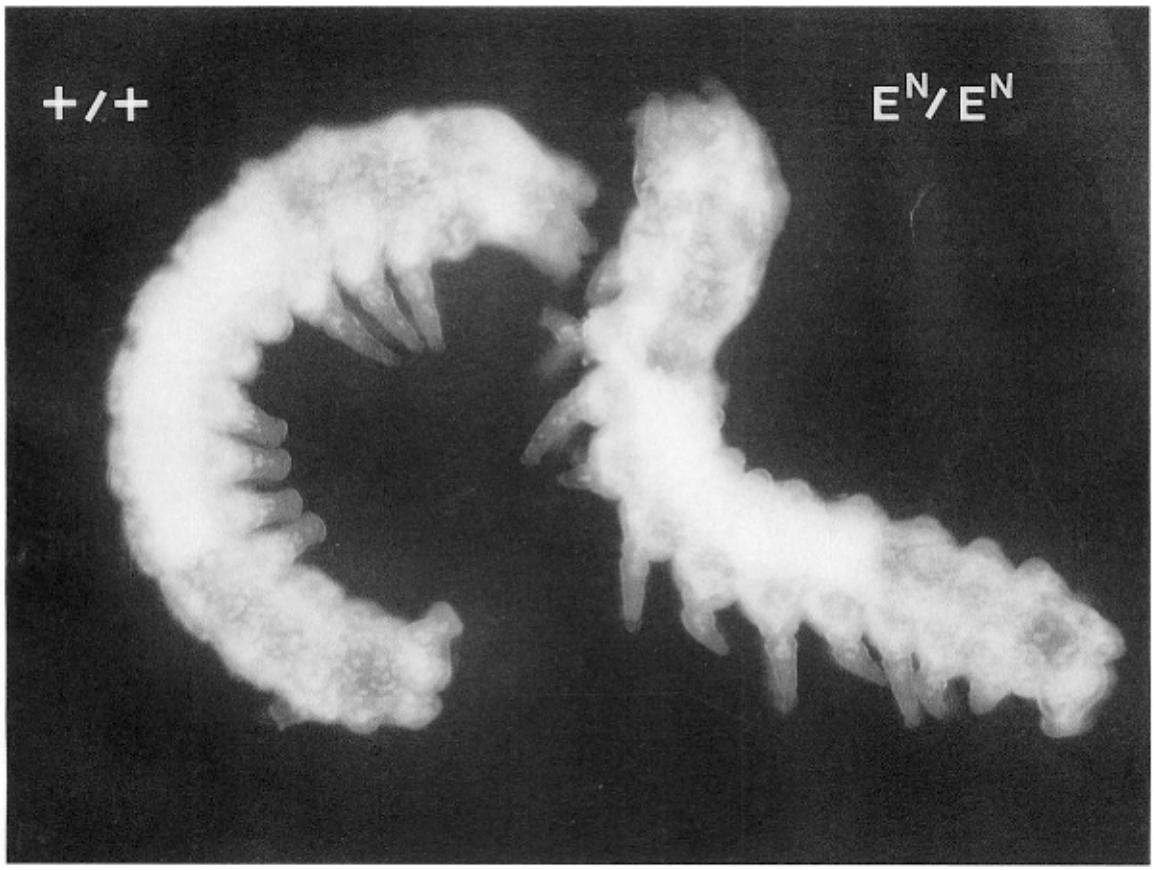
NATIONAL INSTITUTE FOR BASIC BIOLOGY

岡崎国立共同研究機構

基 礎 生 物 学 研 究 所



ANNUAL REPORT

1990

Ikuo Tamura

INTRODUCTION

The National Institute for Basic Biology (NIBB) is a government supported basic research institute in the field of biology. It aims to stimulate and promote studies of biology, by conducting first-rate research on its own, and by cooperating with other universities and research organizations. The Institute concentrates on the studies of cellular functions, growth and development, homeostatic control, and gene expression and regulation in eukaryotic organisms, to elucidate the fundamental mechanisms underlying various living phenomena at the molecular level.

During the past year, we have built up the Laboratory of Gene Expression and Regulation which was inaugurated in 1989 by newly appointing Prof. Takashi Horiuchi (formerly Associate Professor of Kyushu University) who will continue to work on chromosome duplication and reorganization in both prokaryotes and eukaryotes. The other Division of the Laboratory headed by Prof. Yoshiro Shimura has been actively engaged in studies of genes involved in morphogenesis of the plant, *Arabidopsis.*

Two new professors were appointed during the last year. Prof. Yukio Nishimura (former Associate Professor of Kobe University) filled the position of Professor of Cell Mechanisms to promote molecular biological studies on formation of organelles in plant cells. Prof. Yoshiki Hotta of Tokyo University was appointed to be in charge of Division of Cellular Communication (adjunct) to further molecular genetical studies of *Drosoplila* development. On the other hand, four associate professors of the Institute were promoted to professors in other universities during the period. Active exchange of personnel with other universities and institutions, I believe, is essential to keep the Institute active.

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 organized the 24th and 25th Conferences in 1990, entitled "Membrane biogenesis and temperature acclimation of plants" and "Inositol phosphates and Ca signaling" respectively. The conferences were well attended by active scientists in the related fields, from both inside Japan and abroad.

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 1990.

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.

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

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 undertakes the technical education of its staffs.

Research Support Facility

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

Campus

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

GRADUATE PROGRAMS

The NIBB carries out two graduate programs.

1. Graduate University for Advanced Studies

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

The Department consists of the following Divisions and Fields:

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

2. Graduate Student Training Program

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

OFFICE OF DIRECTOR

Publication List

Tasaka, M., Hasegawa, M., Ozaki, T., Iwabuchi, M., and Takeuchi, I. (1990). Isolation and characterization spore coat protein (SP96) gene of *Dictyostelium discoideum.* Cell Differentiation and Development **31**, 1–9.

Takemoto, K., Takeuchi, I., and Tasaka, M. (1990). cAMP regulation of the expression of prespore specific genes, SP96 and Dp87 in disaggregated slug cells of *Dictyostelium discoideum.* Cell Differentiation and Development **31**, 89–96.

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 Associate: Kazuo Ogawa
JSPS Post-doctoral fellow: Luigi De Bellis
Visiting Scientists: Ikuko Hara-Nishimura
Kaori Inoue
Graduate Student: Ryuji Tsugeki

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, while 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 studying the regulatory mechanism which underlies light-induced organelle differentiation. Recently, we have found that the reversible transition of leaf peroxisomes to glyoxysomes takes place during the senescence of the cotyledons (De Bellis and Nishimura. *Plant Cell Physiol.* in press).

As a step to understand the regulatory mechanisms that operate during microbody transition in pumpkin cotyledons (*Cucurbita* sp., Amakuri Nankin), seven microbody enzymes, including two glyoxysomal enzymes, two leaf peroxisomal enzymes and three enzymes present in both microbodies have been purified, and specific antibodies have been prepared for each enzyme. Immunocytochemical analyses demonstrated that glyoxysomes are directly transformed to leaf peroxisomes. In 1990, we have isolated and characterized cDNA clones for the glyoxysomal enzyme malate synthase, the leaf peroxisomal enzyme glycolate oxidase, and an enzyme present in both microbodies, malate dehydrogenase, (Mori *et al. Eur. J. Biochem.* in press; Takeda-Yoshikawa *et al. J. Biol. Chem.* in press). Using *in vitro* translocation experiments with these cDNA clones, we have found that a specific degradation system for glyoxysomal enzymes is induced in the microbodies of cotyledons exposed to light. Studies of

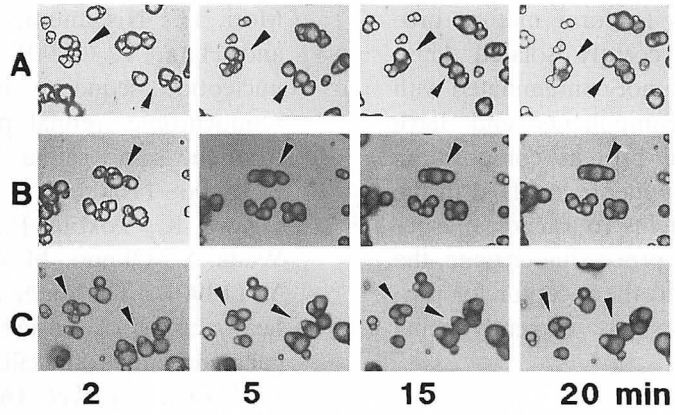


Fig. 1. *In vitro* fusion of isolated protein bodies.

the molecular aspects of this specific degradation system and of the signal transduction processes involved in light-induced microbody transition are in progress.

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

Protein bodies are single membrane-bound organelles which accumulate seed storage proteins. We have found that the protein bodies are closely related to vacuoles which are the lytic compartment 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. We are studying the regulatory mechanisms underlying the reversible transformation between protein bodies and vacuoles, and have focused on the processes of budding and membrane fusion. Protein bodies and vacuoles from various developmental stages of maturing and germinating pumpkin seeds have been isolated. Using isolated protein bodies, we have recently established an *in vitro* system to reconstitute the fusion process (Fig. 1). Experiments employing this *in vitro* system are currently underway to elucidate the molecular mechanisms of membrane fusion during seed germination.

In parallel, the biogenesis of protein bodies and the cellular components involved in the transport of seed storage proteins are also being studied. Protein bodies have been fractionated into three

suborganellar components: membrane proteins, matrix proteins and crystalloid protein. These three classes of proteins are synthesized on rER as preproproteins. After co-translational processing of the signal peptide, proprotein precursors are transported via dense vesicles to vacuoles where specific processing enzyme operates to form mature proteins. We have recently isolated dense vesicles which are not contaminated with other cellular components and have characterized the protein composition. The cellular components involved in the transport of proteins to vacuoles, especially the specific processing enzyme, the dense vesicles, and the receptor for proprotein precursors, are currently being characterized.

Publication List

1. Mori, H. and Nishimura, M. (1990). Purification of mRNA for malate synthase and glycolate oxidase by immunoadsorption of polysomes. *Plant Physiol. Biochem.*, **28**, 505–508.
2. Hara-Nishimura, I., Matsumoto, T., Mori, H., Nishimura, M., Hara, R. and Hara, T. (1990). Cloning and nucleotide sequence of cDNA for retinochrome, retinal photoisomerase from the squid retina. *FEBS Letters*, **271**, 106–110.
3. Ogawa, K., Yokota, E., Hamada, Y., Wada, S., Okuno, M. and Nakajima, Y. (1990). The outer arm dynein β-heavy chains of sea urchin sperm flagella and embryonic cilia are different. *Cell Motil. Cytoskel.*, **16**, 58–67.

DIVISION OF BIOENERGETICS

Professor: Yoshihiko Fujita
Associate Professor: Shigeru Itoh
Research Associates: Mamoru Mimuro
Kaori Ohki
NIBB Post-doctoral Fellow: Katsunori Aizawa
*NIBB Visiting Scientists: Tiang-Qing Gu**
Jonathan P. Zehr#
Fellows: Tohru Ikeya
Yoko Nakamura
Technical Staffs: Akio Murakami
Masayo Iwaki

*(*from Institute of Botany, Academia Sinica, Beijing, China)*
(#from Marine Sciences Research Center, State University of New York, Stony Brook, New York, U.S.A.)

Photosynthetic energy conversion has been investigated at the cellular and molecular levels. Our research is currently focused on (1) regulation of stoichiometry among thylakoid components in response to the light regime for photosynthesis, (2) molecular mechanism of thylakoid protein complexes functioning in the primary process of photosynthetic energy conversion, and (3) nitrogen-fixation in cyanophyte directly coupled with photosynthetic energy conversion.

(1) *Regulation of thylakoid composition:* Previous study has indicated that assembly of photosystem I (PS I), the terminal component of photosynthetic electron transport system (ETS), is regulated in response to the light regime for photosynthesis, so as to balance the two photoreactions under each light regime (cf. Fig. 1). The signal for the regulation was studied by examining the steady-state of photosynthetic electron transport in cells of the cyanophyte *Synechocystis* PCC 6714 under various light regimes in relation to the regulation. Flash-induced oxidation-reduction of Cyt *f* and P700 under steady-state of photosynthesis indicated that the state of Q or modified Q cycle of Cyt b_6-*f* complex is, or is very closely related to, the signal for the regulation. Study of intracellular localization of Cyt oxidase, the terminal of respiratory ETS in this organism, by using chemical electron microscopy revealed that Cyt oxidase is located only in thylakoids but not in cytoplasmic membranes irrespective of growth conditions, indicating that Cyt oxidase is another terminal of thylakoid ETS. Assay of Cyt oxidase in cells grown under various conditions indicated that the level of Cyt oxidase is also regulated, similar to the level of PS I. Thus, it is very probable that levels of the two terminals of thylkoid ETS are simultaneously regulated in response to the state of ETS.

