BIOCHEMICAL STUDIES ON REGULATORY MECHANISMS OF CELL FUSION IN CELLULAR SLIME MOLDS

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Contents

ABSTRACT

Regulation mechanisms of sexual cell fusion were studied on cellular slime molds.

The cellular slime molds have the alternative developmental pathways of sexual or asexual. In heterothallic strains of *Dictyostelium discoideum*, haploid amoeboid cells acquire their fusion competence under particular environmental conditions, such as darkness and excess water, and fuse sexually only between the two mating type cells, HM1 and NC4, to produce diploid zygote which develops into a macrocyst. During maturation of a macrocyst, meiosis is taken place and a number of recombinant offsprings emerge from a mature macrocyst.

It has been known that there are two cell surface glycoproteins, gp70 and gp138, which participate in this sexual cell fusion process. One of these glycoproteins, gp70, is assumed to be working in mutual recognition of cells, because this glycoprotein only appears on fusion-competent HM1 cell surface, but not on NC4. In the chapter 1, experiments were performed to investigate the regulatory mechanisms of gp70 function and the results clarified that gp70 is induced on cell surface only by the existence of excess water. However, if there is light, it induces some other molecules repressing gp70 function on HM1 cell sur-

face. As the result, the functional gp70 expresses only in the dark.

Artificially induced asexual cell fusion were studied in the chapter 2. Strain CK-8 of *Polysphondylium pallidum* has been known to be a killer in cellular slime molds. The cells of this strain produce a cell fusion induction factor(s) besides a killer factor during their growth. I attempted to purify the cell fusion induction factor and discovered that the factor is a highly hydrophobic glycoprotein of approximately 65 KDa in molecular mass, forming a large complex.

This artificially induced cell fusion seemed to be useful to produce artificially many kinds of hybrids and perform better genetic analysis in cellular slime molds. So, I investigated whether hybrids are really formed by the induced cell fusion or not. The results clarified that very few nuclear fusion occurred after the induction of cell fusion. In other words, the factor induces only cell fusion but not nuclear fusion. However, this system inducing cell fusion by the factor would be still useful for the study of the relationship between cell fusion and nuclear fusion and for the analysis of their regulatory mechanisms. Furthermore, it would become a good model system to investigate molecular mechanisms of cell fusion.

GENERAL INTRODUCTION

Cell fusion, such as fertilization of sperm and eggs and myoblast fusion in muscle development, has been studied by many investigators for a long time (Yamaguchi, 1988; Wakelam, 1988). In fertilization, specific glycoproteins adhering to egg were isolated from sea urchin sperm, and a specific protein on the certain virion surface, playing an important role for membrane fusion, is well studied (Stegmann *et al.*, 1989; White, 1990).

Recently, we discovered and isolated glycoproteins involved in sexual cell fusion of cellular slime molds (Urushihara *et al.*, 1988; Ishikawa *et al.*, 1990, Suzuki and Yanagisawa, 1989b; 1990). Cellular slime molds have the two alternative developmental pathways , fruiting-body formation or macrocyst formation. In fruiting-body formation, thousands of haploid amoeboid cells which ceased their proliferation come together to form a multicellular aggregate and subsequently produce a fruiting-body consisting a spore mass and stalk. However, under certain environmental conditions; such as in the dark and in the excess of water, they produce a macrocyst instead of forming a fruiting-body (Nickerson and Raper, 1973; Suzuki and Yanagisawa, 1989a). In this developmental pathway, haploid amoeboid

cells acquire fusion competence and fuse to produce zygotic diploid cells, called giant cells (Saga *et al.*, 1983) which secret a large amount of cyclic AMP (Abe *et al.*, 1984). These giant cells gathers the surrounding cells to form a cell aggregate which is enclosed of a three layer membrane. The giant cells engulf the surrounding cells enclosed with the membrane (Filosa and Dengler, 1972), digest them, and grow into macrocysts (O'Day, 1979). Meanwhile, the diploid nucleus in giant cell begins meiosis (Okada *et al.*, 1986) and genetic recombination occurs. When macrocyst gets mature, about dozens of haploid recombinant amoebae emerge and start to grow, feeding bacteria nearby (MacInness and Francis, 1974).

In *Dictyostelium discoideum*, there are two heterothallic strains, NC4 and HM1, which are opposite in mating type, and if these strain cells are cultivated in a liquid medium in the dark, certain proteins which participate in cell fusion, gp70 (Urushihara *et al.*, 1988; Ishikawa *et al.*, 1990) and gp138 (Suzuki and Yanagisawa, 1989b; 1990), appear on the cell surfaces in the medium, and if Ca^{2+} exists in the medium, fusion-competent NC4 and HM1 cells fuse each other, and zygotic giant cells are produced, and they finally develop into macrocyst.

In the first chapter of this thesis, studies of regu-

lating mechanisms of gp70 appearance by the certain environmental factors were described (Habata *et al.*, 1991).

In the second chapter, investigations concerning to artificial induction of cell fusion was reported. Recently, a very interesting fact concerning cell fusion was discovered in cellular slime molds. A strain CK-8 of *Polysphondylium pallidum*, isolated from forest in northern part of Japan (Mizutani *et al.*, 1990), was found to secret a factor which induces cell fusion (Mizutani *et al.*, 1991). When cells of all strains and species of cellular slime molds, as far as examined, are treated with CK-8 conditioned medium, they become fusion competent and fuse together with any strains or species cells to produce multinucleates. For example, if NC4 cells of *D.discoideum* are treated with the diluted conditioned medium for a minute, washed and suspended in a salt solution containing Ca^{2+}, sixty to seventy percents of the cells fuse to form multinucleates.

This artificially induced cell fusion is completely different in many aspects from ordinary sexual cell fusion in cellular slime molds. For example, if the conditioned medium treated NC4 cells are mixed together with any other strain cells including HM1 cells, a large number of multinucleates are soon produced. Thus, the conditioned medium of CK-8 can produce cell fusion between cells of different

strains or species in cellular slime molds.

In order to study the molecular mechanism of this induced artificial cell fusion, a series of experiments for the identification and purification of the cell fusion induction factor were carried out.

In the third chapter, the multinucleate cells were produced between the two heterothallic opposite mating type strains, NC4 and HM1 by CK-8 conditioned medium, and a possibility occurring nuclear fusion in these cells was examined (Habata and Yanagisawa, 1991). If nuclear fusion occurs in the artificially fused cells, hybrids between any species will be able to form at least in cellular slime molds.

Chapter 1

Environmental control of fusion-related proteins in the sexual development of *Dictyostelium discoideum*

INTRODUCTION

Living organism interact with the surrounding environment. They change their genetic capabilities to overcome environmental inconvenience in a long term. Also they quickly respond to circumstances to achieve most preferable relationship with it. The mode of life cycle, such as sexual or asexual in the cellular slime mold *Dictyostelium discoideum* is totally dependent on environmental conditions. These soil amoebae usually propagate as a single haploid cell but under certain circumstances, in heterothallic strains, cells of opposite mating types fuse to form zygotic cells called giant cells, which subsequently gather and engulf surrounding cells to develop into macrocysts (Blaskovics and Raper, 1957; Erdos *et al.*, 1972; Erdos *et al.*, 1973; O'Day, 1979; Chagla *et al.*, 1980). This process of macrocyst formation is the sexual cycle (MacInnes and Francis, 1974; Okada *et al.*, 1986) and is affected by the environmental factors such as light, excess of water, phosphate and calcium ions (Blaskovics and Raper, 1957; Nickerson and Raper, 1973; Erdos *et al.*, 1976; Chagla *et al.*, 1980). We have studied the mechanism of sexual cell fusion using HM1 and NC4 strains, the opposite mating-types in *D. discoideum*, in consideration of the outstanding advantages of this organism as a simple model system for the analysis of cell to envi-

ronment interactions. Molecular mechanism of sexual cell fusion in *D. discoideum* is becoming uncovered only recently. Involvement of cell surface glycoproteins has been suggested for sexual development (O'Day and Rivera, 1987). Biochemical analysis in conjunction with immunological approaches indicated two membrane proteins to be involved in the fusion process, one is present in HM1 cells but not in NC4 cells and is approximately 70 KDa in molecular weight (gp70) (Urushihara *et al.*, 1988; Ishikawa *et al.*, 1990) and the other, 138 KDa in molecular weight (gp138), is present in both HM1 and NC4 cells (Suzuki and Yanagisawa, 1989b; 1990).

In the present study we examined the effects of the light and excess of water on the expression of the gp70 by culturing HM1 cells in different combinations of these two factors. Excess of water alone was found to induce the gp70 regardless the conditions of light. Light was suggested to induce other protein(s) that suppress the function of gp70.

