It must be remarked that the preparation of stable and highly active thylakoid membranes from cyanobacteria has been a goal that had been pursued for a long time in the field of cyanobacteriology.

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Division of Biological Regulation (Adjunct)

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Growth and development of higher plants are regulated by plant hormones, but they are also greatly influenced by changes in environmental conditions, that provide physical signals to plant cells. In many cases, the environmental signals alter endogenous hormonal balance by changing the rates of biosynthesis, inactivation and transport of plant hormones. To understand the regulatory mechanisms of plant growth as affected by the environmental signals, research in this division has been conducted in three topics; (1) development of the sink activities in growing tissues, (2) mechanisms of gene expression regulated by plant hormones and environmental stress, and (3) acquisition of thermotolerance by non-lethal high temperature. Great progresses have been achieved on the topics 1 and 2 in 1991.

(1) Development of the sink enzymes in rapidly growing tissues. Rapid growth of stem and leaves of seedlings shortly after seed germination is nutritionally supported by sucrose influx from storage tissues of the seed. To maintain the rapid growth, sucrose must be continuously metabolized in growing organs. We have found that dramatic changes of sucrose-metabolizing enzymes, invertase and sucrose synthase, occurred in growing stems and leaves and the changes are correlated with the growth rate of individual organs. The regulatory mechanism underlying in inductive synthesis of invertase and sucrose synthase was studied at the molecular level. Both invertase and sucrose synthase were purified from mung bean seedlings. We found two forms of intracellular acid invertase in mung bean seedlings; one is a heterodimer of 30 and 38 kDa polypeptides and another is a monomer of 70 kDa. The heterodimeric form is not an artifactual product of the monomeric form produced during or after extraction. The relative amounts of the two forms in cells changed during growth of seedlings (Figure 1). cDNAs for the heterodimeric form of acid invertase and sucrose synthase have been cloned and their nucleotide sequences were determined. The heterodimeric acid invertase was found to be synthesized as a single polypeptide containing a leader sequence of 101 amino acids followed by the 30 kDa and 38 kDa portions. No mRNAs for acid invertase and sucrose synthase were found in the embryonal axes in seeds, but the transcripts for the two genes accumulated to the maximal levels within 15 hours of germination, when the enzymatic activities were still negligible (Arai et al. (1992) Plant Cell Physiol., in press). The levels of transcripts were maintained for 3 days, during which period seedlings grew rapidly and both enzymatic activities increased markedly. Presence of mechanisms by which the transcripts are maintained in translationally inactive states is proposed.

(2) Mechanisms of gene expression regulated by plant hormones and tissue wounding. Auxin induces many physiological processes of plants, including cell growth and division. Before such physiological processes are induced by auxin, the synthesis of particular set of proteins is initiated in the affected tissue. Using mung bean stem sections, we have cloned 5 different cDNAs for the auxin-regulated genes by differential screening