(2) *Molecular mechanism of electron transfer in reaction center complexes:* Electron transfer mechanism in the PS I reaction center complex was studied by modification of intrinsic electron transfer components, quinone and iron-sulfur centers. After replacing intrinsic quinone by artificial species or modifying the iron-sulfur proteins, the electron transfer was analyzed by nanosecond-microsecond laser spectroscopy and by cryogenic ESR spectroscopy. Over 100 species of quinones and quinone analogs have been tested for replacement-experiments. About 30 species were found to replace fully the function of intrinsic phylloquinone in spinach and cyanophyte PS I reaction center, indicating that the reaction center complex has a special quinone binding site. Analysis of the relationship between the redox potential and the function of quinone showed that free energy difference between the quinone and the primary electron acceptor or the tertiary acceptor, iron-sulfur center, in PS I complex is primarily important for the reaction. Quinone-reconstitution study was also done for PS II reaction center complex.

(3) *Molecular mechanism of energy transfer in light-harvesting protein complexes:* The study in 1990 was focused on the energy transfer from carotenoids to Chl *a* and structure of prosthetic groups in bacterial reaction center. Study of energy transfer in the fucoxanthin-Chl *a/c* protein from brown algae indicated that the energy transfer from Chl *c* to Chl *a* and from fucoxanthin to Chl *a* is achieved by two independent pathways in respective cases. Structure of prosthetic groups in the reaction center complex of a purple sulfur bacterium *Chromatium tepidum* was deduced by linear dichroism and magnetic circular dichroism spectra. Results indicated that (1) the structure is essentially the same as that in purple non-sulfur bacteria, and (2) magnetic coupling between different energy levels of the special pair is polarization-plane dependent. An occurrence of a "low-lying" antenna component was found ubiquitous in photosynthetic bacteria having the reaction center of quinone-type. This evidence gives rise to a concept of "antenna-reaction center interaction" in terms of energetics of energy transfer and charge separation processes.

(4) *Nitrogen-fixation directly coupled with oxygenic photosynthesis:* The study was focused on the regulation of nitrogenase activity in the non-heterocystous cyanophyte *Trichodesmium* sp. NIBB 1067 in response to the nitrogen-source in the growth environments. Examination of nitrogenase activity and abundance of Fe- and Fe-Mo-proteins of nitrogenase indicated that the regulation of nitrogenase activity occurs at least at two different levels; one, at the level of transcription of *nif* genes or translation of the transcripts, and the other, at the post-translational level, probably modification of Fe-protein. In the latter regulation, photosynthesis appears to be essential for maintaining the active form of Fe-protein. Organization of *nif* genes (*nifH, D* and *K*) in *Trichodesmium* sp. NIBB 1067 was also studied. Current results showed that (1) 3 genes, *nifH, D* and *K*, form a cluster without excision between *nifD* and *K*, and (2) the second copy of *nifH* is not present. These features are different from those reported for the heterocystous cyanophyte *Anabaena* sp. 7120.

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DIVISION OF CELL PROLIFERATION (ADJUCNT)

Professor: Yasuhiro Anraku
Associate Professor: Souichi Nakamura
Research Associate: Hidetoshi Iida
NIBB Post-doctoral Fellow: Hiro Nakamura
JSPS Post-doctoral Fellows:
Junko Nakajima-Shimada
Shuichi Sakaguchi
Tohru Yashihisa

Ca^{2+} is a growth-regulating substance in the cell cycle of mammalian cells. For example, when the availability of Ca^{2+} becomes limiting in the presence of a sufficient amount of growth factors, mouse fibroblastic cells are arrested in the G_1 phase of the cell cycle. Most of the growth factors elicits 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 the role of Ca^{2+} in the cell cycle and mating process of the yeast *Saccharomyces cerevisiae* and on the establishment of measuring systems for $[Ca^{2+}]_i$ in yeast cells, with the hope that combination of cell biological and genetic approaches will uncover the molecular mechanism of Ca^{2+} signaling.

Cell Cycle Control by Ca^{2+}

Unlike in mammalian cells, the role of Ca^{2+} during the cell cycle in the yeast *S. cerevisiae* has been unknown for many years. The main difficulty in investigating its role by direct growth studies was that yeast cells can grow indefinitely in Ca^{2+}-deficient medium. In this project, we performed systematic cell cycle analysis using media containing various concentrations of Ca^{2+}, a Ca^{2+}-ionophore (A23187) and a Ca^{2+}-chelator (EGTA), and established experimental conditions for controlling yeast cell proliferation by lowering the intracellular Ca^{2+} content. Simultanenous addition of 10 μM A23187 and 10 mM EGTA to cells growing in a Ca^{2+}-deficient medium at 22°C caused rapid decrease in intracellular Ca^{2+} content and resulted in transient G_1 arrest followed by block mostly at the G_2/M phase, as revealed by flow cytometry. Recovery from G_1 arrest was not due to coordinated initiation of DNA synthesis and bud emergence: unbudded cells with S or G_2/M DNA were observed. Examination of terminal phenotype suggested that Ca^{2+} was required at all the stages of the cell cycle except for the initiation of DNA synthesis. The intracellular cAMP level decreased within 10 min after addition of A23187 and EGTA. No significant transient G_1 arrest was observed in cells incubated with 8-Br-cAMP, or *RAS2*^{val19} and *bcy1* mutants, which produce a high level of cAMP and have constitutively activated cAMP-dependent protein kinase, respectively. These results indicate that Ca^{2+} is essential for cell cycle progression in yeast and suggest that G_1 events may be mediated by cross-talk between Ca^{2+} and cAMP pathways.

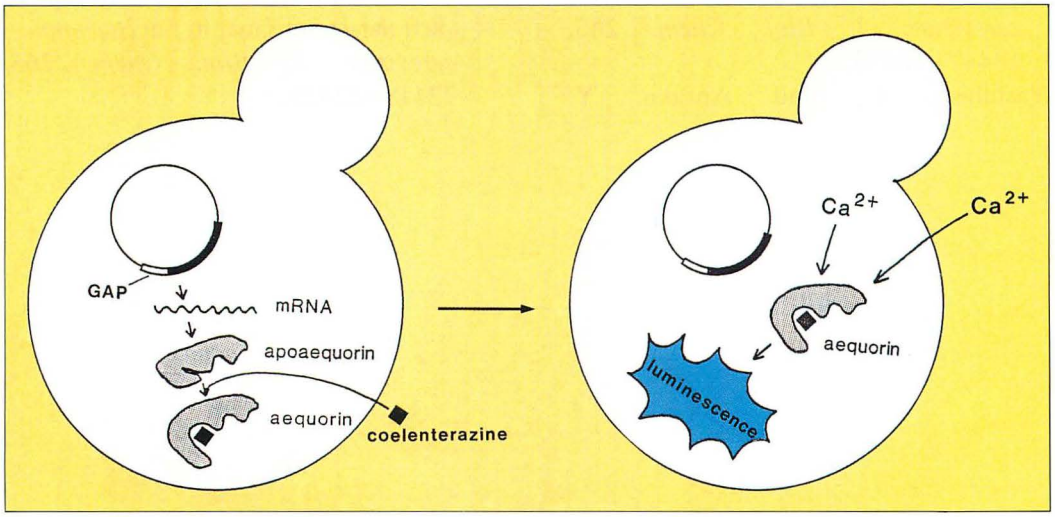
The Role of Ca^{2+} in the Mating Process

In *S. cerevisiae*, the mating process of haploid cells is controlled by the mating pheromones, *a*- and α-factors, that are synthesized and secreted by *a* and α mating type cells, respectively. The mating pheromones induce several responses in cells of opposite mating type. The mating pheromone response pathway of

the yeast has been shown to involve mechanisms similar to those found in mammalian cells, such as the pheromone-receptor interaction and the function of the pheromone receptor-coupled guanine nucleotide-binding regulatory (G) protein. However, nothing is known about the role of extracellular and intracellular Ca^{2+} in this pathway except that Ca^{2+} influx in *a* cells is stimulated by α-factor. Thus, the study in this project was designed to investigate whether Ca^{2+} influx induced by α-factor correlates with changes in $[Ca^{2+}]_i$ that may have a regulatory role for signal transduction of the mating pheromone and to elucidate the role of changes in $[Ca^{2+}]_i$. To measure $[Ca^{2+}]_i$ in individual yeast cells, we employed fura-2 as a Ca^{2+}-specific probe, in conjunction with digital image processing. The results showed that $[Ca^{2+}]_i$ rose to about 500 nM from a basal level of about 100 nM, depending upon an influx of Ca^{2+} which is induced by α-factor. When this influx and consequent rise are prevented by incubating *a* cells with α-factor in a Ca^{2+}-deficient medium, the cells died specifically after they had changed into shmoos, cells morphologically changed by the action of α-factor. The duration of the Ca^{2+} requirement for maintaining viability is short and the requirement is specific to this limited stage. Mating between *a* and α cells was thereby impaired due to cell death at or before the stage of conjugation. These results clearly indicate that induced Ca^{2+} influx followed by a rise in $[Ca^{2+}]_i$ is essential for maintaining viability of yeast cells in the mating process. Taking advantage of the finding that yeast cells die when exposed to α-factor in the absence of a rise in $[Ca^{2+}]_i$, we are isolating mutants defective in Ca^{2+} signaling.

Monitoring of Intracellular Ca^{2+} Using an Apoaequorin cDNA Expression System

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 above. However, the fura-2 method has some drawbacks: 1) fura-2 is sequestered 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 changes in $[Ca^{2+}]_i$ 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), molecular oxygen and coelenterazine (chromophore). The cDNA for apoaequorin of the 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 the *GAL1* or the *GAP* promoters (see Figure) 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 (Nakajima-Shimada *et al.* (1991) Biochem. Biophys. Res. Commun., in press). For an *in vivo* method for monitoring of changes in $[Ca^{2+}]_i$, introduction of



A cartoon indicating expression of the apoaequorin cDNA from the jellyfish *Aequorea victoria* under the control of the *GAP* promoter in yeast cells and regeneration of aequorin from expressed apoaequorin and a chromophore, coelenterazine. The binding of Ca^{2+} to aequorin generates a transient luminescence (λ=470 nm). Intensity of the luminescence correlates with the concentration of intracellular Ca^{2+}. On the plasmid, the open box represents the *GAP* promoter, the closed portion the apoaequorin cDNA, and the line the vector DNA.

coelenterazine into intact yeast cells followed by regeneration of aequorin is an essential step. We found that aequorin can be effectively regenerated by simply incubating yeast cells with coelenterazine. Coelenterazine-incorporated cells responded to extracellular stimuli. A mating pheromone, α-factor, generated extracellular Ca^{2+}-dependent luminescence specifically in *a* mating type cells, 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. These results demonstrate the usefulness of the aequorin system in monitoring $[Ca^{2+}]_i$ response to extracellular stimuli in yeast cells. Using this system, we are investigating mechanisms of Ca^{2+} mobilization in the initiation of the cell cycle triggered by glucose and in the initiation of mating triggered by α-factor.

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DIVISION OF CELL FUSION (ADJUNCT)

Professor: Yoshio Okada
Research Associate: Masahiro Ishiura
Graduate Student: Koide Tsuyoshi
(from School of Medicine, Osaka University)

Molecular and cellular biology of mammalian cells, aided by cell engineering and recombinant DNA technology, are the research projects of this division. In relation to the action mechanism for diphtheria toxin in mammalian cells, the structure and function of the elongation factor 2 (EF2) involved in the protein synthesizing system of eucaryotes are currently being extensively studied at the molecular level using cloned *EF2* genes. Cosmid cloning has been established by constructing simplified cosmid vectors and selecting suitable *Escherichia coli* host cells. Elucidation of the mechanisms for recombination and DNA repair in mammalian cells is also in progress.

1. Cosmid Cloning

(1) Recombinant cosmid DNA suffers from high-frequency deletions in the DNA when propagated in *Escherichia coli* hosts. This deletion occurs even in *recA*-deficient *E. coli* hosts. As reported previously, a *recB recC sbcB recJ* quadruple mutation in the host prevents such deletion (Ishiura *et al.*, 1989, *J. Bacteriol.* **171**: 1068–1074). To clarify the sequence motif(s) which is involved in cosmid-deletion events, we propagated two test cosmids, cMB15 carrying the mouse insert No. 15 and cHEF2-15 carrying the human *EF2* gene (Ishiura *et al.*, 1989, *J. Bacteriol.*), in the *recA E. coli* HB101, isolated the deletion derivatives of the test cosmids, and analyzed the nucleotide sequence surrounding each deletion junction. We found that there are T-stretch (or A-stretch) and/or a specific 8-bp sequence around the deletion junctions.