MATERIALS AND METHODS

Strains and culture methods

Two heterothallic strains, HM1 and NC4, opposite mating-types in *Dictyostelium discoideum*, were used. They were maintained as stock fruiting-body culture on nutrient SM-agar plates (glucose 5.0 g, proteose peptone 10.0 g, yeast extract 0.5 g, $KH_2PO_4 \cdot 12H_2O$ 2.25 g, $K_2HPO_4 \cdot 7H_2O$ 1.5 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, agar 15.0 g in 1 liter) (Sussman, 1966) at 22°C with *Klebsiella aerogenes* as food source. For experiments, growth-phase cells obtained from SM-agar plates were used as solid-cultured cells. Liquid-cultured cells were obtained by inoculating solid-cultured cells (5×10^5 cells/ml for HM1 and 3×10^5 cells/ml for NC4) in Bonner's salt solution (BSS: NaCl 0.6 g, KCl 0.75 g, $CaCl_2 \cdot 2H_2O$ 0.4 g in 1 liter) (Bonner, 1947) with condensed *K. aerogenes* and culturing them in a reciprocal shaker (120 strokes/min) at 22°C for 16 hours unless otherwise stated. The liquid cultivation medium for the HM1 cells was supplemented with 5 % conditioned medium from the mixed culture of HM1 and NC4 cells (Saga and Yanagisawa, 1983). HM1 or NC4 cells thus cultured in a liquid medium in the dark have been demonstrated to be fusion-competent, whilst those cultured on the agar plates in the light, to be fusion-incompetent (Saga *et*

al., 1983). The cells were washed with cold BSS to remove bacteria and kept on ice until use.

Cell fusion assay

A cell fusion assay was conducted as previously described (Urushihara and Yanagisawa, 1987). Briefly, HM1 and fusion-competent NC4 cells were mixed in BSS each at 2.5 x 10^6 cells/ml and incubated in a reciprocal shaker (120 strokes/min) at 22°C for 30 min. The fusion index was calculated as the percentage of cells fused during 30 min of incubation (Saga *et al.*, 1983)

Preparation of antibodies

The same batch of rabbit antiserum was used as in our previous work (Urushihara *et al* ., 1988). This antiserum was obtained by repeatedly injecting membrane components of fusion-competent HM1 cells. Fab fragments were obtained by papain digestion of IgG (Utsumi, 1969) from this antiserum and designated simply as Fab.

Assay for inhibitory activity of Fab

The fusion-inhibiting activity of Fab was assayed as follows. Fusion-competent HM1 cells were incubated with Fab, with or without pretreatment, on ice for 15 min. Fusion-competent NC4 cells were then added to the suspension and

the cell-Fab mixture was further incubated as in a cell fusion assay. Fab activity was detected according to decrease in the fusion indices.

Electrophoresis, electrotransfer of proteins and post-transfer treatment

Slab SDS-polyacrylamide gel electrophoresis was conducted using 7.5 % gels according to the method of Laemmli (1970) with a slight modification. Proteins in the gel were transferred to nitrocellulose membranes (Schleicher and Schuell, Germany) in 5 mM sodium borate at 6 V/cm for 2 hours followed by Western processing using ^{125}I-protein A (New England Nuclear, USA). To absorb Fab, the nitrocellulose membranes with the HM1 proteins were incubated in 1 % polyvinylpyrrolidone K90 for 1 hour and rinsed with BSS. The area in which the gp70 was present was excised from each membrane with the aid of prestained molecular weight markers (Bethesda Research Laboratories, USA) running parallel in adjacent lanes. Each excised membrane portion was incubated with Fab solution in a reciprocal shaker (40 strokes/min) at room temperature for 1 hour. The supernatant Fab solution was assayed for fusion inhibiting activity.

RESULTS

We previously found that HM1 cells cultured in the liquid medium in the dark (liquid-dark HM1 cells) were fusion-competent and possessed gp70, but those cultured on the agar in light (solid-light HM1 cells) are not, nor did the latter possess detectable amount of gp70 (Urushihara *et al.*, 1988). To more clearly understand the mechanism of environmental control of sexual cell fusion, we examined how water and light affects the expression of gp70. For this purpose HM1 cells were cultured under two additional different conditions: in a liquid medium in light (liquid-light HM1 cells) and on an agar plate in darkness (solid-dark HM1 cells).

When antigenic molecules in four types of HM1 cells were analyzed by Western immunoblotting, liquid-light HM1 cells, in addition to liquid-dark HM1 cells, were found to possess gp70 but not solid-light and solid-dark HM1 cells (Fig. 1). These cells without gp70 had a protein band near the position of gp70 but slightly slower in mobility in the gel. Thus, cultivation of cells in liquid conditions induced the expression of gp70 in HM1 cells regardless of light conditions. Although the chemical composition of the liquid and solid culture media was different in the experiments

described above, no specific chemical constituents in the liquid medium was shown to be responsible for the inducement of gp70. Namely, when HM1 cells were cultured in the liquid medium with the same composition as that of the solid medium but without agar, they also possessed gp70 (Fig. 1 lane E). Therefore, cultivation in liquid phase was concluded to induce the expression of gp70.

These results were unexpected if gp70 was responsible for cell fusion between HM1 and NC4 cells, because darkness had been reported to be necessary for liquid cultured HM1 cells to acquire fusion-competency (Saga *et al.*, 1983). A cell fusion assay was thus carried out for HM1 cells cultured under the different conditions to reexamine such a requirement of darkness (Table I). Only liquid-dark HM1 cells fused with fusion-competent NC4 cells, indicating liquid and darkness both to be essential for acquisition of fusion-competency in HM1 cells as the preceding report.

Several explanations would be possible for the above apparent contradiction that liquid-light HM1 cells were incapable of cell fusion despite of having gp70. The first possibility to be considered seems to be that the gp70 in liquid-light HM1 cells is not the functional gp70 and does not have the activity for cell fusion due to, for example, modification in the manner undetectable in Western blotting

of one-dimensional electrophoresis.

To assess this possibility, we first examined whether gp70 from liquid-light HM1 cells could absorb fusion-inhibiting Fab. Total or membrane proteins of HM1 cells were separated by SDS-polyacrylamide gel electrophoresis, blotted to nitrocellulose filter and fractionated by size to partially purify gp70. Such filter pieces were incubated with Fab and the absorbed Fab was assayed for fusion-inhibiting activity. The blot of proteins from liquid-light HM1 cells had the absorption ability as that from liquid-dark HM1 cells (Table II). Interestingly, however, if intact cells were used to absorb Fab, liquid-light HM1 cells showed no such ability. Cell fusion was inhibited by Fab solution absorbed with liquid-light HM1 cells to the same extent as by that absorbed with solid-light HM1 cells (Fig. 2).

These results suggest that the gp70 in intact liquid-light HM1 cells is a target molecule of fusion-inhibiting antibody but exists in a condition such that binding of fusion-inhibiting antibodies is not possible. This protein may not be on the surface in liquid-light HM1 cells or, alternatively, it is sterically hindered so that macromolecules like antibodies are not accessible. It is not clear at this point why the extent of absorption by the blot of liquid-light HM1 cells is not exactly the same level of

liquid-dark HM1 cells.

Liquid-dark HM1 cells were found to lose their fusion-competency by subsequent cultivation in the light (Fig. 3). If cycloheximide was added to the medium during this light-cultivation, the loss of fusion-competency did not occur. This fact suggests *de novo* synthesis of proteins to be necessary for liquid-dark HM1 cells to lose fusion-competency by subsequent light-cultivation. Since liquid-light HM1 cells possess absorption-active gp70 as do liquid-dark HM1 cells, positive degradation system such as induction of proteases to attack gp70 seems to be unlikely. Consequently, it may be assumed that light-cultivation induces the formation of a surface protein(s) that sterically interfere with the function of the gp70. If this is the case, two environmental conditions, water and light, induce different proteins in HM1 cells that have opposite functions in the sexual cell fusion between HM1 and NC4.

DISCUSSION

Among several environmental factors required for macrocyst formation (Blaskovics and Raper, 1957; Nickerson and Raper, 1973; Erdos *et al.*, 1976), the conditions of light and water have been shown to affect the acquisition of fusion-competency in HM1 cells (Saga *et al.*, 1983). We previously identified the gp70 in HM1 cells as a cell surface component involved in sexual cell fusion between HM1 and NC4 (Urushihara *et al.*,1988) and it was possible on the present occasion to analyze the environmental control of its expression. HM1 cells were cultured under different combinations of light vs. dark and liquid vs. solid phases and the presence of the gp70 was examined for each condition. Interestingly, gp70 was detected in liquid-cultured cells regardless of whether cultivation had been conducted in the dark or light. This indicates liquid cultivation alone to be capable of inducing expression of gp70 and darkness not to be required.