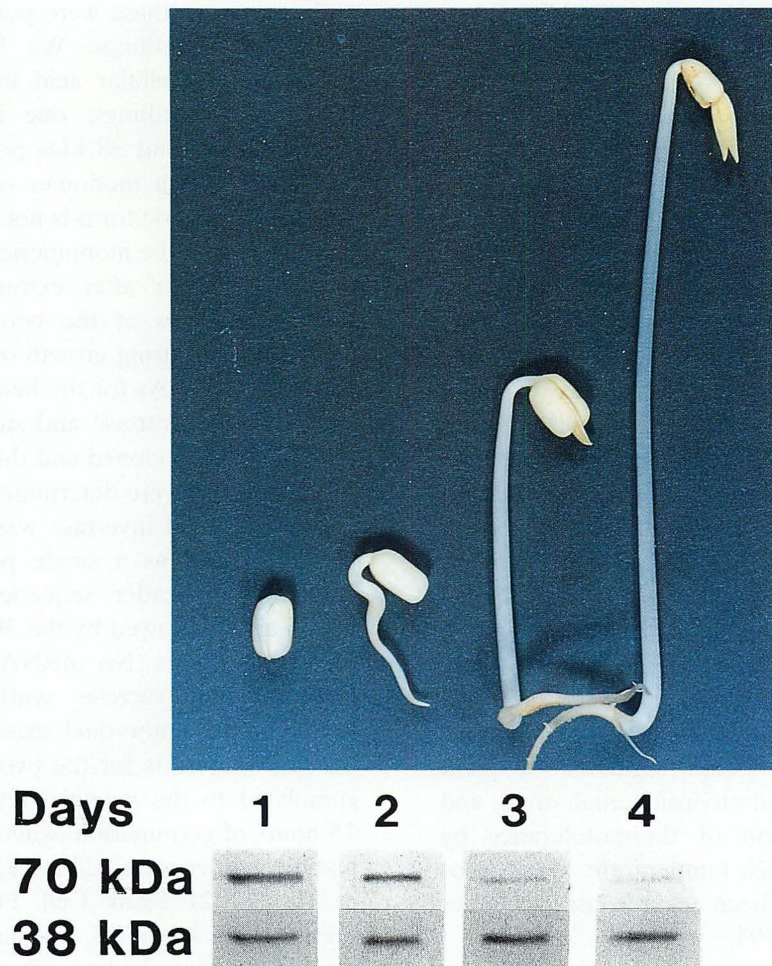

Fig. 1.

(Yamamoto et al. (1992) Plant Cell Physiol., in press). Sequence determination revealed that two of them were new to the literature, and their characterization is under way.

Ethylene biosynthesis is variously regulated by other plant hormones and environmental stress, and in all cases, the regulatory step is formation of 1-aminocyclopropane-1-carboxylic acid (ACC) from S-adenosylmethionine catalyzed by ACC synthase. Thus, ACC synthase is induced by various stimuli. We have found that ACC synthases induced by auxin, wounding and fruit ripening are different molecules. We have cloned cDNAs for the wound- and auxin-induced enzymes from winter squash. Their nucleotide sequences are significantly different, and the expression of respective gene is regulated in a stimulus-specific manner. Tomato was found to contain three different genes for ACC synthases induced by wounding, ripening and auxin, and their genes are expressed differentially by types of stimuli (Figure

Fig. 2.

2; Mori, H. and Imaseki, H. unpublished). Comparative studies on the deduced primary structure of ACC synthases of the wound-, ripening- and auxin-type from different plant sources revealed that there are seven conserved regions among them. An effort to determine the specific sequences in the genes that regulate expression by the specific stimulus is being made.

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Division of Behavior and Neurobiology (Adjunct)

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*Shingo Yoshikawa**
*Kousuke Sumita**
*Ichirou Fujino**
*Norihiko Yamada**
*Yoshimi Ryou**
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*(*from Osaka University)*
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In our research division, we are studying the mechanisms of development, growth and differentiation of the mammalian nervous system at the molecular and cellular levels.

Our studies in last year mainly focused on the second messenger signalling within a cell. In the course of our studies on cerebellar ataxic mutant mice, a membrane glycoprotein P_{400} was found to be enriched in Purkinje cells from normal mice but reduced in Purkinje-cell-deficient mutant mice. The cDNA-cloning and functional expression of the P_{400} protein show that this protein is a receptor for inositol 1,4,5-trisphosphate ($InsP_3$), a second messenger that mediates Ca^{2+} release from intracellular stores such as the endoplasmic reticulum. The increase in the cytoplasmic Ca^{2+} content probably modulates the functions of various Ca^{2+}-associated proteins, such as calmodulin, Ca^{2+}/calmodulin dependent protein kinase, etc, leading to a variety of cellular responses, including metabolism, secretion, contraction, neural excitation and cellular proliferation. We demonstrated that the $InsP_3$ receptor binds to $InsP_3$ with the N-terminal region and forms a homotetrameric structure, exhibiting a Ca^{2+} channel activity in response to $InsP_3$ molecules. Our primary goal is to know how the $InsP_3$-mediated Ca^{2+} release is involved in the neuronal network system, especially in the cerebellar Purkinje cells. In addition, we have recently cloned a cDNA of the Drosophila $InsP_3$ receptor, which probably allows us to elucidate the functional importance of the $InsP_3$-mediated Ca^{2+} release in a wide variety of cell types.

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LABOLATORY OF GENE EXPRESSION AND REGULATION

Head: Yoshihiko Fujita

The Laboratory consists of 2 regular divisions and conducts research into regulatory mechanisms of gene expression in higher plants and animals.