(2) Multi-copy *EF2*-related genes which exist in the mouse genome were analyzed by cosmid cloning. We constructed a cosmid library of mouse L cell genomic DNA with pDC105 vector (Ishiura *et al.*, 1989, *Gene* **85**: 429–435), isolated the *EF2*-related genes by colony hybridization using the *EF2* gene as a probe, and analyzed the nucleotide sequences.

(3) Recombinational hotspots specific to female meiosis in the murine major histocompatibility complex were analyzed by cosmid cloning. We constructed cosmid libraries of the genomic DNAs from recombinant mice with pDC104 vector (Ishiura *et al.*, 1989, *Anal. Biochem.* **176**: 117–127), isolated cosmid clones covering the major histocompatibility complex region, and analyzed the nucleotide sequences sorrounding recombination junctions.

(4) The *Neurospora prd-1* gene which control the period length of circadian conidiation rhythm was isolated by cosmid cloning and sib selection (Nakahsima, H. [Division of Biological Chronology] and Ishiura, M., in preparation). For gene transfer experiments in *Neurospora crassa*, we constructed pDC107 vector by inserting the *Neurospora* benomyl-resistance gene as a dominant selective marker into the pDC1 vector (Ishiura *et al.*, 1989, *Anal. Biochem.*). We constructed a cosmid library of the genomic DNA from wild-type *Neurospora* with pDC107, and isolated about 5,000 single clones and stored them separately. Cosmid DNAs were prepared from a set of clones stored and transferred into *prd-1* mutant cells (sib selection). Single colonies of the benomyl-resistant transformants carrying the transferred cosmid

DNAs were isolated by plating the cells on benomyl-agar plates, and the period length of conidiation rhythm of the transformants was analyzed. By this procedure, we succeeded in isolating single cosmid clone which complemented the *prd-1* mutation.

2. Genetic Analysis of Fabry's disease

We analyzed the case of Fabry's disease of a Japanese family at the molecular level by cloning cDNA which encodes human α-galactosidase A and found that this case of the disease was resulted from a single amino-acid substitution of Ser for Pro-40.

3. Others

(1) Human enzyme synthesizing blood-group A substance was purified to homogeneity from human plasma, and its enzymatic properties were characterized.

(2) Starfish cyclin cDNA was cloned from eggs and sequenced. Expression of cyclin during meiosis was also analyzed.

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4. Shiroishi, T., Hanazawa, N., Sagai, T., Ishiura, M., Gojobori, T., Steinmetz, M., and Moriwaki, K. (1990) Recombinational hotspot specific to female meiosis in the murine major histocompatibility complex. *Immunogenet.* **31**:79–88.
5. Tachibana, K., Ishiura, M., Uchida, T., and Kishimoto, T. (1990) Starfish egg mRNA responsible for meiosis reinitiation encodes cyclin. *Dev. Biol.* **140**:241–252.
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DIVISION OF CELLULAR COMMUNICATION (ADJUNCT)

Professor: Yoshiki Hotta

The division just started its new projects from September 1990, and is currently engaging in the study of cellular communications in the development and differentiation processes of multicellular organisms. The project is aiming at the genetic dissection and molecular analysis of sensory receptors, nervous systems and muscles. Many Drosophila mutations have been isolated and analyzed which harbor defects in the morphology and functions of these structures.

Among them are many visual transduction mutants isolated by their abnormal visual behavior. Their molecular defects have been identified in the phosphatidyl-inositol metabolisms in the eye. It is, therefore, suggested that the visual signal transduction system is closely related to the phospholipid turnover, most likely that of in ositol 1,4,5-trisphosphate (InsP3).

Other genes of interest are the embryonic lethals which are normal in embryonic polarities and segmentations but are causing defects in the early differentiation of mesoderm, neuroblasts, glia and muscle precursor cells. They are currently investigated by using enhancer trap technique, single embryo primary culture method and various other cell biological and molecular technologies.

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.

DIVISION OF REPRODUCTIVE BIOLOGY

Professor: Yoshitaka Nagahama
Research Associates: Michiyasu Yoshikuni
Masakane Yamashita
Minoru Tanaka
JSPS Post-doctoral Fellows: Thomas F. Hourigan
Noriyoshi Sakai
Teresa Telecky
Ian G. Gleadall
Johannes Komen
Graduate Students: Akihiko Yamaguchi
Mika Takahashi
Visiting Scientists: Jennifer L. Specker
Naoki Shibata
Toshiro Hirai
Takeshi Miura
(from Hokkaido University)
Shyuji Ohno
(from Hoshi University)
Chiemi Inoue
(from Okayama 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 (E_2), 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 E_2 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 encoding five steroidogenic enzymes (cholesterol side-chain cleavage enzyme, 17α-hydroxylase, $C_{17,20}$-lyase, aromatase and 20β-hydroxysteroid dehydrogenase) responsible for E_2 and 17α,20β-DP biosynthesis. We have isolated and sequenced the cDNA encoding the rainbow trout aromatase. Northern blot analyses of rainbow trout ovarian follicles at different stages of development have shown that the levels of mRNA specific

for aromatase were abundant in vitellogenic follicles, but undetectable in postvitellogenic follicles, follicles during oocyte maturation, and postovulatory follicles.

Our microinjection experiments have revealed that 17α,20β-DP acts at the oocyte surface: 17α,20β-DP binding was found in oocyte cortices. 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; the highest activity occurs 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 recently purified MPF from unfertilized eggs of carp (*Cyprinus carpio*) using four chromatographic steps (Q-Sepharose FF, p13-Sepharose, Mono S, Superose 12). MPF activity at each purification step was assessed by injecting samples into immature cycloheximide-treated *Xenopus* oocytes. On a Superose 12 gel filtration column, MPF and H1 histone kinase eluted as a single peak with an apparent molecular mass of 100 kd. SDS-PAGE analysis of the active fractions after Superose 12 showed that they contained 4 proteins of 33, 34, 46, and 48 kd. A monoclonal antibody against the PSTAIR sequence of *cdc2* kinase immunoblotted the 33 and 34 kd components of purified carp MPF, indicating that *cdc2* kinase homologs are components of carp MPF. The 46 and 48 kd proteins are endogenous substrates of the kinase. We have also isolated cyclin A and B genes from a cDNA library constructed from mature goldfish oocytes, and their products were expressed in *E. coli*. The deduced amino acid sequences for cyclin A and B of goldfish have a homology of 35%. The 46 and 48 kd proteins were recognized by a monoclonal antibody against *E. coli*-produced goldfish cyclin B, but not by an anti-cyclin A antibody. These results indicate that fish MPF cosists of *cdc2* kinase and cyclin B.

Our PSTAIR monoclonal antibody also cross-reacted with cell homogenates in several species, including mouse, Japanese quail, *Xenopus*, goldfish, medaka, starfish, and a higher plant, lily (Fig. 1). The *cdc2* kinase homolog had several forms which differed in their mobility on SDS-PAGE. In most species examined, the form with the fastest mobility appeared or became dominant when cells entered metaphase. These results suggest that MPF generality discussed earlier is caused by the presence of *cdc2* homolog proteins.

2) Endocrine regulation of male germ cell development and maturation

Using salmonid fishes, we have recently identified two steroidal mediators of male germ cell development (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 cell elements.

Recent studies have revealed a role of 11-ketotestosterone in spermatogenesis

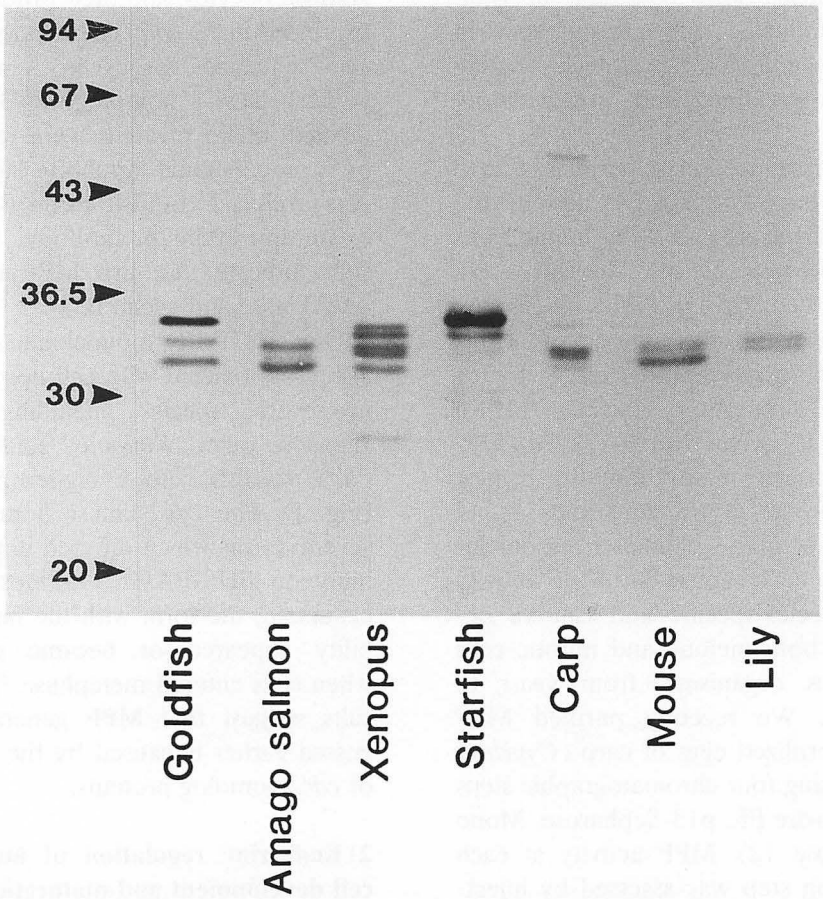

Fig. 1. Immunoblotting with a monoclonal antibody against anti-*cdc2* kinase (PSTAIR). Left to right: unfertilized eggs of goldfish, amago salmon, *Xenopus* and starfish, carp testis, mouse myeloma cell and lily microsporocyte.

and of 17α,20β-DP in sperm motility. In the cultivated male Japanese eel, spermatogonia are the only germ cells present in the testis. We have developed a serum-free, chemically defined organ culture system for eel testes, and used this to investigate the effect of various steroid hormones on the induction of spermatogenesis *in vitro*. It was found that 11-ketotestosterone could induce all stages of spermatogenesis (spermatogonial proliferation, meiotic division and spermiogenesis) *in vitro* within 21 days (Fig. 2). This is the first animal system in which the entire process of spermatogenesis has been induced by hormonal manipulation *in vitro* (Miura *et al.* (1991) *Proc. Natl. Acad. Sci. USA*, in press). Addition of 11-ketotestosterone to the culture medium also caused a marked cytological activation of Sertoli cells, but not Leydig cells, suggesting that the action of 11-ketotestosterone on spermatogenesis is mediated through the action of Sertoli cells. The organ culture system developed for eel testes should aid us in dissecting out the complex of factors involved in spermatogenesis and the roles

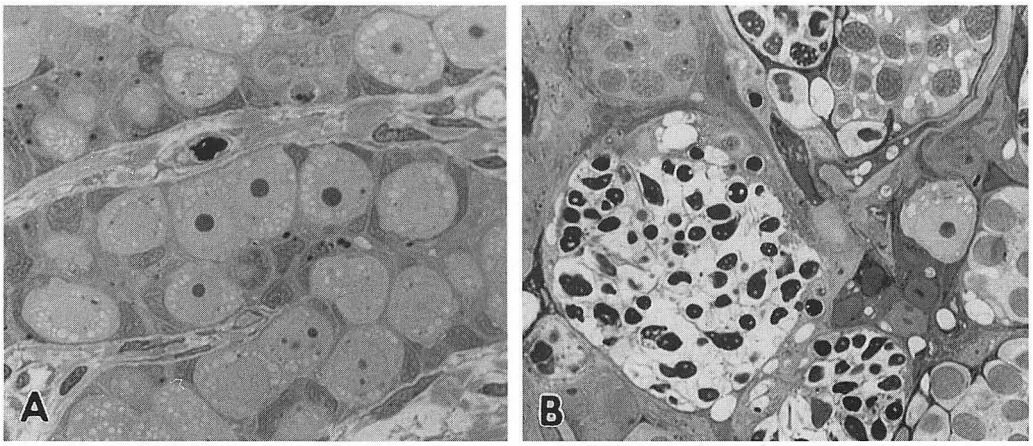

Fig. 2. Light micrographs of toluidine blue-stained 1 μm sections of testes before culture (A) and cultured in media containing 10 ng/ml 11-ketotestosterone for 36 days (B).