Liquid cultivation in the present study differed from solid cultivation in several aspects; for example, 1) compositions of media, 2) frequency of collision between cells and 3) poor circulation of air components. Responsibility of the first difference to the inducement of gp70 was disproved in this study. The second would be of no significance be-

cause macrocysts are formed even on agar plates if cells are submerged in water (Nickerson and Raper, 1973). The third could be a reasonable possibility to explain the effect of liquid cultivation on the induction of gp70 expression but further study is needed to draw any conclusion.

The fact that liquid-light cultured HM1 cells were fusion-incompetent despite the presence of gp70 indicates that gp70 perhaps is not functional in these cells. Since this protein in liquid-light HM1 cells, after SDS-PAGE and blotting could absorb fusion-inhibiting Fab, its modification not detectable in Western profiles would be improbable. Our results that intact liquid-light HM1 cells with gp70 could not absorb Fab would be explained most simply by postulating a fusion-inhibiting protein that covers gp70 and prevents it from functioning in intact HM1 cells. The fusion-competency of HM1 cells was lost by light cultivation, even in a liquid medium, possibly through synthesis of the hypothetical inhibiting protein induced by light. Thus, the effects of light and water on sexual cell fusion in *D. discoideum* consist of the inducement of different proteins which counter-regulate the fusion-competency of HM1 cells. This situation is presented schematically in Fig 4.

The frequency of macrocyst formation shows considerable variation among cellular slime mold strains (Nickerson and

Raper, 1973). Environmental regulation in those strains is often not always as rigid as in HM1 cells. For example, NC4 cells acquire fusion-competency when cultured in a liquid medium even in the light, though to the lesser degree than in the dark (Saga *et al.*, 1983). The homothallic strain AC4 can form macrocysts even on SM-agar plates in the light (Nickerson and Raper, 1973; Urushihara *et al.*, 1990). Very intense light and less wet plates are necessary for this strain to form fruiting-bodies. Strains in *D. mucoroides* have a much greater tendency to form macrocyst than *D. discoideum* strains (Nickerson and Raper, 1973). The comparative biochemistry of the molecules involved in cell fusion among the variety of the cellular slime mold strains would be interesting in consideration of the manner they respond to the environment.

Chapter 2

Partial purification and identification of cell fusion induction factor produced by the cellular slime mold *Polysphondylium pallidum* CK-8

Introduction

The strain CK-8 of *Polysphondylium pallidum* is a killer in cellular slime molds, isolated from forests in Japan (Mizutani *et al.*, 1990). CK-8 cells kill cells of other strains and species of the cellular slime molds, but not CK-8 itself and its opposite mating type CK-9. Our previous works discovered that growth-phase cells of CK-8 produced and secreted a proteinaceous killer factor, approximately 10-12 KDa in molecular weight (Mizutani *et al.*, 1990). If cells of other species, such as strain NC4 of *Dictyostelium discoideum* are cultivated in conditioned medium (CM) of a CK-8 culture containing the killer factor, almost all the cells are died within 2 hours.

In addition to this killer factor, CK-8 cells are known to release other factors. When the fraction of CK-8 CM containing molecules larger than 50 KDa is separated by ultrafiltration and added into a culture of NC4, growth of NC4 cells are almost completely inhibited without killing (Mizutani and Yanagisawa, 1990). Furthermore, if the cells are treated with the diluted fraction of CK-8 CM, cell fusion is induced at a very high frequency, 40-60 % of the treated cells (Mizutani *et al.*, 1991). These effects, cell growth inhibition and cell fusion induction, are both induced by heat-sensitive and trypsin-sensitive proteina-

ceous molecule(s) of which molecular mass is more than 50 KDa (Mizutani *et al.*, 1991).

Cells treated with the CK-8 fraction containing molecules larger than 50 KDa become to be capable to fuse with untreated cells of other strains or species of cellular slime molds at a high frequencies (Mizutani *et al.*, 1991), however nuclear fusion does not successively occur in the fused cells (see Chapter 3, Habata and Yanagisawa, 1991). Even if it occurs, frequency is very low, the same as in spontaneous cell fusion (see Chapter 3, Habata and Yanagisawa, 1991). During macrocyst formation in the heterothallic strains of cellular slime molds, sexual cell fusion only takes place between two opposite mating types, and nuclear fusion subsequently occurs to form diploid zygotes, called to giant cells. The fusion induced by CK-8 CM is clearly asexual. There are no mutual recognition system between cells and no occurrence of successive nuclear fusion (See Chapter 3), however, both cell fusions, the sexual and the induced cell fusion, require Ca^{2+} (Saga *et al.*, 1983; Mizutani *et al.*, 1991). Our previous work also showed that the induced cell fusion always accompanied growth inhibition of cells (Mizutani *et al.*, 1991).

For artificial induction of membrane fusion, certain virus particles, such as HVJ (Sendai virus)(Asano and

Asano, 1984), influenza virus (Wharton *et al.*, 1986) and HIV (AIDS virus) (McCune *et al.*, 1988) are well known. However, induction of cell fusion by factor produced by a living micro-organism had never been reported until we reported recently (Mizutani *et al.*, 1991). In this part, to understand the mechanism of cell fusion induction of the factor, identification and purification of the factor was firstly attempted and the relationship between induction of cell fusion and inhibition of cell growth was also investigated.

Materials and Methods

Strains and culture methods

Strain CK-8 of *Polysphondylium pallidum*, isolated from forest soil in Japan (Hagiwara, 1989) and strain NC4 of *Dictyostelium discoideum* were used. These strain cells were successively cultivated on SM-agar plates (Sussman, 1966) with *Klebsiella aerogenes* at 22°C in *D.discoideum* and 25°C in *P.pallidum*, respectively.

Preparation of CK-8 conditioned medium

Conditioned medium containing the cell fusion inducing factor and cell growth inhibition factor was prepared as follows. Growth-phase cells of CK-8 were collected from SM agar plates, suspended in Bonner's salt solution (BSS, 5×10^5 cells/ml)(Bonner, 1947) with bacteria and cultivated at 25°C on a reciprocal shaker (120 strokes/min). After cultivation for 18 hours, the culture was centrifuged (10,000 rpm, 10 min) and filtered though a cellulose acetate membrane (0.45 μm pore size, Toyo Roshi Co., Japan) to remove cells and bacteria. The filtrate obtained was used as conditioned medium (CM).

Purification of the factors

All experiments for purification described below were car-

ried out at 0-4°C.

1.Precipitation by Ammonium sulfate

Ammonium sulfate powder was gradually added to CK-8 CM with stirring to 33 % at a final concentration. After 1 hour, the solution was centrifuged (10,000 rpm, 30 min) and ammonium sulfate was further added to the supernatant obtained (50 %). After centrifugation of the supernatant, ammonium sulfate was again added to supernatant (66 %). Pellet were collected from each above step, suspended in BSS and dialyzed against BSS for 12 hours. Activities of the cell fusion induction and the cell growth inhibition of the dialyzed solutions were then assayed.

2.Hydrophobic column (Phenyl-Sepharose and Phenyl-Superose) chromatography

The fraction of 33-66 % ammonium sulfate was dialyzed overnight against a buffer A (10 mM Na-phosphate pH 7.2, 0.5 mM dithiothreitol;DTT, 0.1 mM phenylmethylsulfonylfluoride;PMSF, 0.02 mM leupeptin) and loaded after the addition of 25 % ammonium sulfate to Phenyl-Sepharose column (1 cm x 10 cm, Pharmacia LKB, Sweden) which was equilibrated with buffer A containing 25 % ammonium sulfate. The column was washed with buffer A containing 25 % ammonium sulfate then eluted with double density gradient solution of ammonium sulfate (25→0 %) and ethylene glycol (0→50 %)

in buffer A. To apply to a Phenyl-Superose column (0.5 cm x 5 cm) of FPLC system (Fast Protein Liquid Chromato system, Pharmacia LKB, Sweden), the fraction was centrifuged (14,000 rpm, 10 min) and supernatant obtained was loaded on the column. Elution was performed by the same procedures described above. Each fraction obtained was dialyzed and activities of the cell fusion induction and the cell growth inhibition were assayed.