Division I of Gene Expression and Regulation

Professor: Yoshiro Shimura
Associate Professor: Kiyotaka Okada
Research Associate: Hideaki Shiraishi
Post-doctoral Fellow: Masako K. Komaki
Graduate Students: Kazuhito Akama
Hiroyuki Okamoto
Koji Sakamoto
Azusa Yano
Toshiro Ito
(from Kyoto University)
Technical Staff: Hideko Nonaka

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 crucifer, *Arabidopsis thaliana*. This plant is called "botanical *Drosophila*", because it has some remarkable features, such as a small genome size (7×10^7 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 fragments are mapped on 5 chromosomes. Experimental techniques such as transformation, regeneration of transgenic plants, and gene tagging have been improved. Using this plant, we have isolated and characterized many mutants defective in flower development and morphogenesis or in root growth responsive to the environmental stimuli. We have also cloned several genes which would be related to the flower development or to the root growth response to the stimuli.

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.*, Development, 104, 195–203, 1988; Okada *et al.*, Cell Differ. Dev., 28, 27–38, 1989). The analysis of double mutants constructed from these single mutants revealed mostly additive phenotypes of the parental mutants. In some cases flowers with novel and highly complex structures were observed, indicating simultaneous expression and interaction of the two gene products (Komaki *et al.*, in preparation).

Floral bud formation on the inflorescence axis was studied using the *pin-formed* mutant (Okada *et al.*, 1991). In some cases this mutant forms no floral buds at the top of inflorescence axis. But in other cases flowers with no stamens and wide petals are formed. These phenotypes of the mutant are exactly the same with wild type plants cultured in

the presence of chemcal inhibitors on auxin polar transport. The polar transport activity and the amount of free IAA in the mutant inflorescence axis are decreased to about 10% of wild type. These observations suggest that normal level of the polar transport activity in the inflorescence axis is required in early developmental stages of floral-bud formation. In addition we measured the level of endogenous free auxin (indole acetic acid) in several parts of a Brassica plant (Ueda *et al.*, J. Plant Physiol. 137, 628–630; J. Ueda: Osaka Prefectural University).

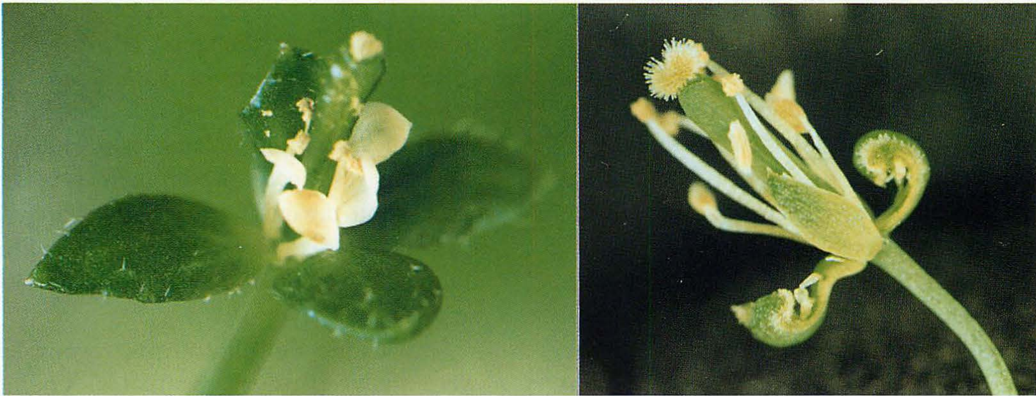
We have constructed a relatively simple model which may explain the action of the homeotic genes (Komaki *et al.*, in preparation). The model explains the flower structure of double mutants constructed by crossing a series of the known homeotic mutations.

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.*, in preparation). Attempts have also been made to isolate *Arabidopsis* cDNA and genomic DNA clones which have homologous sequences with DEF A, a homeotic gene of *Antirrhinum majus*. The homology between the DEF A and the coding region of the isolated homolog of *Arabidopsis* was about 60% (Okamoto *et al.*, in preparation). The map position of the isolated gene is being investigated to examine whether the gene corresponds to any known mutants of *A. thaliana*.