Leydig cells and Sertoli cells play in this process.

Sperm maturation is the development of spermatozoa from non-functional gametes to mature spermatozoa fully capable of vigorous motility and fertilization. Although the importance of hormones, such as androgens, for this process is generally accepted in vertebrates, the specific role played by specific hormones has not yet been clarified. In salmonid fishes, spermatozoa taken from the testes are immotile, but acquire motility during their passage through the sperm duct. Using male masu salmon, we found that gonadotropin-induced testicular production of 17α,20β-DP is responsible for the acquisition of sperm motility. However, neither 11-ketotestosterone nor testosterone were effective. We also obtained evidence that 17α,20β-DP acts to increase sperm duct pH, which in turn increases the cAMP content of sperm, allowing the acquisition of motility. This appears to be the first example in which the sequence of endocrine mediators and related mechanisms resulting in the acquisition of sperm motility have fully been established. Thus, 17α,20β-DP, which was identified as the steroidal mediator responsible for the acquisition of sperm motility, has now been proven to be the common endocrine mediator of oocyte and sperm maturation in salmonid fishes.

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

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DIVISION OF CELL DIFFERENTIATION

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Kensuke Suzuki (from Nagoya City University)

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 and results in transcribing a specific set of genes, like the silk fibroin, fibroin L chain, P_{25}, sericin-1, and sericin-2 genes. In 1990, some of the regulatory factors for these genes were identified as homeobox- or POU domain-containing proteins. 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 segment identities. In 1990, the *E* complex genetically known as a cluster of homeotic genes on the sixth chromosome was identified as the *Bombyx* equivalent of the bithorax complex accommodating the *Bombyx Ultrabithorax, abdominal-A*, and *Abdominal-B* homeobox genes. The *Nc* locus which is located about 1.4 cM apart from the *E* complex was also identified to accommodate the *Bombyx Antennapedia* homeobox gene.

Several *cis*-acting elements and *trans*-acting factors of the fibroin and sericin-1 genes that are transcribed in the posterior silk gland and middle silk gland, respectively, have been identified by the development and use of the cell-free transcription systems from various tissues of different developmental stages coupled with techniques of footprinting and gel shift assays. Those factors are FF1, FF2, SGF-1, -2, -3, -4, -5, and FBF-A1; some of them function specifically on the respective genes, and some act on both genes. Tissue distribution of these factors was found also complex; an active form of FF1 which constructs in the presence of FF2 a gel shift complex with the fibroin gene enhancer element I (-238/-73) is present specifically in the posterior silk gland but an inactive form is present ubiquitously, SGF-2 is present specifically in the posterior silk gland, and SGF-3 is present most abundantly in the middle silk gland, in lesser amount in the posterior silk gland, and not detectable in cultured cells derived from embryos.

From the posterior silk gland extracts FF1 has been purified to homogeneity which, in association with a partially purified FF2, specifically binds to -205/-185 of the fibroin gene (T. Suzuki *et al.* (1991a) *J. Biol. Chem.*, in press). An antibody raised against the purified FF1 inhibited the transcription enhancement governed by the enhancer I (T. Suzuki *et al.* (1991b) *J. Biol. Chem.*, in press) that accommodates 6 copies of homeodomain protein binding sequences, TCAATTAAAT or its variants. The FF1 binding region accommodates 2 copies of these. An introduction of two-base substitution mutation on all 6 repeats destroyed the transcription enhancement function. Competition assays

using oligonucleotides of the wild type and mutant types on the gel shift complexes suggested that SGF-2, -3, and -4 are homeo- or POU-domain containing proteins.

Besides the enhancer I and the proximal upstream element, at least two other parts serve for the preferential transcription of the fibroin gene in the posterior silk gland extract; one is the core-promoter and the other is the intronic modulator (the enhancer II). The enhancer II is composed of AT-rich sequences and multiple octamer-like sequences. By footprint experiments these sequences were protected differentially in extracts prepared from different tissues and cells, suggesting its interactions with cell type specific factors. The core-promoter was indispensable for the preferential transcription and functioned synergistically with the enhancer elements.

cDNA clones that contain homeobox sequences homologous to *Drosophila Antennapedia, engrailed,* and *invected* were obtained from the middle silk gland of the fifth larval instar. By using a PCR technique, a cDNA clone containing a POU-domain sequence, POU-M1, homologous to *Drosophila* Cf1-a or rat POU-III Brn-2 was obtained (see Fig. 1). The POU-M1 protein synthesized *in vitro* from the cDNA binds to the SC element of the sericin-1 gene, the key element controlling the sericin-1 gene transcription, as does SGF-3. Footprint and gel shift assays using various elements and their mutant oligonucleotides indicated that the POU-M1 protein is indistinguishable from SGF-3. Messenger RNA for POU-M1 is present at a high concentration in the fourth moulting stage and becomes lower in the fifth larval instar in the middle silk gland. In the posterior silk gland the mRNA was not detectable in the early stage of the fifth instar but a trace amount was detected in the fourth moulting stage.

cDNA clones covering the entire open reading frame for *Bombyx engrailed, invected,* and *Antennapedia* were obtained and sequenced. Developmental expression patterns of the respective mRNAs were analyzed by Northern blotting. The *Bombyx Antennapedia* mRNA was 4.8 kb long in the middle silk gland RNA from the wild type larvae, while an additional mRNA 1.8 kb long was detected in the middle silk gland RNA from the *Nc*/+ heterozygote larvae. Cloning and sequencing of the 1.8 kb mRNA revealed that it lacks the YPWM, homeobox, and carboxyl end regions. A Southern blot analysis on genomic DNAs indicated that the missing sequence on the mRNA is deleted on the *Nc* DNA. From these results we conclude that the *Bombyx Antennapedia* gene is mapped to the *Nc* locus which is located about 1.4 cM apart from the *E* complex on the 6th chromosome. As described previously by N. Itikawa in 1944, the *Nc*/*Nc* homozygote is embryonic lethal showing defects in the thoracic segments (see Fig. 2). The silk glands in the lethal embryos were found shrunk and compacted in the affected thoracic regions, while those from wild type embryos were extended into abdominal regions. At this moment we can not determine whether the effect on the silk glands was caused directly by the *Nc* mutation or simply by the physical abnormality of the thorax segments.

Candidate clones for SGF-2 are being screened from the posterior silk gland cDNA library by the DNA-protein binding screening method. PCR and sequencing analyses revealed that homeobox sequences homologous to *Drosophila Deformed, proboscipedia, labial,*

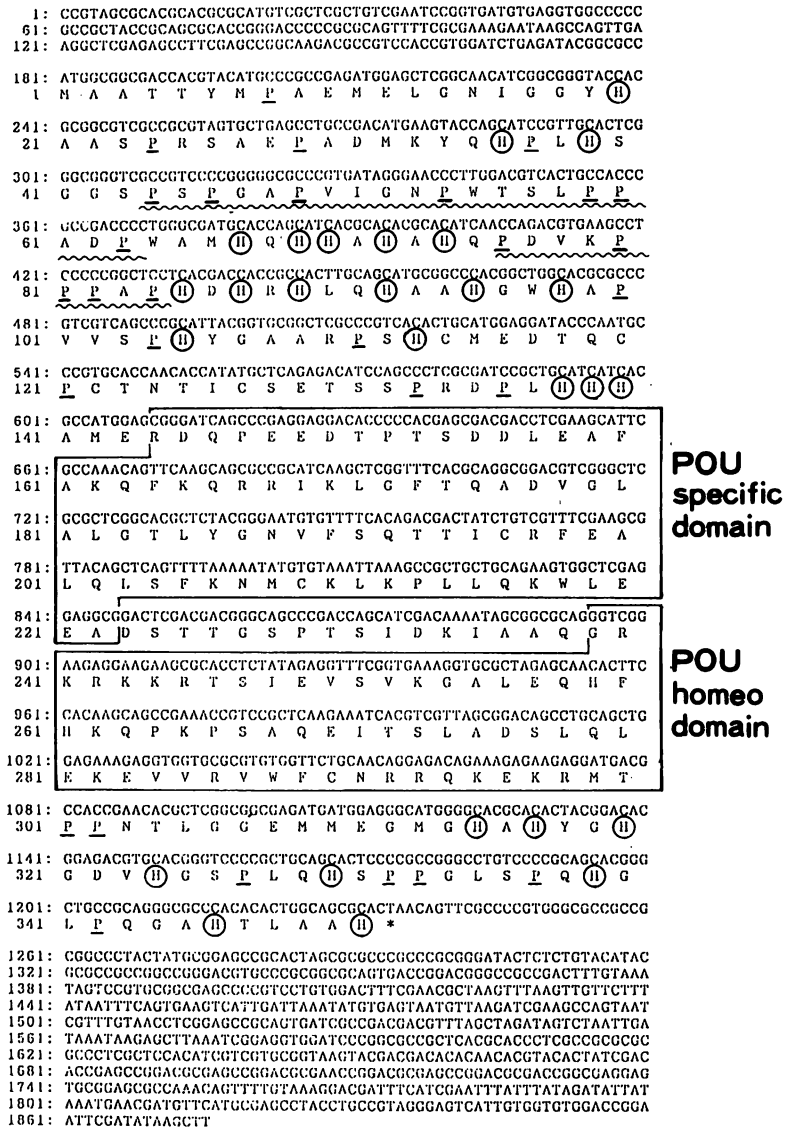

Fig. 1 The DNA sequences of cDNA encoding the POU-M1 protein The amino-acid sequences were deduced from the DNA sequences. Entire open reading frame sequence accommodates repeats of histidine-rich sequences followed by proline-rich sequences in the N-terminal half, POU specific domain and POU homeodomain in the middle, and histidine- and proline-rich sequences in the C-terminus.

caudal, and others are expressed in the posterior silk gland. They are homologous genes mostly expressed at around the head region of *Drosophila*. Since the *Bombyx* silk gland is derived from the labial segment, it is important to identify the roles of these genes and others mentioned above in the silk gland differentiation and/or the silk gene transcription.

The *E* complex in *B. mori* is a homeotic gene complex specifying the

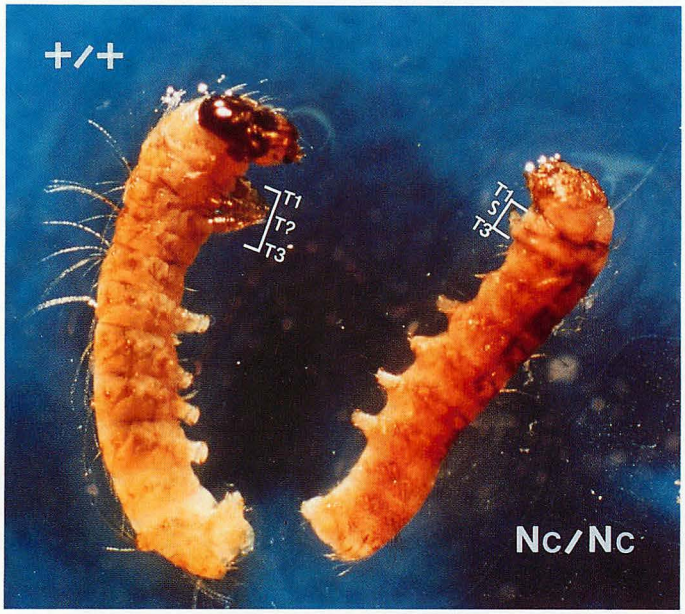

Fig. 2 Morphological appearances of the wild type embryo and the *Nc/Nc* homozygote embryo