3.Lectin conjugated column (ConA-agarose) chromatography

The fraction containing the factor isolated by addition of 33-66 % ammonium sulfate was dialyzed overnight against buffer A and centrifuged (14,000 rpm, 10 min). The supernatant was applied to a ConA-agarose column (1 cm x 5 cm, Honen Corporation, Japan) which was equilibrated with buffer A. After washing the column by the buffer A, ConA-bound fraction was eluted by the buffer containing 400 mM methyl α-D-mannopyranoside. Fractions isolated was dialyzed against BSS and activities for the cell fusion induction and the cell growth inhibition were assayed.

4.Gel filtration (Superose 6)

The ConA-bound fraction was concentrated by using Centricon-10 (Amicon div. W.R.Grace & Co., USA), sonicated and centrifuged (14,000 rpm, 10 min). The supernatant was then loaded on Superose 6 column (1 cm x 30 cm, Pharmacia,

Sweden) equilibrated with buffer B [buffer A + 0.2 M KCl, 0.2 mM ethylene glycol-bis(β-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA)] and eluted with buffer B. Each fraction (1 ml) was dialyzed against BSS and activities of the cell fusion induction and the cell growth inhibition were assayed.

5.Anion exchange column (MONO Q) chromatography

The active fractions (fraction No. 3-6) of Superose 6 column were collected and dialyzed overnight against buffer C (20 mM Tris-HCl pH 7.8, 0.5 mM DTT, 0.1 mM PMSF, 0.02 mM leupeptin) and centrifuged (14,000 rpm, 10 min). The supernatant was loaded to a MONO Q column (0.5 cm x 5 cm, Pharmacia LKB, Sweden) equilibrated with buffer C. The column was washed by buffer C and then eluted with KCl gradient (0→0.4 M KCl gradient and 1.0 M KCl in buffer C). Each fraction (1 ml) was dialyzed against BSS and activities of the cell fusion induction and the cell growth inhibition were assayed.

Assay for the activities of cell growth inhibition and cell fusion induction

The CK-8 CM and the fractionated samples described above were dialyzed overnight against BSS containing 0.1 mM PMSF and 0.02 mM leupeptin at 4°C, then used for assays.

Growth-phase NC4 cells were taken from SM agar plates and suspended (1 x 10^6 cells/ml) in the diluted CK-8 CM and the diluted fractionated samples (1:100 with BSS). Immediately after suspending cells, they were collected by centrifugation (2,000 rpm, 2 min) and washed with BSS and Ca^{2+}-free BSS. The cells were then resuspended (5 x 10^5 cells/ml) in Ca^{2+}-free BSS to prevent cell fusion, and cultivated with bacteria on a reciprocal shaker (120 strokes/min) at 22°C. After 18 hours of cultivation, the relative activity of cell growth inhibition of each sample was estimated by using the following formula:

$$\frac{\log N' - \log N^f}{\log N' - \log N} \times 100 \ (\%)$$

where N', N^f and N indicate the number of cells after 18 hours of cultivation in CK-8 CM, fractionated samples, BSS treatment, respectively.

For assay of cell fusion, growth-phase NC4 cells were treated with the diluted CK-8 CM (1:100 with BSS) and the diluted fractionated samples (1:100 with BSS). After treatment, cells were cultivated with bacteria in a low-Ca^{2+} BSS (0.54 mM) to acquire fusion competence without any actual cell fusion. When they acquired fusion-competence, they were washed with Ca^{2+}-free BSS and 2.7 mM $CaCl_2$ was added to undergo cell fusion (Mizutani *et al.*, 1991).

After 60 min of shaking, small samples were taken to determine the fusion index. Prior to examination, ethylenediaminetetraacetic acid (EDTA) was added to the sample at a final concentration of 10 mM to dissociate unfused cells and prevent further cell fusion during counting. The fusion index was calculated as follows:

$$\frac{\text{No. of total cells at } T_0 - \text{No. of total cells at } T_{60}}{\text{No. of total cells at } T_0} \times 100(\%)$$

where T_0 and T_{60} indicate 0 and 60 min after shaking.

Polyacrylamide gel electrophoresis (PAGE) and immunoblot analysis

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was performed by the modified method of Laemmli (1970). Piperazin di-acrylamide (Bio-rad, USA) was used instead of N,N'-methylene bis-acrylamide in gel polymerization. The CK-8 CM and the fractionated samples were lyophilized, suspended in a sample solubilizing buffer (5 % SDS, 8 % glycerol, 2 mM DTT in 0.2 M Tris-HCl pH 6.8, 0.01 % bromo phenol blue; BPB) and boiled for 3 min. Electrophoresis was performed under a constant current of 10 mA until the BPB dye reached the end of the gel.

PAGE containing Softes 12 (Lion Corp., Japan) instead of SDS was also performed. Softes 12 is mild detergent

which can not dissociate interactions among subunits (Koide *et al.*, 1987). Softes 12-PAGE was performed without DTT at $4^{o}C$.

Silver staining: To visualize separated proteins, gels were fixed with ethanol/acetic acid (50-10 %) and stained with silver-staining kit Wako (Wako, Japan).

Immunoblotting: Proteins in gels were transferred on to nitrocellulose membrane (0.45 μm, Schleicher and Schuell, Germany) at 6 V/cm for 2 hours in 5 mM sodium borate (Urushihara *et al.*, 1988). The nitrocellulose membrane was then incubated in 1 % polyvinylpyrrolidone K90 (PVP) for 1-16 hours to block further binding of proteins. The membrane was washed with 20 mM Na-phosphate pH 7.8 containing 0.15 M NaCl (PBS), and incubated with 1/200 diluted CK-8 CM neutralizing antiserum in PBS containing 2 % bovine serum albumin (BSA-PBS) for 1 hour. After washing with PBS containing 0.1 % tween 20 (Tween 20-PBS), the membrane was incubated with 0.5 μCi/ml ^{125}I-protein A (New England Nuclear, USA) in BSA-PBS for 1 hour, washed with Tween 20-PBS, dried and then exposed a X-ray film (XAR-5, Kodak, USA).

Protein assay

Protein concentrations of CK-8 CM and the fractionated samples were determined by using Bio-rad Protein assay kit

(Bio-rad, USA) with bovine serum albumin as the standard.

Antibody preparation and immunodepletion

For preparation of antigen, CK-8 CM was subjected to SDS-PAGE. After electrophoresis, the top part (2 mm) of a 4 % stacking gel containing active protein was sliced out and dialyzed overnight against PBS.

The sliced gel was homogenized and injected subcutaneously into a rabbit. The injections were given first three times at a week intervals and additional booster thereafter a month. After the third injection, booster was given between the intervals and antiserum were collected a week thereafter.

To conduct a assay for antiserum-neutralizing activity, antiserum was coupled to 200 μl of Protein A-sepharose (Pharmacia, Sweden) in a microfuge tube for 1 hour at 4^{o}C. Antiserum-Protein A complex was washed with PBS, incubated with the CK-8 CM (100 μl) for 2 hours at 4^{o}C and centrifuged (3,500 rpm, 3 min) to recover supernatant of CK-8 CM. The supernatant was assayed for activities of the cell fusion induction and the growth inhibition.

For antiserum-absorption assay, PVP-incubated nitrocellulose membrane on which proteins of CK-8 CM blotted (see *PAGE and immunoblot analysis*) was rinsed with BSS.

The membrane was cut transversally into several pieces of an equal size. The molecular weight range of these pieces was determined using prestained molecular-weight marker (Bethesda Research Laboratories, USA) running parallel in adjacent lanes. Each piece was further cut to fit each well of multiwell titer plates and incubated with the antiserum for 1 hour at room temperature. The supernatant of antiserum was then assayed for the neutralizing ability to determine the antibody absorption activity of each membrane blot.

Results

Isolation and purification of the cell fusion induction factor and the cell growth inhibition factor

It was previously reported that the cell fusion induction factor and the cell growth inhibition factor in CK-8 CM seem to be proteinaceous (Mizutani and Yanagisawa, 1990; Mizutani *et al.*, 1991). To isolate and purify these factors, first, fractionation by ammonium sulfate was carried out. The activities of cell fusion induction and growth inhibition of the four fractions obtained by the addition of less than 33 %, 33-50 %, 50-66 % and more than 66 % ammonium sulfate were assayed. As shown in Table III, the activities were both detected in the two precipitated fractions by addition of 33-50 % and 50-66 % ammonium sulfate.