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).

Gravitropic and phototropic responses were analyzed using agar plates. Young seedlings grown on vertical agar plate have roots which grow srainght 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 side, roots grow to the opposite direction of incoming light. Mutants which show abnormal graviresponse or photoresponse were isolated (Okada *et al.*, in preparation).

In order to analyze the obstacle-escaping mechanisms, a similar system which provides a constant obstacle-touching stimulus to root tips was devised. Seedlings were grown on vertical agar plates. When the angle of the agar plate is shifted to 45 degrees, the root-tips bend downward under the influence of gravity and encounter the agar surface. Due to incapability of penetrating into the agar, this touching stimulus induces root tip rotation whose direction is periodically reversed. As the result of this response, roots form a wavy growth pattern on the inclined agar surface. Mutants which form abnormal wavy patterns were isolated. Genetic analysis has shown that at least six genes (*wav*1–6) are involved in this growth response (Okada & Shimura, Science, 250, 274–276, 1990). Several mutants also show abnormal gravitro-



Flowers of a homeotic mutant, *ap2–1*, of *Arabidopsis thaliana*. A single mutation of the *apetala2* gene causes temperature-dependent abnormalities on flowers. Sepals are converted to leaves, and petals carry pollen sacs at lower temperature (left). At higher temperature, sepals and petals are converted to carpels and stamens respectively (right).

pism 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-dimentional gel electrophoresis. Amounts of at least 11 spots were shown to be increased by the stimuli (Sakamoto *et al.*, in preparation).

Attempts are being made to idenify 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.*, in preparation).

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DIVISION OF GENE EXPRESSION AND REGULATION II

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

We are attempting to examine mechanisms involved in chromosomal replication, recombination and repair. Homologous recombination and replication, especially initiation and termination process, have been given special attention.

On procaryotic and eucaryotic genomes, there is a site, named termination (*ter*) site or fork blocking site, at which the DNA replication fork is severly impeded. We directed attention to the entire DNA replication termination system in *Escherichia coli* and clarified the following: To block progress of the DNA replication fork in *E. coli*, two factors are required, one factor is the *ter* sequence in the *E. coli* system and the consensus 22 bp sequence is 5′-(A/T)(G/T)TAGTTACAACAPy(A/T/C)C-(A/T)(A/T)(A/T)(A/T)(A/T)(A/T)-3′. The sequence is represented by (◣) and has activity which inhibits travel of the replication fork in a specific direction. In the above orientation, only the fork, traveling from the right, is inhibited. The six *ter* sites in the *E. coli* genome, are arranged as shown in Figure 1. The *ter*-binding protein (Ter protein or TBP), another factor essential for termination reaction, is coded by a gene called *tau* (*tus*), the map position of which is at 35.5 min on the *E. coli* linkage map. At the promoter region of this gene locates one of the five *Ter* sites, *TerB*. Ter protein, with a molecular mass of 36k daltons, is a basic protein and binds specifically to the *ter* sequence. Using an *in vitro oriC* reconstructed system, the Ter protein dependent termination reaction can occur *in vitro* at the *ter* site, in a polar fashion, on the genome of the *oriC-terC* chimera plasmid. Ter protein bound at the *ter* sequence blocks the replication fork by impeding the action of helicase, with polarity *in vitro*.

The following progress was made by members of this division in 1991.

(1) *E. coli ter*-Ter protein complex can function in eucaryotic DNA replication systems *in vitro* (Hidaka *et al.* (1992) *J. Biol. Chem.* in press).

We found that the *E. coli ter*-TBP complex impedes action of the three *E. coli* helicases, DnaB, Rep and UvrD proteins. To generalize the intrinsic nature of the orientated *ter*-TBP complex against eucaryotic helicases, we tested the potential of the complex to inhibit the action of two helicases, Simian Virus 40 (SV40) large tumor (T) antigen, and Helicase B, derived from SV40 and mouse FM3A cells, respectively. The complex impedes the unwinding of the tested helicases in a specific orientation, with the same polarity observed in case of blockage of a replication fork, and as a result, there was a block of SV40 DNA replication in both crude and purified enzyme systems, *in vitro*. As the specificity in polarity of inhibition extends to heterologous systems, there may be common structure/mechanism features in helicases.