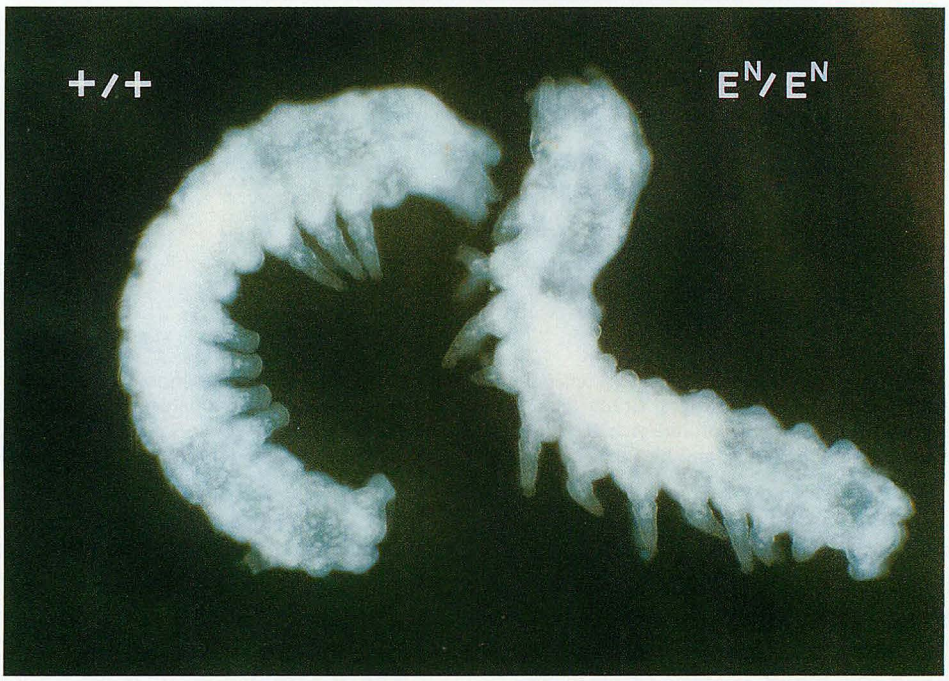

Fig. 3 Morphological appearances of the wild type embryo and the E^N/E^N homozygote embryo

identities of the larval abdominal segments. We presumed that the *E* complex might be similar in structure and function to the *Drosophila* bithorax complex, which specifies from the mesothoracic segment to the eighth abdominal segment in *Drosophila melanogaster*. To test the assumption, we isolated and sequenced three homeobox genes from *Bombyx*. The deduced amino-acid sequences of homeodomain are almost identical to *Drosophila Ultrabithorax, abdominal-A*, and *Abdominal-B* in the bithorax complex. Interesting mutants E^N and E^{Ca} were described by N. Itikawa in 1943 and an E^N/E^N homozygote embryo is shown in Fig. 3. Analyses on E^N and E^{Ca} chromosomes indicate that the *Bombyx Ultrabithorax* and *abdominal-A* genes are deleted in the embryos homozygous for E^N, and the *Bombyx abdominal-A* gene is deleted in the embryos homozygous for E^{Ca}. From these results we conclude that the *E* complex consists of the *Bombyx Ultrabithorax, abdominal-A*, and possibly *Abdominal-B* genes, which may play similar roles to their analogues in the *Drosophila* bithorax complex.

Continuation of these projects would reveal us how the head, thoracic, and abdominal segments are designed, and specifically how the silk gland differentiates from the labial segment and results in transcribing a specific set of genes.

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DIVISION OF MORPHOGENESIS

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The metaplastic changes of some ocular tissues during regeneration of lost parts of the urodele eye are perhaps the clearest examples of differentiation from a once-specialized tissue into another. Actually the lens and neural retina in the newts can be completely regenerated through transdifferentiation of pigmented epithelia. Hence evidence derived from analysis of these phenomena might make a valuable contribution to a much deeper understanding of the mechanisms both of cell differentiation and probably of cellular transformation during carcinogenesis in vertebrate tissues as well. From such a view point members have engaged in cellular and molecular analyses of transdifferentiation utilizing the lens transdifferentiation of the vertebrate pigmented epithelial cells *in vivo* and *in vitro* as a model system, in order to understand the regulatory mechanisms of stabilization in cell differentiation of animal tissue cells, which must be one of fundamental requisites for stable maintenance of multicellular organization of tissues and organs. The following results and development have been achieved by members of this division in 1990.

(1) Factors controlling the lens transdifferentiation of pigmented epithelial cells *in vitro*

Since Itoh and Eguchi introduced a culture method by which the lens transdifferentiation of pigmented epithelial cells of chicken embryos is greatly enhanced, we have utilized this method to study biochemical and molecular biological aspects of the transdifferentiation phenomenon. This culture method uses phenylthiourea and testicular hyaluronidase as important components of the culture medium. When different product lots of commercially available preparations of testicular hyaluronidase were compared, we noticed that they show large variation in their ability to enhance the transdifferentiation, though they have similar activity to degrade hyaluronate. We tried to show whether the hyaluronidase activity itself is important in enhancing the transdifferentiation or whether there are other factors which are really effective.

We purified crude preparation of testicular hyaluronidase by column chromatography and obtained a pure specimen of hyaluronidase, however it showed no activity to enhance the transdifferentiation. According to recent reports that the basic fibroblast growth factor (bFGF) is important in controlling cellular differentiation, we examined whether there is a bFGF-like component in the crude hyaluronidase. We could purify a 18kd protein whose amino acid sequence at N-terminal is the same as that of bFGF. Further, we could show that bFGF purified from bovine brain is also effective in enhancing the transdifferentiation.

Since bFGF alone can not enhance the transdifferentiation and phenylthiourea is needed as well, there must be a special condition of cells where bFGF can exert its effect. As described in the next section, a possibility that the activity of

A chimeric chicken by microinjection of the dissociated blastoderm cells of a black chicken into the blastoderm cavity of a white leghorn embryo.

transforming growth factor (TGF) is precisely controlled through its binding protein in the course of transdifferentiation is also raised. The role of growth factors and their mode of action is our next point of concern.

(2) Gene regulation in the process of lens transdifferentiation from pigmented epithelial cells

To investigate the molecular mechanisms of gene-regulation in the course of transdifferentiation, (i) pigmented epithelial cell- and lens-specific genes were isolated and the expression of these genes was followed in the process of transdifferentiation: (ii) the genes of which the expression is qualitatively and quantitatively regulated during the transdifferentiation were isolated and the function of these genes was investigated.

We have already reported that the lens transdifferentiation from pigmented epithelial cells proceeds through a bipotent intermediate cell state in which the expression of the *c-myc* gene is activated, but neither the expression of pigmented epithelial cell- nor lens-specific genes are activated. Recent analyses of one of the transcriptionally repressed genes during the dedifferentiation process showed that a serine protease inhibitor gene (pP344) is strongly expressed in fully-differentiated pigmented epithelial cells and the product of this gene may play a role in the metabolism of extracellular matrix and the maintenance of the pigmented epithelial cell phenotype. We have also succeeded in isolating the pP64 gene of which the expression is qualitatively regulated at the level of RNA splicing during transdifferentiation. The deduced amino acid sequence of the product of this gene shows high similarity to the human TGF binding protein. These data indicated that the primary structure of the TGF binding protein is regulated by

RNA splicing depending upon cell state during transdifferentiation. Now, investigation are extending to the role of TGF and the TGF binding protein in the process of transdifferentiation.

(3) Establishment of a multipotent cell line derived from human pigmented epithelial cells

Adult human pigmented epithelial cells isolated from an 80-year-old donor readily transdifferentiated when cultured in our established medium consisting of Eagle's minimum essential medium, fetal bovine serum (10%), testicular hyaluronidase (250U/ml medium), and phenylthiourea (0.15 mM). The progeny of completely dedifferentiated pigmented epithelial cells was able to express either lens cell or pigment cell phenotype depending upon given culture conditions. Seven clones with stable growth potential from progeny of dedifferentiated cells were maintained for about one year by repeated subculturing. In two of these clones cells were found to preserve their multipotentiality (at least bipotential) for differentiation. Cells of one of these two clones, designated as 80HdePEC-C6, synchronously synthesized melanin as in melanosomes when maintained in the condition permissive for pigment cell differentiation. The sister cell population of this clone could express lens cell phenotype when reaggregated and maintained in the condition permissive for lens cell differentiation, but not in the monolayer cell culture condition even with the permissive medium. Forty-six per cent of cell population in this clone possessed the normal karyotype as human somatic cells and expressed stable growth potential. In addition, they have maintained their morphology as typical epithelial cells and formed a typical polygonal monolayer cell sheet characteristic to the epithelial cell when attained confluency. This permanent cell line should provide powerful model systems for studying the decision mechanism in cell differentiation of multipotent undifferentiated cells.

(4) Production of the blastoderm chimera of the chicken

We have already succeeded in the introduction of cloned genes into the chicken by the microinjection method in which the cloned genes are microinjected into fertilized eggs and these eggs are incubated *in vitro* in recipient egg shells to hatching. We have also tried to produce transgenic chickens by the blastodermal cell transfer method. We introduced cloned genes into blastodermal cells which are supposed to be embryonic stem cells of the chicken and transferred them into another embryos at the blastoderm stage to produce the blastoderm chimera. Recently, we have succeeded in the efficient production of the blastoderm chimera and the F1 progeny from donor blastodermal cells. Now, we are trying to establish stable embryonic stem cell lines *in vitro* to introduce cloned genes into germ lines.

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DIVISION OF DEVELOPMENTAL BIOLOGY (ADJUNCT)

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Institute Research Fellow: Takefumi Kawata
Visiting Scientist: Michael F. Filosa
(from Toronto University)
JSPS Post-doctoral Fellow: Keiko Takemoto
Graduate Students:
Hajime Nakao (from Kyoto University)
Maki Minami (from Kyoto University)
Takahiro Morio (from Kyoto University)

This division is devoted to the study of the regulatory mechanisms of gene expression involved in cell proliferation and differentiation in higher plants and a cellular slime mold. We have focused on the following two projects:

(1) Regulation of cell cycle-dependent transcription of plant genes.

Histone genes are mostly expressed in the S phase of the cell cycle, coupled to DNA synthesis. The molecular mechanisms of S phase-specific gene expression are unknown. To elucidate the regulatory mechanisms of transcription of plant histone genes, we have investigated *cis*-acting elements and *trans*-acting factors of wheat (*Triticum aestivum*) histone H3 and H4 genes. Until now, we have identified three positively-acting *cis*-elements, the hexameric (ACGT CA), octameric (CGCGGATC), and nonameric (CATCCAACG) motifs, in the proximal region of the H3 promoter. *In vivo* transcription experiments with deletion mutants of the H3 gene suggest that some of these motifs may contribute to the S phase-specific transcription of the H3 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 *trans*-acting factors. The former two specifically bind to the hexameric motif and the latter one to the nonameric motif. The cDNA clones encoding HBP-1a or HBP-1b have been isolated from a wheat cDNA library in λgt11 using South-western method. Sequence analyses of the cDNAs and DNA binding experiments with bacterially expressed HBP-1a and -1b have revealed that both proteins have the characteristic properties as the bZIP-type transcription factors. In wheat seedlings, there is a parallel relationship between the level of the transcripts from the HBP-1a or -1b and H3 genes. Recently, HBP-1a has been shown to function as a repressor in non-proliferating cells in transient expression experiments using rice cultured cells. Analyses of other cDNA clones, cross-hybridizable to HBP-1a or -1b cDNA, have suggested that HBP-1a and -1b are members of two distinct protein families, respectively. Since 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 are now trying to isolate cDNAs for the protein counterparts from various plants and have succeeded in the isolation of some HBP-1b counterparts.

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

The development of the cellular slime mold *Dictyostelium discoideum* is characteristic in that after tissue formation, two types of cells, the prestalk and prespore cells, differentiate in a fixed proportion at the anterior and posterior regions of the tissue, respectively. In these few years, we have concentrated our effort to understand the regulatory mechanisms of gene expression in the process of prespore cell differentiation.

We have isolated two prespore specific genes, Sp96 and Dp87. The Sp96 gene codes for one of the main spore coat proteins. Each of these two genes is unique in the genome and northern-blot analyses have indicated that their mRNAs are accumulated only in the prespore cells at the same stage of development. *In vitro* 'run on' assays have shown that the expression of the two genes is mainly controlled at the transcription level and their transcription is more directly regulated by extracellular cAMP.