Since the activities were not obtained in a single fraction, the following hydrophobic columns, Phenyl-sepharose and Phenyl-superose, were independently employed separately to purify further the fraction precipitated by addition of 33-66 % ammonium sulfate. After applying the fraction to Phenyl-sepharose column, double density gradient solution of 25 → 0% ammonium sulfate and 0 → 50% ethylene glycol was used for elution. The both activities were detected in highly hydrophobic fractions eluted with low concentration of ammonium sulfate and high concentration

of ethylene glycol (Fig. 5 A). However, the activities still distributed broadly. Next, Phenyl-superose column on FPLC system (Fast Protein Liquid Chromato system, Pharmacia, Sweden) was attempted for further purification. Elution pattern obtained from Phenyl-superose column chromatography was almost the same as that of Phenyl-sepharose (Fig. 5 B). These results indicate, however, at least that the factors are both hydrophobic.

Since hydrophobic columns did not work well, ConA-agarose column was attempted to use. The fraction obtained by 33-66 % ammonium sulfate precipitation was dialyzed against buffer A, centrifuged and the supernatant was applied to a ConA-agarose column. As shown in Table IV, the activities were both detected in ConA-bound fraction.

As next step, ConA-bound fraction was applied to a gel filtration column, Superose 6 (FPLC system) for further purification. The ConA-bound fraction was concentrated by Centricon-10, sonicated and subjected to the column. A typical result was shown in Fig. 6. The activity of cell fusion induction was found mostly in the fraction between 500-1,000 KDa. The activity of growth inhibition was also detected in the same fraction as cell fusion induction (data not shown). This fraction was then dialyzed against buffer C and further applied to a MONO Q anion exchange

column. Although the fraction was separated into several peaks (Fig. 7), again the both activities were appeared in the identical single broad peak eluted by 0.2-0.3 M KCl (data of growth inhibition not shown). It should be noticed that activities for the cell fusion induction and cell growth inhibition were generated identical factor(s).

The results of purification procedures are summarized in Table V. When specific activity was calculated on the activity of growth inhibition, the specific activity was increased approximately 300 fold after applying MONO Q column.

Identification of cell fusion inducer and growth inhibitor

In order to identify the molecule involved in induction of cell fusion, SDS-PAGE was carried out by a modified method of Laemmli as described in "Materials and Methods". Each fraction obtained by Superose 6 column was applied to SDS-PAGE, using 7.5-12 % gradient running gel, and separated proteins were dyed with silver-staining. Fig. 8 shows the results of fractions from Superose 6. There were no protein band which is specifically found in the fractions possessing cell fusion induction activity in the running gel. However, one such band was detected in the top part of stacking gel (4 % of acrylamide) of the fractions 3-6.

Next, fractions were separated by using a MONO Q column and applied to SDS-PAGE in the same way as described above. The particular band was not found again in this case in the gradient running gel (7.5-12 %), but existed in the stacking gel (Fig. 9 A). When the concentration of acrylamide in running and stacking gels were changed to 3-12 % and 3 %, respectively, the specific band was detected in high molecular weight region, larger than 300 KDa, of running gel in corresponding fractions of MONO Q column chromatography as shown in Fig. 9 B.

Since the presumptive molecule involved in cell fusion induction activity was detected as a single band at the top portion of 4 % stacking gel, this fraction of unstained 4 % stacking gel was sliced, mashed and injected into a rabbit to obtain antiserum. CK-8 CM was then depleted by the antiserum. Both activities of cell fusion induction and growth inhibition were completely lost after the depletion. Degree of the depletion of the activities was dependent on concentrations of the antiserum (data not shown).

Immunoblotting tests were carried out by using the above antiserum on CK-8 CM and the active fractions isolated by the each purification step. As indicated in Fig. 10, at least four bands recognized by the serum were detected at the positions of larger than 300 KDa, 160 KDa, 140 KDa and

65 KDa in the all samples. To identify the functions of the each protein and the relationship among these, blot-absorption experiments were performed. The active ConA-bound fraction was concentrated, separated by a 3-12 % gradient gel and transferred onto a nitrocellulose membrane. The membrane was sliced into four pieces containing each molecule blotted; larger than 360 KDa, 230-360 KDa, 115-230 KDa and less than 115 KDa. The antiserum which neutralized the cell fusion induction activity in CK-8 CM was incubated with each sliced piece. As shown in Table VI, pieces containing the bands of larger than 330 KDa and 65 KDa could absorb the activity of the antiserum.

The results of the previous experiments, gel filtration by using Superose 6, indicated that the activity of cell fusion induction was mainly present between 500-1,000 KDa (Fig. 6). Fig. 6 also showed that the activity was also present in much small molecules, less than 200 KDa. These results seem to suggest a possibility that a small unit of the active molecule, approximately 65 KDa, might form large active complexes.

To test this possibility, two dimensional electrophoresis was performed. The fraction possessing the cell fusion activity which was separated by a Superose 6 column was applied to PAGE using the mild detergent Softes 12 which does

not dissociate a complex into subunits. Only broad ranged high molecular proteins, larger than 300 KDa, were recognized after reaction with the antiserum. When these molecules were applied to SDS-PAGE, several small molecular weight bands which react with the antiserum appeared. Molecular weight of the main band of those was approximately 65 KDa (Fig. 11). This results support the possibility that 65 KDa small molecules would form complexes, larger than 300 KDa.

All the results obtained from a series of experiments clarified that the protein involving in cell fusion induction in cellular slime molds might be a large complex, larger than 300 KDa in general, which would consist of small subunit molecules, approximately 65 kDa.

Discussion

The results obtained from a series of experiments for the purification suggested that the activities of cell fusion induction and growth inhibition in CK-8 CM are probably generated by the identical molecules. The molecular characters of this factor are 1) very strong and wide hydrophobicity, 2) wide molecular mass in high molecular weight range (500 - 1,000 KDa in molecular weight) on Superose 6, 3) ConA-bound and 4) a broad range in elution (0.2 - 0.3 M KCl) from MONO Q.

Since it has not been reported that cells of cellular slime molds secreted a single protein larger than 300 KDa and several bands lower than 200 KDa were detected in the active 500 - 1,000 KDa fraction of Superose 6 in SDS-PAGE, the factor would exist not as a single protein but as a complex including many kinds of proteins in CK-8 CM. Of many kinds of proteins in this postulated complex, only a part of proteins but not all proteins in the complex, may have the activity of cell fusion induction and growth inhibition. The results of blot-absorption experiment indicate that the presumptive proteins for the activity are the high molecular protein larger than 300 KDa and the protein of 65 KDa in SDS-PAGE (Fig. 10 and Table VI). Though the relationship between high molecular protein and 65 KDa protein is not

clear, the high molecular protein are supposed a multimer or aggregate of 65 KDa protein by its strong hydrophobicity. If the complexes are formed in variable sizes, it is natural that the activities were eluted in wide and high molecular fractions from Superose 6. More, the some characters of factor would change gradually according to their sizes and the activities would be broadly detected because of broad elution of the factor from hydrophobic and anion exchange columns. In order to make sure this hypothesis, I am now attempting to analyze the relationship between the high molecular protein and 65 KDa protein, clone the gene of active proteins and analyze process of the protein synthesis and secretion.

If the factor exists as a large complex in CK-8 CM, how does such a large complex interact with cells of cellular slime molds? Since diluted CK-8 CM are enough for cells to take the effects of cell fusion induction and growth inhibition at a minute, the factor in CK-8 CM would interact with cells with high-affinity in a short time. Therefore, firstly, a receptor for the factor is supposed to be on the cell surface of cellular slime molds. CK-8 CM is effective in killing many strains and species of cellular slime molds (Mizutani *et al*, 1990) and in cell growth inhibition (Mizutani and Yanagisawa, 1990), if such a receptor really

exists, it would exist widely in many strains and species in the cellular slime molds, may be not specific receptor for the factor of CK-8, for other molecules. Second model mechanism would suppose the non-specific adsorption of the factor on cell surface in a short time by their strong hydrophobicity. After the adsorption, the factor would interact with phospholipid layer or proteins in plasma membrane and change their characters. The changes in plasma membrane would make cellular mechanism different, followed by ceasing their proliferation. More these changes may induce cells to be fusion-competent.

However, even if the factor interacts with cells by any mechanism, why the factor does not take effect on the cells of CK-8 itself and its opposite mating type CK-9? There may be a mechanism of self-defence for the factor in the cells of CK-8 and CK-9. For example, yeast $K1^{+}$ killer strain of *Saccharomyces cerevisiae* has linear double stranded RNA plasmid (M-dsRNA) coded a gene of K1 killer toxin, and strains of *S. cerevisiae* which do not have this plasmid are sensitive for K1 toxin. In the K1 killer system, a resistance for the toxin protein is a competition with non-toxic precursor (Boone *et al*, 1986), genes of the toxin and a resistance for the toxin are coded on same plasmid. Also in the cellular slime molds, cells of CK-8 and CK-9 would have

a immunity system for the CK-8 factor, which is similar to the system of yeast, and may be co-related with mating type.