(2) Structural analysis of eucaryotic replication fork blocking site (Kobayashi *et al.* (1992) *Mol. Gen Genet.* in press).

In addition to procaryotes, eucaryotic (yeast, higher plants and humans) cells were found to have the DNA replication fork blocking site. All these sites heretofore identified are located at the region within the rRNA gene cluster and the blocking activity exhibits polarity. To compare eucaryotic and procaryotic

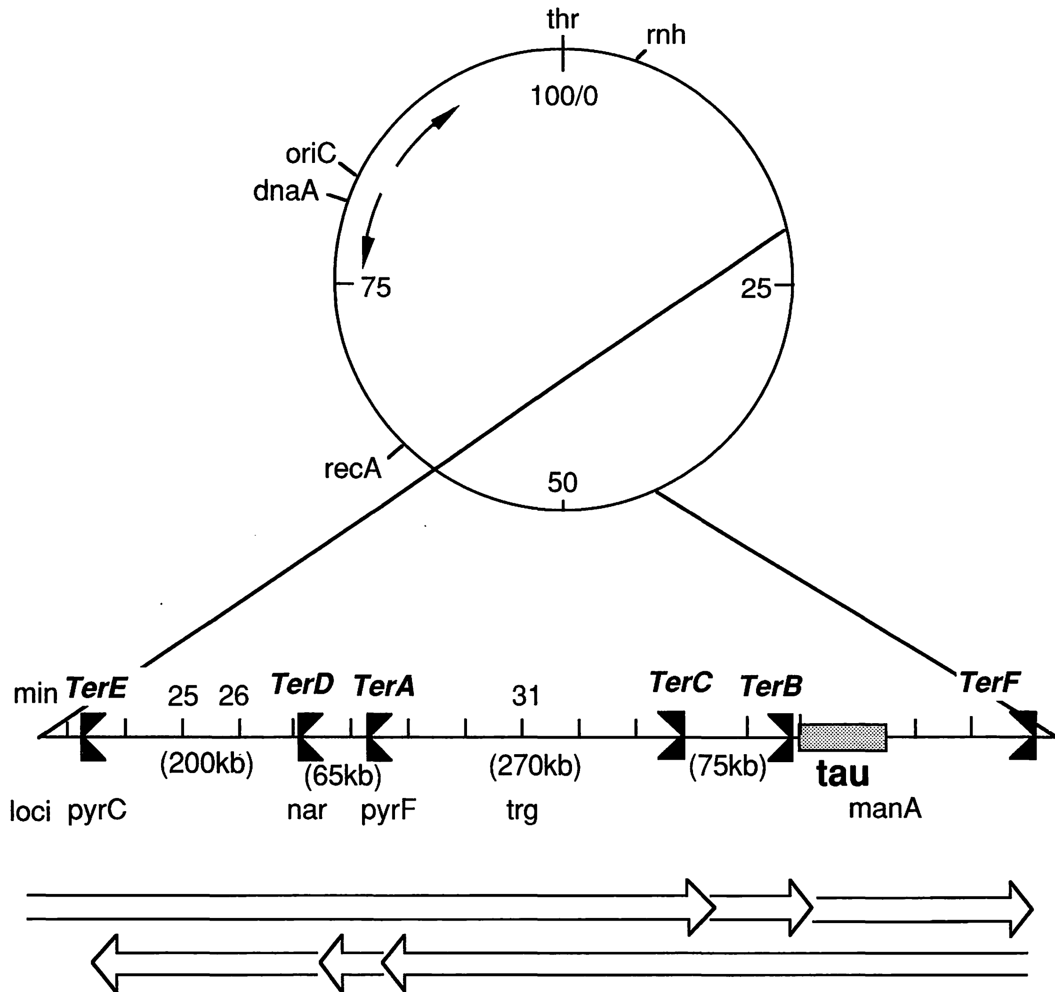

Fig. 1. Arrangement of DNA replication terminus (*Ter*) sites and *tau* gene, a structural gene for Ter binding protein, on the *E. coli* genome. Upper part shows circular and expanded *E. coli* chromosome map and lower arrows mean direction of the DNA replication fork blocked at the *Ter* sites.

blocking systems, it is essential to elucidate structure of the site and the molecular mechanism of fork blocking rection.