To determine the *cis*-regulatory regions of the Sp96 and Dp87 genes, we have used a transformation assay method. In the case of Sp96, we constructed mutant mini-genes with the 5′ upstream, and 3′ downstream sequences, then introduced into *D.discoideum* cells. Northern analyses indicated that the region from position −686 to −494 is important for temporal and cell type-specific transcription of the Sp96 gene. This *cis*-regulatory region coincides with one of the DNase-I hypersensitive sites which appears in the 5′ upstream region of the Dp87 gene after tissue formation. We have identified the *cis*-regulatory region of the Dp87 gene in transformation experiments using chimeric genes which contain different length of the 5′ upstream sequence and the coding sequence of the CAT gene. The region from −447 to −356 was found to be indispensable for the stage and cell type-specific transcription. To confirm that this region really contains the *cis*-regulatory elements of the Dp87 gene, a DNA fragment with sequences up to −447 was jointed upstream of the *Escherichia coli* β-gal gene, introduced into *D.discoideum* cells, and the expression of the chimeric gene in transformed cells was examined by a histochemical staining method of β-galactosidase. Positively stained cells were randomly distributed in the cell mass during the late aggregation stage of development. The number of positive cells dramatically increased at the mount stage at which the accumulation of Dp87 mRNA occurs. The positively stained cells dispersed in the cell mass during the early aggregation stage, and after the tip formation, no positively stained cells were observed in the tip and prestalk regions in migrating slugs. These observations indicate that the 5′ upstream region up to −447 bp is sufficient for the prespore-specific transcription of Dp87 gene, and further support that the distribution pattern of the prestalk and prespore cells in slug is caused by sorting-out of differentiated cells.

We have also identified nuclear factors which specifically bind to the *cis*-regulatory regions of these genes by gel retardation assays. The DNA-binding proteins, specific to these *cis*-regulatory regions, were found only in the nuclear extract from the slug cells. Competition experiments using synthetic oligonucleotides were carried out to assign the precise binding sequences. These studies indicated that the binding sequence in the Sp96 gene is highly homologous to that in the Dp87 gene. Competition assays using the Dp87 or Sp96 motifs suggested that the DNA-binding factors which interact with the homologous *cis*-regulatory regions are probably similar to each other. It is still unclear whether the nuclear proteins are *trans*-regulatory factors of the two prespore-specific genes. The purification of these proteins and the isolation of their cDNA clones are now in progress.

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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 SENSORY PROCESSING

Professor: To be appointed

DIVISION OF CELLULAR REGULATION

Professor: Norio Murata
Associate Professor: Hidenori Hayashi
Research Associates: Takao Kondo
Ikuo Nishida
*NIBB Visiting Professor: Antoni R. Slabas**
NIBB Post-doctoral Fellow: Hiroyuki Ohta
JSPS Post-doctoral Fellow: Hajime Wada
Visiting Scientists: Toshio Sakamoto[1]
Yoshitaka Nishiyama[2]
Yasushi Tasaka[3]
Zoltan Gombos[4]
Prasanna S. Mohanty[5]
George C. Papageorgiou[6]
Csaba Lehel[7]
Suleyman I. Allakhverdiev[8]
Mahir D. Mamedov[9]
Chin B. Lee[10]
Graduate Student: Hiroyuki Imai

*(*from University of Durham, England)*
([1]from The University of Tokyo)
([2]from The University of Tokyo)
([3]from Green Research Center, Ltd.)
([4]from Hungarian Academy Sciences, Hungary)
([5]from Jawaharlal Nehru University, India)
([6]from National Research Center Demokritos, Greece)
([7]from Hungarian Academy Sciences, Hungary)
([8]from USSR Academy of Science, USSR)
([9]from Moscow State University, USSR)
([10]from Dongeui University, Korea)

Two important objectives of the current research efforts of this division are to understand (I) the molecular mechanisms of low-temperature tolerance and adaptation in higher plants and microalgae, and (II) the structure and function of proteins of photosynthetic membranes.

I. The molecular mechanisms of low-temperature tolerance and adaptation in higher plants and microalgae.

During this year three major lines of research have been pursued.

(1) *Desaturation of fatty acids in microalgae and low-temperature tolerance* (i) Understanding of the mechanism of low-temperature tolerance is one of our long-term objectives. A cyanobacterial low-temperature sensitive mutant has been isolated which has reduced low-temperature tolerance. The mutant was defective in desaturation of fatty acids at the delta-12 position. A gene (designated as *desA*) whose product is crucial for desaturation of fatty acids at the delta-12 position has been cloned from the cyanobacterium, *Synechocystis* PCC 6803, using the low-temperature sensitive mutant. Restoration of the mutant with the cloned wild-type gene resulted in recovered low-temperature tolerance (see Figure). Alteration of the fatty-acid composition by genetic manipulation could play a crucial role in our understanding of low-temperature tolerance. Experiment has been performed in this area. Transformation of wild type and another desaturation mutant (Fad6), which is defective in desaturation of fatty acids at the delta-6 position, of *Synechocystis* PCC6803, with a disrupted *desA* which had been interrupted by kanamycin-resistance gene cartridge changed the fatty-acid unsaturation of membrane lipids of the recipients. This result demonstrates that the fatty-acid unsaturation of membrane lipids can be controlled by genetic manipulation of *desA* in *Synechocystis* PCC6803. Other genes responsible for plant-type desaturation of fatty acids are being cloned from cyanobacteria and higher plants. (ii) A low-temperature sensitive strain of the cyanobacterium, *Anacystis nidulans* R2-SPc, was transformed with *desA* from *Synechocystis* PCC6803. The introduction of this gene increased the tolerance of the recipient to low temperature. This result demonstrates that the low-temperature tolerance of cyanobacteria can be enhanced by genetic manipulation of fatty-acid unsaturation. (iii) Effects of fatty-acid unsaturation on thermal properties of photosynthetic activities have been

studied with use of cyanobacteria transformed with the disrupted *desA* gene of *Synechocystis* PCC6803 which causes alteration in fatty-acid unsaturation. It was found that the temperature profile of the heat inactivation of photosystem 1 and photosystem 2 was not affected by the change in fatty-acid unsaturation. Tolerance to photoinhibition at low temperature was decreased in transformation with fatty-acid unsaturation.

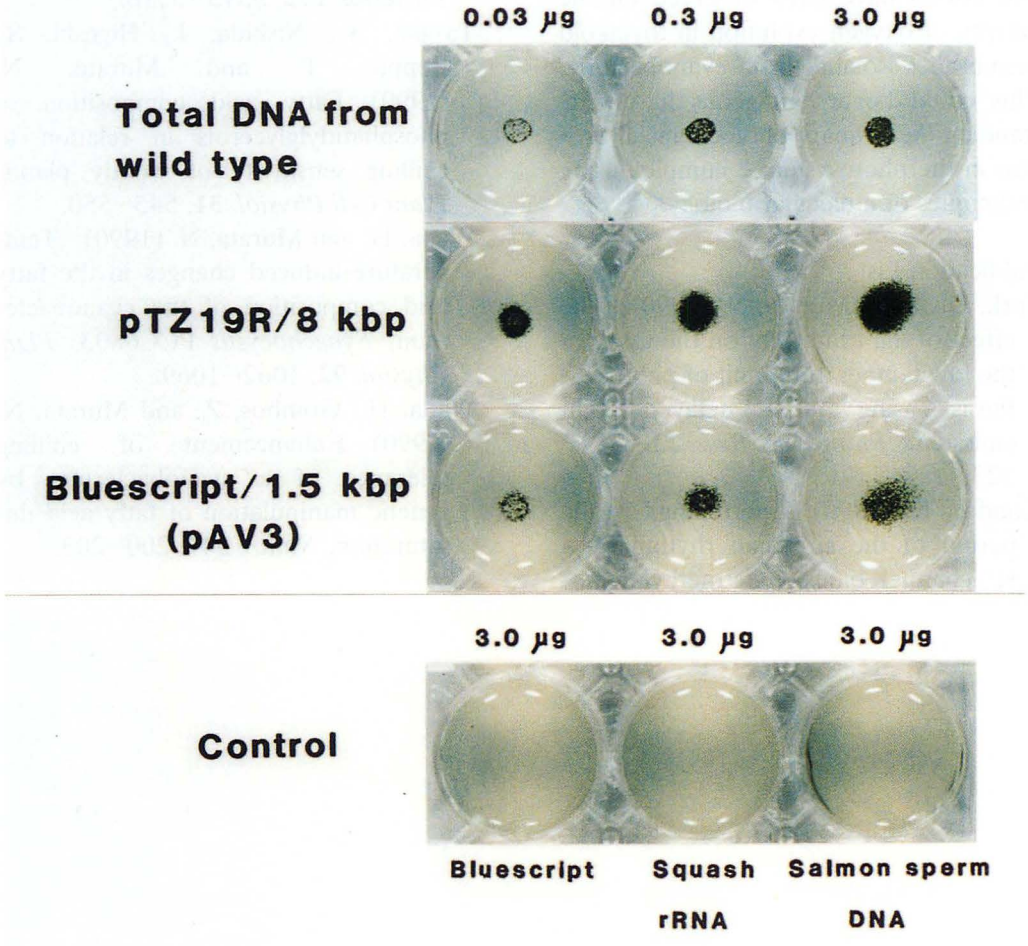
(2) *Acyltransferase from low-temperature tolerant plant.* The enzyme, glycerol-3-phosphate acyltransferase, produces special lipid molecular species that are suspected to be responsible for the low-temperature sensitivity of higher plants. In order to evaluate the role of this enzyme in low-temperature sensitivity of higher plants, the enzyme has been previously purified by our group from a low-temperature-sensitive plant, squash, and a cDNA clone encoding its precursor protein has been obtained. In order to complement and extend our knowledge in acyltransferase and their role in low-temperature tolerance we have isolated the genomic gene (designated as *pls1* gene) for the acyltransferase from a low-temperature-tolerant plant, *Arabidopsis.* A cDNA clone corresponding to the mRNA for the *pls1* gene has also been isolated and characterized. The *pls1* gene (3,397 bp) contains 11 introns (1,412 bp in total), in its open-reading frame (1,377 bp), and one intron (94 bp) in the 3′-untranslated region. The open-reading frame encodes a polypeptide of 459 amino-acids residues. It is predicted that the protein has a leader sequence of 90 amino-acid residues which is cleaved to produce the mature protein of 369 amino-acid residues. In accordance with this prediction the *Arabidopsis* mature protein exhibited enzymatic activity when the cDNA gene corresponding to the 369 amino-acid residues is over-expressed in *E. coli* as a fusion protein, with a small phage leader peptide.

(3) *Plant transformation with acyltransferase gene.* Tobacco plants were transformed with Ti-plasmids that were constructed with the squash and *Arabidopsis* cDNAs for acyltransferases under control of the 35S CaMV promoter and the NOS terminator. Evaluation of transformants with respect to fatty-acid composition of their leaf lipids and low-temperature sensitivity is in progress.

II. The structure and function of proteins of photosynthetic membranes.

Photosynthesis research is focused on the machinery of the oxygen-evolving complex of photosystem 2. This complex is embedded in the chloroplast thylakoid membrane and consists of approximately 20 different protein components. In order to elucidate the molecular mechanism of photosynthetic oxygen evolution, the following subjects are being investigated using techniques of biochemistry, physicochemistry and molecular biology: (i) Purification and elucidation of the properties of the structural components of the complex. (ii) Three-dimensional interaction of the protein components within the complex. (iii) The functional role of each component on oxygen evolution. (iv) Identification of low-molecular weight components of the oxygen-evolving complex and their genes.

In these subject areas, the protective effect of glycine betaine on the photosystem 2 activity was most extensively investigated. Glycine betaine is a nontoxic osmolite found in halophilic higher plants and microbial plants. Spinach photosystem 2 complex loses its oxygen-



Complementation of a desaturation mutant (Fad12) of *Synechocystis* PCC6803. The cells of Fad12 were applied to an agar plate and DNA solutions were spotted on the center of agar plates. Then the agar plates were incubated at low temperature in the light. The cells of Fad12 were able to grow very slowly at low temperature (see control). When the total DNA from wild type and the recombinant plasmids containing *desA* gene (pTZ19R/8 kbp and pAV3) were applied onto agar plates, the transformants, that recovered growth at low temperature, appeared on the plates as a spot.

evolving activity by dark incubation at room temperature for a few hours. The loss of the activity is greatly depressed in the presence of glycine betaine at high concentration (*e.g.* 1 M). Several analyses indicate that glycine betaine protects the Mn cluster, which serves an essential role in oxygen evolution, by maintaining the structure and interaction of the hydrophilic protein subunits in the photosystem 2 complex. Similar protective effects have been observed on the activity of oxygen evolution in thylakoid membranes isolated from cyanobacteria. This could be advantageous in understanding the primary photochemical process in the photosystem 2 complex using techniques of molecular biology.

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DIVISION OF BIOLOGICAL REGULATION (ADJUNCT)

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Satoru Tokutomi
Hitoshi Mori
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(Nagoya University)*

Growth and development of higher plants are regulated by plant hormones, but they are also greatly influenced by changes in environmental conditions, which 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 aspects; (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.