It has been reported that a toxin produced by a killer strain of yeast *Kluyveromyces lactis* arrests the growth of sensitive yeast cells of other species, as it arrests *S.cerevisiae* at G1-phase specifically (Sugisaki *et al.*, 1983; White *et al.*, 1989). This toxin of *K.lactis* is a large complex formed from two kinds of subunits.(Sugisaki *et al*, 1983). It is very interesting that the growth inhibitor of *K.lactis* is also a large complex such as cell growth inhibitor of CK-8.

It has been reported that CK-8 has three effects, cell killing (Mizutani *et al*, 1990), cell growth inhibition (Mizutani and Yanagisawa, 1990) and cell fusion induction (Mizutani *et al*, 1991). In this part, when the isolation and purification of the cell fusion inducer were carried out, the activity of cell growth inhibition was always detected in the same active fractions of the cell fusion induction on any steps of purification and never separated. Therefore it was suggested that the cell fusion inducer and growth inhibitor are identical molecules. In this case, even if only cytokinesis was inhibited and nuclear division continued without cell fusion, multinucleates would be observed and it may be incorrectly concluded that cell

fusion occurs. However, the observation in viral staining of cells and the synchronous fusion by adding Ca^{2+} strongly suggest that multinucleates by CK-8 factor are clearly caused by cell membrane fusion (Mizutani *et al*, 1991). According to these results, an identical factor has two kinds of activities of cell fusion induction and growth inhibition, so growing cells of CK-8 secrete at least two factors which are active for the cells of other strains and species. In addition to the killer factor, why does CK-8 produce and secrete the another factor which has two activities of cell fusion induction and growth inhibition? Since these factors are not active for CK-8 itself and opposite mating type CK-9 and killing is also observed in mix culture with cells of other strains and CK-8 on agar plates as well as treatments in CK-8 CM (Mizutani *et al*, 1990), secreting the killer factor would be an advantage of growth and development of CK-8 and CK-9 cells in competition with other strains and species. In addition to cell killing, the cell growth inhibition is effective for cells of other strains in diluted CK-8 CM (1:100 with BSS) at a short time treatment (2-3 min), so this activity is also thought as an additional advantage of gaining more wide fields for growth and development of CK-8 and CK-9 cells. More, the cells of which the growth are inhibited by CK-8 CM form multinucleates by

cell fusion (Mizutani *et al.*, 1991), after cell fusion the nuclear fusion hardly occurs and the multinucleates gradually disappear (See Chapter 3, Habata and Yanagisawa, 1991), so the activity of cell fusion induction is also able to be an advantage of competition with other cellular slime molds in the same fields.

Since the cell fusion by CK-8 factor is induced without nuclear fusion, the plasma membrane fusion occurs alone except the nuclear fusion in this cell fusion system. Therefore this cell fusion induction by CK-8 factor is a very simple system to analyze the process that the cells become fusion-competent by an exogenous factor. More, this cell fusion event would become a useful system to analyze a relationship and differences between cell membrane fusion and nuclear fusion in their control mechanism.

The cell fusion induction by CK-8 factor occurs between the cells inhibited their growth. In other words, in this system, the cells may have to inhibit their growth to become fusion-competent. For example, in the conjugation of yeast, yeast cells are arrested at G1 phase by sexual pheromone of opposite mating type as exogenous signal and able to become gametes (Wilkinson and Pringe, 1974), and in myogenesis myoblast fusion occurs between myoblasts arrested at G1 phase (Buckley and Konigsberg, 1973). So

there may be some relevance between these cell fusions and cell cycle as well as between growth inhibition and cell cycle. The cell fusion by CK-8 factor may be also correlate with cell cycle.

Chapter 3

Nuclear behavior in artificially induced multinuclear cells of *Dictyostelium discoideum*

Introduction

The killer strain CK-8 of *Polysphondylium pallidum* kills other strains or species of cellular slime molds, but does not kill its own or opposite mating type strains. This strain was recently isolated from a forest soil in the northern part of Japan (Mizutani *et al.*, 1990).

Growth-phase cells of CK-8 secrete a proteinaceous killer factor with molecular mass of approximately 10 kDa into the culture medium. In addition, they produce another glycoproteinaceous molecule, larger than 50 kDa, which strongly inhibits cell growth of other strains or species of cellular slime molds (Mizutani and Yanagisawa, 1990). Since these factors are different in molecular mass, they are able to be separated by filtration through a proper-sized membrane filter. For example, if the conditioned medium of CK-8 cells is separated into two fractions, one containing molecules less than 50 kDa and the other larger than that, by using Centriflo (CF50A, Amicon Div. W.R.Grace & Co.), the killer factor is contained in the former fraction (F1) and the cell-division inhibitor in the latter (F2) (Mizutani and Yanagisawa, 1990).

More recently, it was discovered that if growth-phase cells of other strains or species of cellular slime molds, such as NC4 of *Dictyostelium discoideum*, are treated with

the diluted F2 fraction (1:100 with salt solution) for minutes, the cells become fusion competent within several hours of successive cultivation and begin to fuse (Mizutani *et al.*, 1991). In this case, more than 70 % of cells participate in fusion and numerous large multinuclear cells are produced. Such cell fusion occurs, even if treated NC4 cells are cultivated together with untreated other strain or species cells (Mizutani *et al.*, 1991). Thus, F2 fraction of CK-8 contains a cell-fusion induction factor, in addition of the cell-division inhibitor.

During macrocyst formation in the cellular slime mold *D.discoideum*, large zygotic cells, called giant cells, are formed by sexual cell fusion (O'Day, 1979; Saga and Yanagisawa, 1982; Saga *et al.*, 1983). When cells of the two heterothallic strains NC4 and HM1, which are the opposite mating types, are cultivated in a medium containing bacteria in the dark for 15-18 hours, they become to be fusion competent and fuse each other to form giant cells (Saga *et al.*, 1983). If cells of the each strain are pre-cultivated separately in the dark for 15-18 hours and then mixed, they fuse immediately after the mix and numerous large multinuclear cells containing more than a dozen of nuclei, are produced within 30 min (Saga *et al.*, 1983). In such cells, only two haploid nuclei fuse and all others degenerate to

develop normally into macrocysts (Okada *et al.*, 1986).

Since the multinuclear cells artificially produced by the diluted F2 treatment looked very similar to the multinuclear giant cells formed by sexual cell fusion, we wondered whether if the multinuclear cells were produced artificially between NC4 and HM1 by the diluted F2 treatment, nuclear fusion would also take place and normal macrocysts are formed or not. If nuclear fusion occurs and macrocysts are formed, F2 fraction can be used for producing hybrids between different strains or species in cellular slime molds, and the F2 treatment will become a useful method for genetical and biochemical studies in these organisms. In the present experiments, we examined whether or not nuclear fusion would occur in the artificially produced multinucleates and subsequently macrocysts were produced or not.

Materials and methods

Strains and cultivation.

Strains NC4, NP-2, XP-55 and HM1 of *D. discoideum*, and a strain CK-8 of *Polysphondylium pallidum* were used in the experiments. NP-2 and XP-55 are derivatives from NC4. The former is a recessive temperature sensitive growth mutant (Kessin *et al.*, 1974) and the latter is a recessive *Bacillus subtilis* sensitive growth mutant (Newell *et al.*, 1977; Ross and Newell, 1979). HM1 is the opposite mating type strain of NC4. CK-8 is a wild isolate from forest soils in Japan (Hagiwara, 1989; Mizutani *et al*, 1990). Cells were cultivated on SM-agar plates (Sussman, 1966) with *Klebsiella aerogenes* at 22^{o}C for *D.discoideum* and 25^{o}C for *P.pallidum*. Fruiting-bodies formed were maintained at 4^{o}C as stocks.

Preparation of the fraction containing cell-fusion induction factor.

Growth-phase CK-8 cells were taken from agar plates, suspended (5 x 10^{5} cells/ml) in a Bonner's salt solution (BSS)(Bonner, 1947) containing bacteria. After 18 hours cultivation on a shaker at 25^{o}C, the cell suspension was filtrated through a nitrocellulose membrane (pore size 0.45μm, Advantec-Toyo, Japan) following centrifugation

(10,000 rpm, 10 min) to remove the cells and bacteria. The filtrate was then separated by a Centriflo (CF50A, Amicon Div. W.R.Grace & Co. U.S.A.) into two fractions; one containing molecules less than 50 kDa (F1) and the other larger than 50 kDa (F2). F2 was used as the cell-fusion induction fraction.

Synchronous cell fusion.