We attempted to localize more precisely *Saccaromyces cerevisiae* fork blocking site. We named this "sog" site, which was identified using two dimensional (2D) agarose gel electrophoresis. First we cloned the sog site into a YEp-type plasmid, a derivative of 2 μm plasmid, and analyzed the replication intermediates using 2D gel electrophoresis (see Fig. 2). The blocking activity of the sog site remained even on the plasmid not involved in any rRNA gene and inhibited fork movement in the same polar fashion as on the genome. We named this method "sog assay". Using this assay, we narrowed the region required

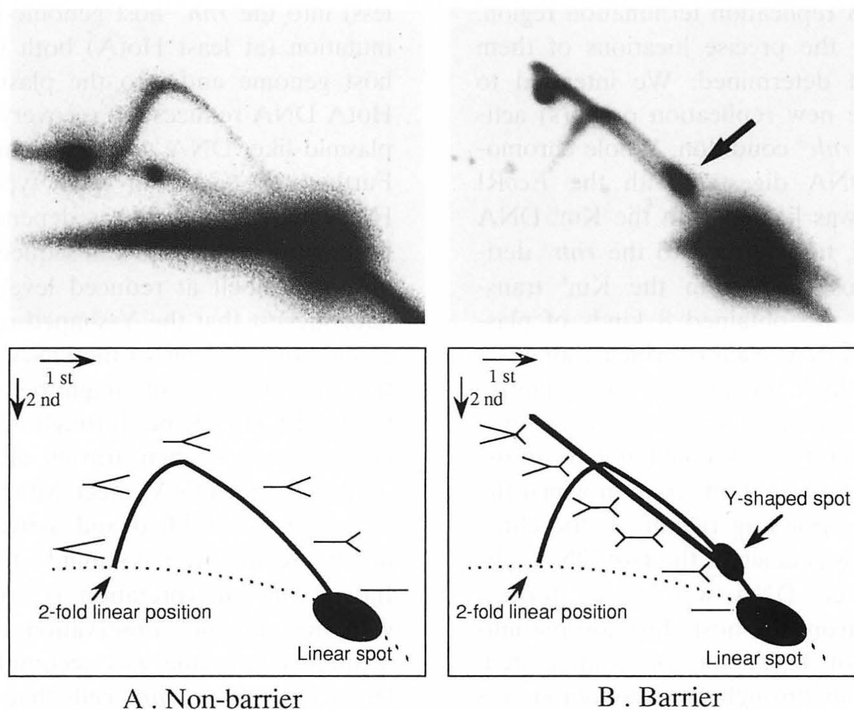

Fig. 2. Detection of DNA replication intermediate molecules using 2-D(dimensional) agarose gel electrophoresis.
A. A DNA fragment without replication fork barrier. A large spot and an arc, shown in the upper gel pattern, correspond to a linear DNA fragment and its replication intermediates, molecular structure of which are shown schematically along the spot and the arc in the lower figure.
B. A DNA fragment with replication fork barrier. In addition to the pattern of A, a new spot on the arc and a line starting from the spot are seen. The new spot corresponds a specific Y-shaped molecule, at a specific site (barrier) on which the DNA replication fork is blocked. The molecular structures of this and other intermediates are also shown schematically in the lower figure.

for sog activity to a 109 bp fragment which locates at an interspacer region placed between the transcriptional termination sites of 35S and 5S rRNA genes. It is also close to the enhancer region for 35S transcription and overlaps with the site essential for recombinational hotspots, *HOT1.* These results suggest that in addition to fork blocking function, sog site might be link to control of 35S transcription and trigger homologous recombination.

(3) Homologous recombinational hotspot activity near the replication termination sites in *E. coli*; termination event and Chi sequence dependency.