(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 and accompanies massive accumulation of cell wall materials which are mainly various polysaccharides. To maintain the rapid growth, sucrose must be continuously metabolized. We have found that a dramatic change of sucrose-metabolizing enzymes, invertase and sucrose synthase, occurred in stems and leaves which are correlated with their growth. However, the contribution of these two enzymes to growth changes in a tissue-specific manner. Rapid growth of hypocotyl (stem below the cotyledons) in the dark was correlated with rapid increases in both invertase and sucrose synthase, whereas only sucrose synthase increased in slowly expanding plumule (young etiolated primary leaves). In the light, rapid decreases in both enzyme activities were followed by cessation of hypocotyl growth, and invertase but not sucrose synthase increased concomitant with growth of epicotyl (stem above the cotyledons) and expansion of leaves. Both invertase and sucrose synthase have been purified from mung bean seedlings, and their respective cDNAs have been cloned (M. Arai, H. Mori and H. Imaseki, unpublisehd results). Expression of these genes are being examined in relation to growth capacity of the various tissues.

(2) Mechanisms of gene expression regulated by plant hormones and tissue wounding. Auxin induces many physiological processes in 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. Sequence analysis revealed that two of them were new to the literature, and their characterization is under way (K.T. Yamamoto, H. Mori and H. Imaseki, unpublished results). Ethylene biosynthesis is variously regulated by other plant hormones and environmental stress, and in all cases, the regulatory step is the formation of 1-aminocyclopropane-1-carboxylic acid (ACC) from S-adenosylmethionine. However, the ACC synthase induced by auxin is different from that induced by tissue

wounding, and the cDNA for the wound-induced ACC synthase from winter squash fruits has been cloned and sequenced (1). The gene is induced only by tissue wounding, but not by other plant hormones. Subsequently, the basic structure of the gene was determined. The gene has four introns and the initiation site of transcription is 361 base upstream of the initiation codon (H. Mori, N. Nakajima and H. Imaseki, unpublished results). To compare genes for the enzymes induced by different stimuli, cloning of cDNAs for the ripening-associated enzyme and the auxin-induced enzyme is under way.

(3) Mechanisms of acquisition of thermotolerance by non-lethal high temperature. When plant seedlings are exposed to a lethal high temperature (53°) for a short period (20 min) and returned to a growth temperature (28°), they are irreversibly damaged and eventually die. However, a short (1 hour) exposure to a non-lethal high temperature (40°) provides plants tolerance to the subsequent exposure to an otherwise lethal high temperature. This acquisition of thermal tolerance was correlated with changes in physicochemical properties of the membranes. Lethal high temperature caused the formation of lysophosphatidylethanolamine (PE) in the membrane lipids of the control plants but not in the thermotolerant plants. PE was found to increase membrane permeability in isolated protoplasts (S. Tokutomi and H. Imaseki, unpublished results), and the thermotolerance acquired by non-lethal high temperature is related to a mechanism by which the formation of PE is suppressed at lethal high temperature.

Publication List

1) Nakajima, N., Mori, H., Yamazaki, K. and Imaseki, H. (1990) Molecular cloning and sequence of a complementary DNA encoding 1-aminocyclopropane-1-carboxylate synthase induced by tissue wounding. *Plant Cell Physiol.* **31**, 1021–1029.

DIVISION OF BEHAVIOR AND NEUROBIOLOGY (ADJUNCT)

Professor: Katsuhiko Mikoshiba
Associate Professor: Masaharu Ogawa
Research Associates: Taka-aki Tamura
Teiichi Furuichi
Visiting Scientists: Kousuke Sumita[*]
Ichirou Fujino[*]
Norihiko Yamada[*]
Yoshimi Ryou[*]
Masuo Gotoh[**]
Tetsuichirou Saitoh[+]
Makoto Nakamura[#]
Visiting Research Fellow: Yasutaka Makino[@]
Graduate Students: Atsushi Miyawaki[*]
Shingo Yoshikawa[*]
Takafumi Inoue[*]
Michisuke Yuzaki[++]

(* *from Osaka University)*
(** *from Kanazawa University)*
(+ *from University of Tokyo)*
(# *from Hokkaido University)*
(@ *from Ono Pharmaceutical Co., Ltd.)*
(++ *from Jichi Medical School)*

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. The main themes of our division are:

(1) 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. 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 cerebellar neuronal network system, especially in the 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.

(2) Studies on the mechanisms of brain specific gene expression. We have been studying the structure and function of the promoters of the myelin basic protein gene by using *in vitro* transcription system derived from animal tissues. Our aim is to understand the molecular mechanisms of tissue-specific gene expression in the nervous system.

Publication List

1) Aoyama, A., Tamura, T. and Mikoshiba, K. (1990) Regulation of brain-specific transcription of the mouse myelin basic protein gene: Function of the NF-1-binding site in the distal promoter. *Biochem. Biophys. Res. Commun.* **167**, 648–653.
2) Asaga, H., Inoue, T. and Mikoshiba, K. (1990) Two-dimensional gel electrophoretic analysis of the cerebella from neuropathologically-mutant weaver, nervous and staggerer mice. *Develop. Growth and Differ.* **32**, 235–244.
3) Furuichi, T., Shiota, C. and Mikoshiba, K. (1990) Distribution of inositol 1,4,5-trisphosphate receptor mRNA in mouse tissues. *FEBS Lett.* **267**, 85–88.
4) Ikenaka, K., Fujino, I., Morita, N., Iwasaki, Y., Miura, M., Kagawa, T., Nakahira, K. and Mikoshiba, K. (1990) Reliable transient promoter assay using fluorescein-di-β-D-galactopyranoside substrate. *DNA and*

Cell Biol. **9**, 279–286.

5) Ikenaka, K., Nakahira, K., Takayama, C., Wada, K., Hatanaka, H, and Mikoshiba, K. (1990) Nerve growth factor rapidly induces expression of the 68kDa neurofilament gene by posttranscriptional modification in PC 12h-R cells. *J. Biol. Chem.* **265**, 19782–19785.
6) Ino, H., Nakade, S., Niinobe, M., Ikenaka, K., Teshima, T., Wakamiya, T., Matsumoto, T., Shiba, T., Kawai, N. and Mikoshiba, K. (1990) Characterization of binding sites for spider toxin, [^{3}H]NSTX-3, in the rat brain. *Neurosci. Res.* **8**, 29–39.
7) Inoue, T., Tamura, T., Furuichi, T. and Mikoshiba, K. (1990) Isolation of complementary DNAs encoding a cerebellum-enriched NF1 family that activate transcription from the mouse myelin basic protein promoter. *J. Biol. Chem.* **265**, 19065–19070.
8) Kondo, T., Ikenaka, K., Kato, H., Ito, K., Aimoto, S., Hojo, H. and Mikoshiba, K. (1990) Long-term enhancement of synaptic transmission by synthetic mast cell degranulating peptide and its localization of binding sites in hippocampus. *Neurosci. Res.* **8**, 147–157.
9) Maeda, N., Niinobe, M. and Mikoshiba, K. (1990) A cerebellar Purkinje cell marker P_{400} protein is an inositol 1,4,5-trisphosphate ($InsP_3$) receptor protein — Purification and characterization of $InsP_3$ receptor complex. *EMBO J.* **9**, 561–567.
10) Miura, M., Tamura, T. and Mikoshiba, K. (1990) Cell-specific expression of the mouse glial fibrillary acidic protein gene: Identification of the cis-trans-acting promoter elements astrocyte-specific expression. *J. Neurochem.* **55**, 1180–1188.
11) Mikoshiba, K., Okano, H., Aruga, J., Tamura, T., Miura, M., Ikenaka, K. and Nakagawa, T. (1990) Chimeric and molecular genetic analysis of myelin deficient (shiverer and mld) mutant mice. *Proc. New York Acad. Sci.* "Myelination and dysmyelination". **605**, 166–182.
12) Miyawaki, A., Furuichi, T., Maeda, N. and Mikoshiba, K. (1990) Expressed cerebellar-type inositol 1,4,5-trisphosphate receptor, P_{400} has calcium release activity in a fibroblast L cell line. *Neuron* **5**, 11–18.
13) Nakahira, K., Ikenaka, K., Wada, K., Tamura, T., Furuichi, T. and Mikoshiba, K. (1990) Structure of the 68 KDa neurofilament gene and regulation of its expression. *J. Biol. Chem.* **265**, 19786–19791.
14) Tamura, T., Aoyama, A., Inoue, T., Miura, M. and Mikoshiba, K. (1990) A new transcription element in the JC virus enhancer. *J. Gen. Virol.* **71**, 1829–1833.
15) Tamura, T., Sumita, K., Hirose, S. and Mikoshiba, K. (1990) Core promoter of the mouse myelin basic protein gene governs brain specific transcription *in vitro*. *EMBO J.* **9**, 3101–3108.

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 OF GENE EXPRESSION AND REGULATION I

Professor: Yoshiro Shimura
Associate Professor: Kiyotaka Okada
Research Associate: Hideaki Shiraishi
Post-doctoral Fellows: Makako K. Komaki
Graduate Students: Kazuhito Akama
Hiroyuki Okamoto
Koji Sakamoto

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 might 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 (dwarf: mutants with short inflorescence axis), 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; Shiraishi *et al.*, in preparation). 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.*, in press). 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 chemical inhibitors on auxin polar transport. The polar

Fig. 1. Inflorescences of *Arabidopsis thaliana* wild type (right) and the *pin-formed* mutant (left). No floral buds are formed on the inflorescence axis of the mutant.

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.*, in press; 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.

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 straight downward on the agar surface. When the plates were put aside, roots bend 90 degrees and grow to the new direction of gravity. If the plates were covered with black cloth and illuminated from 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

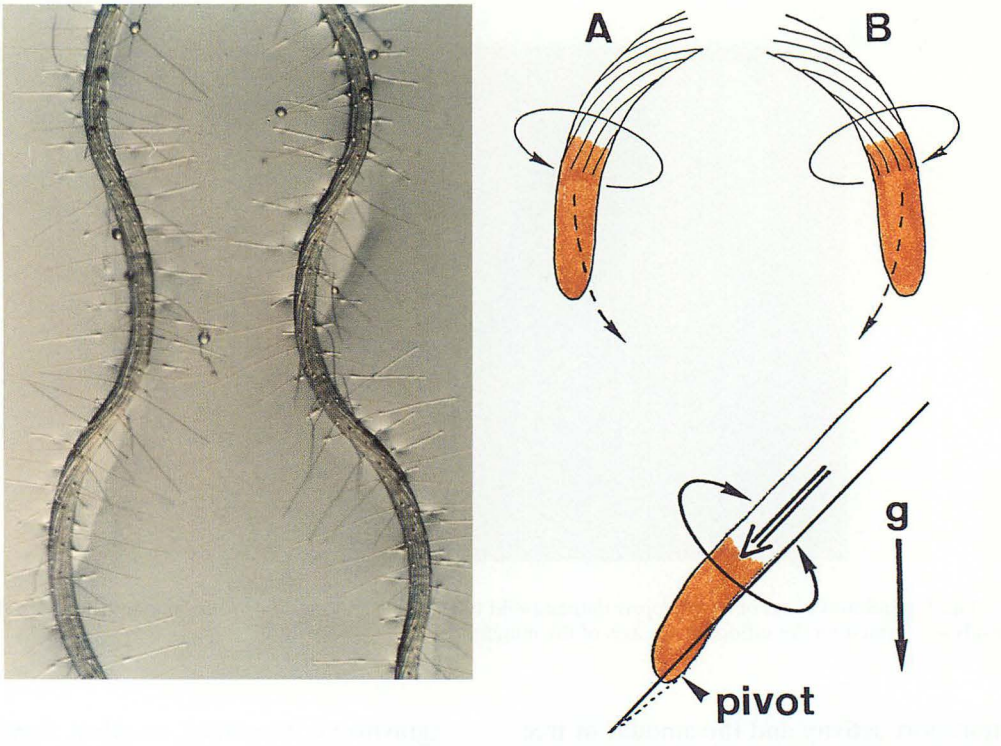


Fig. 2. Waving growth of roots of *Arabidopsis thaliana* wild type on the angled agar plates. Although the roots grow straightly downward on the surface of vertical agar plates, the roots form a wavy pattern on agar plates angled to 45 degrees. The wavy pattern is due to periodical reversion of root-tip rotation induced by obstacle-touching stimulus perceived at the root tip. A. Right-hand rotation causes counterclockwise curve. B. Left-hand rotation causes clockwise curve.