Growth-phase cells of *D.discoideum* were suspended (1 x 10^6 cells/ml) in the diluted F2 fraction (1 : 100-200 with BSS), centrifuged immediately after suspending of cells (2,000 rpm, 2 min), transferred into low Ca^{2+}BSS culture (0.54 mM Ca^{2+}) containing bacteria and cultivated on a shaker at 22°C. These cells acquire fusion competence after 6-7 hours cultivation, but can not actually fuse in low Ca^{2+}BSS (Mizutani *et al.*, 1991). After 18 hours cultivation, the cells were taken, washed to remove bacteria and resuspended (2.5 x 10^6 cells/ml) in BSS (2.7 mM Ca^{2+}). Then, fusion competent cells began to fuse and all fusions were completed within 60 min (Mizutani *et al*, 1991). The frequency of cell fusion was estimated by the following formula.

$$\frac{\text{Total cell No. at } T_0 - \text{Unfused cell No. at } T_n}{\text{Total cell No. at } T_0} \times 100\ (\%)$$

T_0 and T_n indicate 0 and n hours after transfer

of cells into BSS.

Vital staining of cells.

For direct observation of cell fusion, cells of two different strains were each stained with either Neutral Red or Nile Blue following a treatment with diluted F2 fraction. To stain cells, either of 0.02 % stock solutions of the dyes dissolved in distilled water were added to cell suspensions at a concentration of 2 % and incubated at 22^{o}C for 15 min on a shaker. The cells stained with the different colors were washed, mixed in an equal number in BSS. Colors of large cells produced by the treatment were examined.

Fluorescent staining of nuclei

To examine the number of nuclei in large cells produced by the F2 treatment, The cells were washed thoroughly to remove bacteria, placed on a slide, dried, fixed with 100 % ethanol at -20^{o}C for 5 min and stained with 1 μg/ml Heochst 33342 (Heochst). These were then observed by fluorescent microscopy.

Determination of DNA contents of nuclei.

Cells were suspended in NP-40 lysis buffer (Firtel and Lodish, 1973) and incubated at 0^{o}C for 3 min. After cells were lysed, nuclei were collected by centrifugation

(7,000 rpm, 3 min), fixed with 70 % ethanol and stained with 4-6-diamidino-2-phenylindole (DAPI) by the method of Kuroiwa *et al* (1981). Relative DNA contents of each nucleus were determined by the method of Coleman *et al* (1981). Red blood cells from 18 days chick embryos were used as a standard for DNA content.

Detection of hybrid formation.

Cell fusion in the asexual cycle of *D.discoideum* was detected by the method of Katz and Sussman (1972). Cells of the mutant strain NP-2 (a temperature sensitive) and XP-55 (a *Bacillus subtilis* sensitive) were suspended in BSS, mixed in an equal number, placed on non-nutrient agars containing 100 μg/ml streptomycin and incubated to form fruiting-bodies. Spores were taken from the formed fruiting-bodies, plated on SM agars with *B. subtilis* and incubated at 27^{o}C. After one week incubation, the frequency of plaques appeared on bacterial surface was examined.

Results

Since multinuclear cells produced by the diluted CK-8 fraction, F2, looked very similar to multinuclear giant cells formed by the sexual cell fusion, we produced the multinuclear cells by treating NC4 and HM1 cells with the diluted F2 fraction and examined whether they could develop into macrocysts or not. Nuclear behaviors in these cells during incubation were also investigated.

Cell fusion between NC4 and HM1.

Growth-phase cells of NC4 and HM1 were treated separately with the diluted F2 fraction (1:100 with BSS) for 3 min, washed and transferred into low Ca^{2+}BSS (0.54 mM Ca^{2+}) containing bacteria to cultivate at 22°C. When the cells acquired fusion competence, 18 hours after the initiation of cultivation, they were mixed in an equal number and transferred into standard BSS (2.7 mM Ca^{2+}) to allow them to fuse. They began to fuse immediately after the transfer and approximately 70 % of the cells participated in fusion within 60 min. Numerous multinuclear cells containing more than several nuclei were consequently produced. When F2 treated NC4 cells were mixed with even untreated HM1, about 50 - 60% of cells participated in fusion. Staining cells of the two different strains with the different dyes, Neutral

Red or Nile Blue, clearly showed that fusion occurred randomly between them. Multinuclear cells produced were then incubated in BSS with or without bacteria for 10 days to examine whether they develop into macrocysts or not. Results were negative. None of them developed into macrocysts.

Disappearance of the multinuclear cells.

In order to investigate the cause that the artificially induced multinucleates do not develop into macrocysts, some cytological observations were performed during incubation. A cell suspension containing the multinuclear cells, was incubated at 22^{o}C with bacteria and small samples were taken at different times to count the numbers of multinucleates and total cells, then the ratio of multinuclear cells to total was estimated. As shown in Fig. 12, while the number of total cells increased, the ratio of the two kinds of cells quickly decreased. This decrease, however, possibly is overestimated, if mononuclear cells divide faster than multinuclear cells.

To eliminate such a possibility, the cells were then incubated without bacteria in low Ca^{2+}BSS to inhibit their proliferation and fusion. The results indicated again the same rate of decrease of the ratio, although total cell number did not increase during incubation (Fig. 13).

When NC4 and HM1 cells were separately suspended in

low Ca^{2+}BSS and incubated without bacteria, they remained constantly in their numbers at least for 20 hours (data not shown). Therefore, the decrease of the multinuclear cell number may be depending on their lysis.

Behavior of nuclei in the multinuclear cells

To observe behavior of nuclei in the artificially produced multinucleates, they were taken at different times during incubation, stained with the fluorescence Heochst 33342 to examine by fluorescence microscopy. The results were shown in Fig. 14, the cells produced immediately after the transfer, from low Ca^{2+}BSS to standard BSS to allow fusion, contained more than a dozen of nuclei (Fig. 14 B). However, after 2 hours (Fig. 14 C) and 6 hours (Fig. 14 D) of incubation in BSS without bacteria, they became to contain only 3 - 6 nuclei. Meanwhile, the ratio of multinuclear cells to total cells also decreased, and after 20 hours (Fig. 14 E), most of them became mononucleates. Thus, the number of nuclei in each multinucleates quickly decreased during incubation and the ratio of the cells markedly decreased with time.

These results again suggest that quick lysis of the multinuclear cells is at least one of the causes for decrease of their number during incubation. Large multinuclear

cells possibly lyse more quickly than normal cells. However, there are still other possibilities for the decrease of the multinucleates, like performing of cell division without nuclear fission or nuclear fusion without cell division. For example, if cell division is carried out without nuclear fission, the number of the multinucleates will decrease. This seemed to occur, but at present we could not show an exact cytological evidence for this. The other possibility for the decrease is that if two nuclei fuse each other and all others degenerate like the sexually produced multinuclear cells do (Okada *et al.*, 1986), the number of nucleus will also decrease. This possibility was then examined in the next experiments by measuring DNA content of each nucleus in the multinucleates.

DNA content of nucleus.

Small samples were taken from the cell suspension containing the multinucleates at various times during incubation and suspended in NP-40 lysis buffer (Firtel and Lodish, 1973). A relative DNA content of each separated nucleus was measured. The results indicated that no diploid or multiploid nuclei did appear even after 20 hours incubation (Fig. 15). Thus, no nuclear fusion seems to occur in the multinuclear cells.

From these results, we concluded that in the artifi-

cially produced multinuclear cells, no nuclear fusion forming diploid took place and that most of them lysed during incubation. This would be the reason that the artificially produced multinuclear cells does not form macrocysts.

Formation of hybrids.

In the asexual cycle, cell fusion occurs very rarely and hybrids are able to isolate if appropriate mutants are used (Katz and Sussman, 1972; Loomis and Ashworth, 1968; Loomis, 1969; Williams *et al.*, 1974). We concluded above that nuclear fusion forming diploids did not take place in the artificially produced multinucleates. However, there is still another possibility that it occurs at cytologically unrecognizably low frequency. To substantiate this point possibility, the following genetic experiments were performed .

Since no cell fusion occurs between different strain cells, two derivatives NP-2 and XP-55 from NC4 were used. NP-2 is a mutant which can grow with either *K.aerogenes* or *B.subtilis* at 22^{o}C, but not at 27^{o}C, and XP-55, can grow with *K.aerogenes* at 27^{o}C, but not with *B.subtilis*. These are depending on a each single recessive mutation. When they were separately treated with diluted F2 fraction, cultivated in low Ca^{2+}BSS for 18 hours at 22^{o}C. Growth-phase cells were

collected from each suspension and mixed in an equal number during in BSS. Approximately 50-60 % of cells participated in fusion and consequently a large number of multinuclear cells were produced. They were then spread on SM agar plates with *B.subtilis* and incubated at 27°C for one week. Under these conditions, both of them are not able to grow, but if hybrid diploids are formed, they are able to grow to produce plaques. The results showed that the frequency forming plaques were approximately 7.4×10^{-6}.