In wild type *E. coli* cells, an unique origin, *oriC* and initiator protein, DnaA protein, are essential for DNA initiation of replication. When either of these is defective, the cells die. However in these mutants, when a ribonuclease H defective mutation (rnh^-) is introduced, the cells survive. The replication system, which is *oriC*- and *dnaA*-independent, is apparently activated under rnh^- conditions. de Massy et al. reported that in rnh^- conditions, DNA replication initiates at several new origins in which two most active origins were located in

the DNA replication termination region. However the precise locations of them were not determined. We intended to clone the new replication origin(s) activated in *rnh*⁻ condition. Whole chromosomal DNA digested with the *Eco*RI enzyme was ligated with the Km^r DNA fragment, transformed to the *rnh*⁻ derivative host and from the Km^r transformants, we obtained 8 kinds of plasmid-like DNA, each of which contains a specific DNA fragment, termed "HotA-H", derived from the *E. coli* genome. Because Hot DNA could not be transformed into a mutant strain in which the Hot corresponding region on the chromosome was deleted, the Hot DNA, obtained as ccc DNA, was formed through excision from the host chromosome into which Hot DNA was once integrated, rather than through an autonomous replication, thus the Hot fragments are hyper-recombinogenic. All but one (HotH) of the Hot DNA fragments locates at the DNA replication termination region on the *E. coli* genome. All Hot fragments so far tested have Chi activity. In 3 Hot (A, B and C) groups, introduction of *tau*⁻ mutation (*tus*⁻; terminationless) into the *rnh*⁻ host genome or Chi⁻ mutation (at least HotA) both into the host genome and into the plasmid-like HotA DNA reduces the recovery of Hot plasmid-like DNA to less than 1/10. Furthermore even in wild type cells, HotA activity, which was dependent on termination event and Chi sequence, was observed albeit at reduced level. These data suggest that the Y-shaped molecule formed by blockage of the DNA replication fork at the *ter* site might be entrance for RecBCD enzyme, through which the enzyme passes, then travels along the double helical DNA, meet with the Chi sequence, is modified and activates the nearby region to recombine efficiently. Indeed this interpretation is consistent with our previous observation that the Y-shaped molecule was accumulated in larger amounts in *rnh*⁻ cells than in wild type cells, because in *rnh*⁻ cells, DNA replication initiating at unidentified replication origins, which are located at termination region, would be immediately blocked at the Ter sites and thus these Y-form DNA molecules would be accumulated.

TECHNOLOGY DEPARTMENT

Head: Hachiro Honda

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Chief: Hiroyuki Hattori

Research Support Facilities
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Chieko Nanba
Toshiki Ohkawa
Kaoru Katou
Tomoki Miwa
Mariko Saitoh
Kimiko Yamamiya

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Kazuhiko Furukawa (Unit Chief)
Yoshimi Matsuda
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Yoko Fujimura
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Koji Hayashi

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

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

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

RESEARCH SUPPORT FACILITY

Head of Facility: Yoshitaka Nagahama
Research Associates:
Masakatsu Watanabe (Spectrograph)
Yoshio Hamada (Tissue and Cell Culture)
Kenta Nakai (Computer Facility)

The facility maintains large-scale experimental equipment and facilities for growing and maintaining biological specimens. The facility is shared among the research members, and has five laboratories.

The Large Spectrograph Laboratory: This laboratory has the largest spectrograph in the world, dedicated mainly to action spectroscopical studies of various light-controlled biological process (the Okazaki Large Spectrograph, OLS). 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 to 1,000 nm onto its focal curve of 10 m in length. The fluency rate (intensity) of the monochromatic light is more than twice as much as that of the tropical sunlight at noon at each wavelength.

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 micro-beam irradiator (up to 200 $\mu m \cdot s^{-1}$ in tracking speed, down to 2 μm in beam diameter) with an infrared phase-contrast observation system.

Cooperative Research Program for The Okazaki Large Spectrograph

More than 30 projects every year are conducted by visiting scientists including foreign scientists as well as those in the Institute under the NIBB Cooperative Research Program for the use of the OLS.

Action spectroscopical studies for various regulatory and damaging actions of light on living organisms have been conducted.

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Yasumasu, I., Tazawa, E., and Fujiwara, A. (1991) Photo-activation of respiration in the presence of CO in sperm of several marine invertebrates. *Comp. Biochem. Physiol.*, **100B**, 141–147.

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Y. (1991) The shape of fluence-response curves for the light-induced transcript accumulation in pea seedlings. *Photochem. Photobiol.*, **54**, 495–497.