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, 1990). Several mutants also show abnormal gravitropism and/or phototropism. These results indicate that root gravitropic, phototropic and obstacle-escaping responses share at least in part a common genetic regulatory mechanism.

Attempts are being made to identify and isolate the genes responsible for the mutants using the transformation systems mediated by the Ti-plasmid vectors or by a direct gene-tagging system. For such experiments, it is absolutely necessary to develop a good, efficient system of transformation and transgenic plant regeneration. We have tested several combinations of *A. thaliana* ecotypes and *Agrobacteria* strains and established an efficient system (Akama *et al.*, in preparation).

Publication List

Okada, K. and Shimura, Y. (1990) Reversible root tip rotation in *Arabidopsis* seedlings induced by obstacle-touching stimulus. *Science* **250**, 274–276.

DIVISION OF GENE EXPRESSION AND REGULATION II

Professor: Takashi Horiuchi
*Visiting Graduate Students: Masumi Hidaka**
*Takehiko Kobayashi**

(from Kyushu University)*

The laboratory was set up in December 1990. Main subjects of investigation will be the molecular analysis of DNA replication, recombination and repair in procaryotes and eucaryotes. DNA replication termination processes and mechanisms of homologous recombination enhanced by initiation and termination events will receive particular attention. These studies are expected to elucidate the links between DNA replication, chromosomal partition and recombination.

TECHNOLOGY DEPARTMENT

Head: Hachiro Honda

Common Facility Group
Chief: Hiroyuki Hattori

Research Support Facilities
Chieko Nanba
Masahiro Kawaguchi
Mamoru Kubota
Takashi Yamamoto
Tomoki Miwa

Radioisotope Facility
Kazuhiko Furukawa (Unit Chief)
Yoshimi Matsuda
Mitsuaki Higashi

Center for Analytical Instruments
Hiroko Kajiura (Unit Sub-chief)
Hisashi Kojima
Yukiko Koyama

Glassware Washing Facility
Toshio Ohkawa
Hisaya Uemura

Research Support Group

Cell Biology Group
Akio Murakami (Unit Sub-chief)
Masayo Iwaki
Maki Kondo

Developmental Biology Group
Shinji Adachi (Unit Sub-chief)
Sachiko Fukada
Kaoru Katou
Miyuki Ohkubo

Regulation Biology Group
Yoko Fujimura
Shoichi Higashi

Gene Expression and Regulation Group
Hideko Nonaka

The Technology Department is a supporting organization for reseachers 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 (Large Spectrograph)
Yoshio Hamada (Tissue and Cell Culture)

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 wavelength ranged from 250 nm to 1,000 nm onto its focal curve of 10 m in length. The fluence 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 microbeam irradiator (up to 200 μm s^{-1} in tracking speed, down to 2 μm in beam diameter) with an infrared phase-contrast observation system.

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 actions of light on living organisms have been conducted.

Publication List

Furusawa, Y., Suzuki, K., and Sasaki, M. (1990) Biological and physical dosimeters for monitoring solar UV-B light. *J. Radiat. Res.* **31**, 189–206.

Iino, M. (1990) Phototroposm: mechanisms and ecological implications. *Plant Cell Environ.* **13**, 633–650.

Matsunaga, T., Mori, T., and Nikaido, O. (1990) Base sequence specificity of a monoclonal antibody binding to (6-4) photoproducts. *Mutat. Res.* **235**, 187–194.

Mori, T., Matsunaga, T., Chang, C.-C., Trosko, J. E., and Nikaido, O. (1990) In situ (6-4) photoproduct determination by laser cytometry and autoradiography. *Mutat. Res.* **236**, 99–105.

Nakagawa, T., Oda, J., Koizumi, H., Furukawa, T., Yasui, C., and Ueda, T. (1990) Ultraviolet action spectrum for intracellular free Ca^{2+} increase in human epidermal keratinocytes. *Cell Struct. Funct.* **15**, 175–179.

Nakamura, S., Watanabe, M., Hatase, K., and Kojima, M. K. (1990) Light inhibits flagellation in a *Chlamydomonas* mutant. *Plant Cell Physiol.* **31**, 399–401.

Nakahigashi, K., Komine, Y., Watanabe, M., and Inokuchi, H. (1990) An *E. coli* promoter that is sensitive to visible light. *Jpn. J. Genet.* **65**, 381–386.

Ogura, T., and Takahashi, S. (1990) Observation of the Fe^{II}-O_2 stretching raman band for cytochrome oxidase compound A at ambient temperature. *J. Amer. Chem. Soc.* **112**, 5630–5631.

Ogura, T., Takahashi, S., Shinzawa-Ito, K., Yoshikawa, S., and Kitagawa, T. (1990) Observation of the Fe^{4+}=O stretching Raman band for cytochrome oxidase compound B at ambient temperature. *J. Biol. Chem.* **265**, 14721–14723.

Sugai, M., and Furuya, M. (1990) Photo-inhibition of red-light induced spore germination in *Pteris vittata:* cyanide, azide and ethanol counteracts restorable inhibitory action of near UV and blue-light but not that of far UV. *Plant Cell Physiol.* **31**, 415–418.

Tada, M., Watanabe, M., and Tada, Y. (1990) Mechanism of photoregulated carotenogenesis in *Rhodotorula minuta.* VII. Action spectrum for photoinduced carotenogenesis. *Plant Cell Physiol.* **31**, 241–246.

Tokutomi, S., Mizutani, Y., Anni, H., and Kitagawa, T. (1990) Resonance Raman spectra of large pea phytoch rome at ambient temperature: difference in chromophore protonation between red- and far red-absorbing forms. *FEBS Lett.*, **269**, 341–344.

Torikai, A., Ohno, M,, and Fueki, K. (1990) Photodegradation of poly (methyl methacrylate) by monochromatic light: Quantum yeild, effect of wavelength and light intensity. *J. Appl. Polym. Sci.* **41**, 1023–1032.

Ueda, T. (1990) Photosensory transduction in the slime mold *Physarum polycephalum. Trends Photochem. Photobiol.* **1**, 25–29.

Ueda, T., Nakagaki, T., and Yamada, T. (1990) Dynamic organization of ATP and birefringent fibrils during free locomotion and galvanotaxis in the plasmodium of *Physarum polycephalum. J. Cell Biol.* **110**, 1097–1102.

Van Volkenburgh, E., Cleland, R. E., and Watanabe, M. (1990) Light-stimulated cell expansion in bean (*Phaseolus vulgaris* L.) leaves. II. Quantity and quality of light required. *Planta* **182**, 77–80.

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 measuring computerized-videomicrographs of the motile behavior of the cells at the cellular and subcellular levels. Photoreceptive and signal transduction mechanisms of algal gametogenesis are also studied by action spectroscopy.

Publication List

Tada, M., Watanabe, M., and Tada, Y. (1990) Mechanism of photoregulated carotenogenesis in *Rhodotorula minuta.* VII. Action spectrum for photoinduced carotenogenesis. *Plant Cell Physiol.* **31**, 241–246.

Nakamura, S., Watanabe, M., Hatase, K., and Kojima, M. K. (1990) Light inhibits flagellation in a *Chlamydo-*

monas mutant. *Plant Cell Physiol.* **31**, 399–401.

Van Volkenburgh, E., Cleland, R. E., and Watanabe, M. (1990) Light-stimulated cell expansion in bean (*Phaseolus vulgaris* L.) leaves. II. Quantity and quality of light required. *Planta* **182**, 77–80.

Nakahigashi, K., Komine, Y., Watanabe, M., and Inokuchi, H. (1990) An *E. coli* promoter that is sensitive to visible light. *Jpn. J. Genet.* **65**, 381–386.

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.

Laboratory Computer Facility: The NIBB's computing is handled by a network of Digital Equipment Corporation's VAX 11/780 and VAX 2,000 with Floating Point System's AP120B and 5310 array processors. An Ethernet interfaces the network with a number of laboratory PC's. An extensive software system for time-series analysis developed in-house has been operating for the last several years. A limited number of image processing routines are also available.

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 glass-houses with precise 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
Associate Professor: Kohji Hasunuma (moved)
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 provide users appropriate guidance for radioisotope handling.

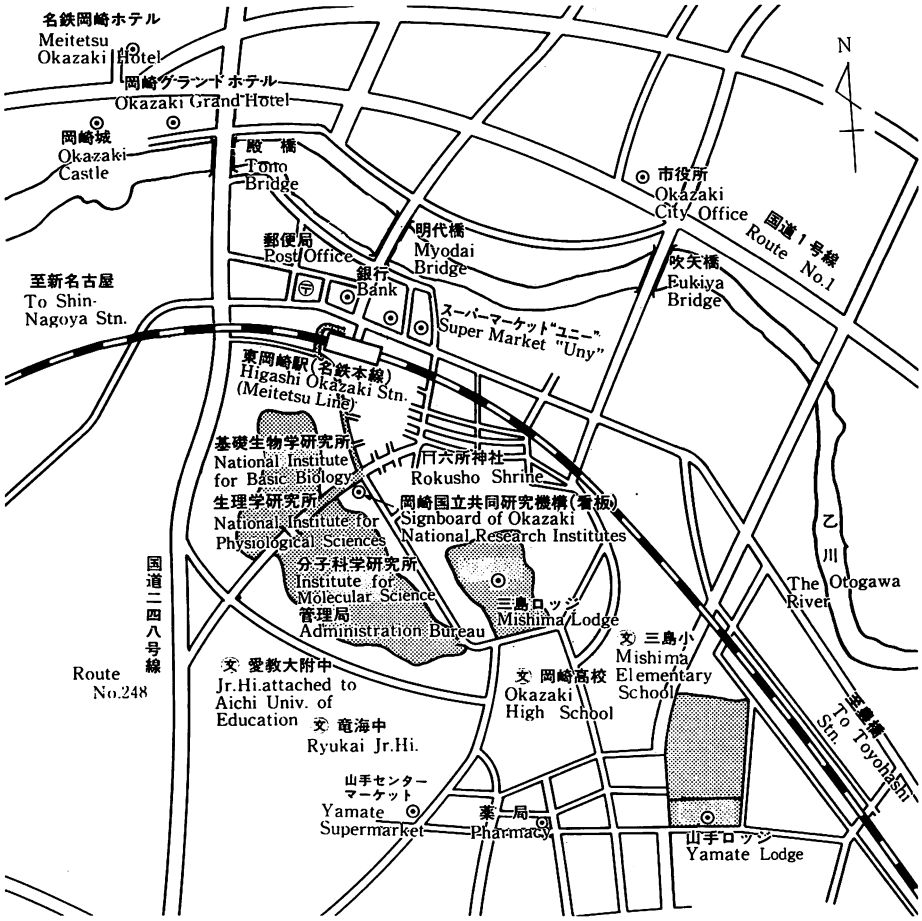
The faculty of the facility conducted his own research on the analysis of signal transduction system in plants and fungi. The study in 1990 was mostly focused on higher plants. Analysis of proteins in the plasma membrane of etiolated pea seelings indicated that (1) red light stimulates ATP-GTP binding to 4 peptides in the membranes, (2) among them, a 37kDa peptide was found to react with the antibody against the α-subunit of transducin, and red light causes loss of this peptide from the membrane, and (3) ADP-ribosylation of this peptide by the action of pertussis toxin is stimulated by red light. These features suggest that transduction of light signal in pea seedlings involves the action of ATP-GTP binding proteins.

CENTER OF FACILITY FOR ANALYTICAL INSTRUMENTS (managed by NIBB)

Head: Norio Murata

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

1. Section for Chemical Analysis
   - Amino-Acid Analyzer
     - HITACHI 835
   - Gas Chromatograph
     - SHIMADZU GC-7APTF
   - 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
     - SHIMADZU RF-5000
   - Spectrophotometer
     - HITACHI 330
   - Spectropolarimeter
     - JASCO J-40S
4. Section for Physical Analysis
   - Analytical Ultracentrifuge
     - HITACHI 282
   - Differential-Scanning Calorimeter
     - SEIKO DSC100
   - EPR Spectrometer
     - BRUKER ER 200D
   - GC-Mass Spectrometer
     - HITACHI M-80
   - GC/LC-Mass Spectrometer
     - JEOL DX-300
   - NMR Spectrometer
     - BRUKER AMX 360wb
   - Viscometer
     - CONTRAVES RM-30
5. Section for Microscopic Analysis
   - Film Data Analysis System
     - NAC MOVIAS GP-2000
   - Image Analyzer
     - KONTRON IBAS-I & II
   - Microscope Photometer
     - CARL ZEISS MPM 03-FL
   - Two-Dimension Microdensitometer
     - JOYCE LOEBL 3CS



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