The faculty of the Large Spectrograph Laboratory conducts its own work. Photoreceptive and signal-transduction mechanisms of phototaxis of single-celled, flagellate algae are studied action-spectroscopically by analysing 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.

Publication List

Kawai, H., Kubota, M., Kondo, T., and Watanabe, M. (1991) Action spectra for phototaxis in zoospores of the brown alga *Pseudochorda gracilis*. *Protoplasma*, **161**, 17–22.

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Tissue and Cell Culture Laboratory: This is a facility for tissue and cell culture. This laboratory is equipped with safety rooms which satisfy the P2/P3 physical containment level. This facility is routinely used for DNA recombination experiments.

The faculty of the Tissue and Cell Culture Laboratory also conducts its own work. 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.

Computer Facility: In order to meet various computational needs in the NIBB, various kinds of computers are maintained in this laboratory. They are: VAX/VMS machines (VAX11/780 and microVAX II), UNIX workstations (SPARCstation IPX and IPC and DECstation2100), and personal computers (PC-9801 and Macintosh machines).

All of them are linked to the ethernet LAN of Okazaki National Research Institutes as well as many personal computers within this institute. Thus, from each laboratory, computers of this laboratory can be accessed. In addition, a NetWare server machine (QuarterL) is used to integrate PCs in the NIBB and it enables the sharing of PostScript printers. Several softwares for DNA and amino acid sequence analyses are installed in our computers.

As for its own work, efforts to develop new methodology for sequence analysis have been initiated. For example, an expert system has been constructed in order to predict various protein localization sites from amino acid sequence information only. Now, a software to predict splice sites in mRNA precursors is also under development.

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

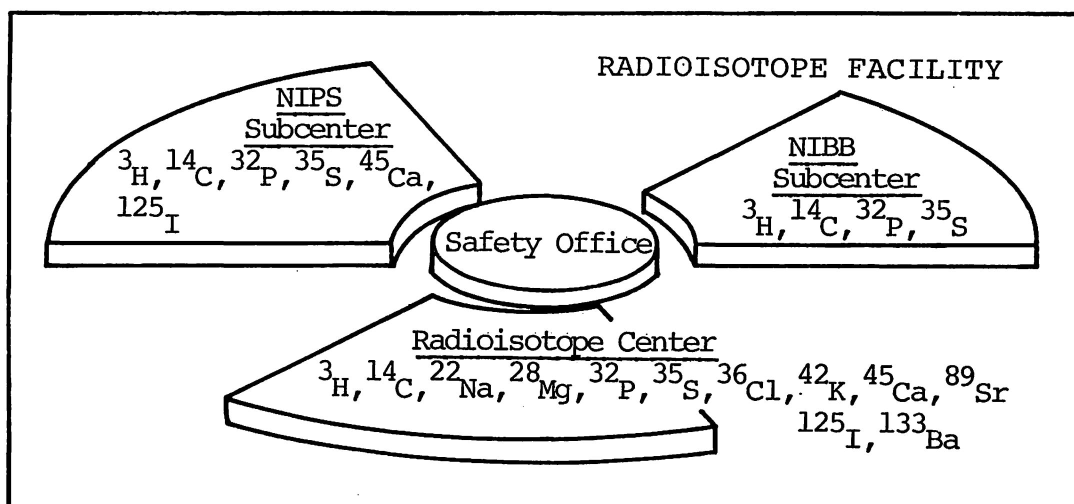
Experimental Farm: This facility consists of two 20 m^2 glasshouses with precision temperature and humidity control, a limited farm, two greenhouses (45 m^2, 88 m^2) with automatic sprinklers and window control, two open aquariums (30 t, 50 t) and several smaller tanks. The facility also includes a building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

Plant Cell Culture Facility: Autotrophic and heterotrophic culture devices are equipped for experimental cultures of plant and microbial cells.

RADIOISOTOPE FACILITY (managed by NIBB)

Head of Facility: Yoshihiko Fujita
Technical Staffs: Kazuhiko Furukawa
Yoshimi Matsuda
Mitsuaki Higashi

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

The Center 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
   - 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
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
   - Microsope Photometer
     - CARL ZEISS MPM 03-FL
   - Two-Dimension Microdensitometer
     - JOYCE LOEBL 3CS