To compare this hybrid formation frequency to that in the asexual cycle, growth-phase cells of NP-2 and XP-55 were then suspended together in BSS in an equal number, placed on a plain agar and incubated at 22°C. When fruiting-bodies were formed, their spores were collected and plated on SM agars with *B.subtilis* to incubate at 27°C. The plaque formation frequency was approximately 6.5×10^{-6} after one week. Thus, the hybrid formation frequency in the artificially produced cells was similar to that in the asexual cycle.

From all the experimental results presented here, nuclear fusion seems to do not usually take place in the artificially formed multinucleates and the cells lysed during incubation. No macrocysts are then formed.

Discussion

In the sexual cycle of the cellular slime mold *D.discoideum*, cells fuse between the opposite mating type strains, such as NC4 and HM1, and a large number of multinuclear giant cells are formed (Saga and Yanagisawa, 1982). Nuclear fusion takes place here on at a certain time after cell fusion (Okada *et al.*, 1986). However, in the artificially produced multinucleates, no nuclear fusion occurs and consequently no macrocysts were formed even if the two opposite mating type NC4 and HM1 cells were fused. This suggests that cellular conditions of the artificially produced multinucleates would be different from those of the sexually formed multinucleates, although the both cells are very similar in morphology. Probably, sexual cell fusion would occur only at a time when the cellular conditions are also ready for nuclear fusion.

It is also reasonable to assume that nuclear fusion may be influenced by the nuclear stage of cell cycle or by the degrees of synchrony of nuclei in the heterocaryons. However, if this is true, then nuclear fusion must be taken place at least at some frequency. Since only growth-phase cells were used they contained all stages of nuclei. In the present experiments, however, no nuclear fusion occurred. Therefore, nuclear fusion seems to be not influenced by

nuclear stage of cell cycle and consequently be not influenced by the degrees of synchrony of nuclei.

As described before, F2 fraction contains both the cell-fusion inducer and a cell-division inhibitor (Mizutani *et al.*, 1991), and those factors are not yet separated each other. However, they are probably different molecules, because, either diluted or undiluted cell-division inhibitor can work, but cell-fusion inducer should be highly diluted. This problem, however, will be clarified by isolation of the molecules in the future work.

Concerning to multinucleate formation, if the inhibitor inhibits only division of cells but not of nuclei, then consequently multinucleates will be produced. But this seems to be not the case as reported in our previous paper (Mizutani *et al*, 1991). The multinucleates would produce only by cell fusion.

Para-sexual cell fusion during fruiting-body formation has been used for most genetical works in *D.discoideum*, however, sexual cell fusion during macrocyst formation has not. This is because, macrocyst maturation take a long time and their germination are very low at the rate. Furthermore, segregation ratios of offsprings obtained from germinated macrocysts are often abnormal and difficult to get reproducible results (Wallace and Raper, 1979). These reasons left

genetics of cellular slime molds behind other organisms. Many attempts were made to break through the barrier, but not succeeded. For example, mass production of cell fusion by using polyethylene glycol or Sendai virus which were most widely used agents for cell fusion in many organisms had been attempted, however, they did not work for cellular slime molds (Neumann *et al.*, 1980).

Discovery of mass production of cell fusion by F2 fraction had been expected as a good method for making hybrids and useful for genetical studies in cellular slime molds. However, the present results showed that almost no hybrid was produced.

Thus, the method producing multinuclear cells in mass could not be useful for genetical studies of cellular slime molds, but should be useful for biochemical and cytological studies of these organisms, such as studies of the regulatory mechanisms between nuclear fusion and cell fusion or studies of the molecular mechanisms of membrane fusion.

GENERAL CONCLUSION

The molecular mechanisms of cell fusion and the induction mechanism of cell fusion by the external environmental factors were studied in cellular slime molds. Haploid myxamoeboid cells of cellular slime molds behave differently under different environments. If there are light and less water, they behave like somatic cells of multicellular organisms. They grow and proliferate by fission by feeding bacteria. However, if there are dark and excess water, they behave like germ cells in multicellular organisms. They acquire sexual fusion competence and fuse each other to form zygotic diploid cells.

In the most commonly using strains of *D.discoideum*, NC4 and HM1, there are known to be at least two glycoproteins, gp70 and gp138, which involve in their sexual cell fusion. Gp70 is induced on the cell surface of HM1 only when excess water exists. The experiments described in the first chapter showed that there might be another molecule which represses function of gp70 and that expression of the molecule is induced by light. Thus, the two environmental factors, excess water and light, seems to work independently for acquiring fusion competence through different molecules.

Induction of cell fusion by the factor secreted from CK-8 cells of *P. pallidum* is also a kind of effects of exter-

nal factors. In the second chapter, the experiments revealed that the cell fusion induction factor might be complexes of hydrophobic glycoproteins of which are approximately 65 KDa in molecular mass. Analyzing action mechanisms of this complex would give a key for understanding general mechanism of cell fusion. Furthermore, the results in the chapter strongly suggest that the molecules for cell fusion induction and cell growth inhibition would be identical. If this is the case, then this system will be one of the most useful system to study relationship between cell growth and cell fusion.

In the third chapter, the results indicated clearly that the condition medium of CK-8 are only capable to induce cell fusion but not subsequently nuclear fusion. Therefore, this factor can not produce hybrids and consequently no genetical analysis could be performed using this system. However, this system will be still useful for studying the mutual relationship between cell fusion and nuclear fusion.

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Tables

Table I Fusion-competency of HM1 cells cultured under different conditions

Culture condition	cell fusion (%)		
	expt 1	expt 2	expt 3
Liquid-dark	81.8	83.8	73.8
Liquid-light	2.6	ND	9.9
Solid-dark	ND	1.8	9.8
Solid-light	1.2	ND	8.2

HM1 cells cultured under the indicated conditions were mixed with fusion-competent NC4 cells and incubated for 30 min at 22^{o}C to obtain fusion indices.

ND : not done

Table II Absorption of fusion-inhibiting Fab by gp70

Used cell for blot-absorption	Absorption (%)	
	expt 1	expt 2
Liquid-dark HM1	53.8	91.6
Liquid-light HM1	34.6	73.5
Solid-light HM1	0.0	30.0
None[a]	13.3	33.5

Proteins of $2x10^7$ cells (expt 1) or membrane fraction from $6x10^7$ cells (expt 2) were separated in preparative 7.5 % polyacrylamide gels and transferred to nitrocellulose membranes. The area containing gp70 was excised and incubated with 0.6 mg (expt 1) or 0.5 mg (expt 2) of Fab as described in Materials and Methods. After the incubation, the Fab solution was assayed for fusion inhibiting activity. The extent of absorption was determined as the ratio of decreases in fusion indices (FIs) in the presence of absorbed Fab to of non-absorbed Fab, which was calculated by the following formula:

$$\frac{\text{FI with absorbed Fab} - \text{FI with non-absorbed Fab}}{\text{FI without Fab} - \text{FI with non-absorbed Fab}} \times 100\ (\%)$$

[a] A control filter without protein blot.

Table III The activities of cell fusion induction and growth inhibition in fractions separated by addition of ammonium sulfate

fraction	expt 1		expt 2	
	cell fusion	growth inhibition	cell fusion	growth inhibition
CK-8 CM	68.5%	100.0%	63.5%	100.0%
0-33% $(NH_4)_2SO_4$	0.0	0.0	20.9	24.5
33-50% $(NH_4)_2SO_4$	72.5	99.3	75.1	94.1
50-66% $(NH_4)_2SO_4$	76.3	103.7	78.0	108.8
>66% $(NH_4)_2SO_4$	49.6	52.2	7.5	22.5

Different concentration of ammonium sulfate was added to CK-8 CM, Each precipitate was suspended in BSS and dialyzed against BSS and assayed for the activities. The activities of cell growth inhibition of each fraction was indicated as a relative activity to CK-8 CM.

Table IV The activities of cell fusion induction and growth inhibition in fraction separated by ConA-agarose column

fraction	expt 1		expt 2	
	cell fusion	growth inhibition	cell fusion	growth inhibition
CK-8 CM	68.3%	100.0%	72.2%	100.0%
33-66% $(NH_4)_2SO_4$	62.7	103.3	73.1	156.1
ConA-unbound	15.4	32.9	29.8	56.4
ConA- bound	54.0	99.2	71.3	159.7

The fraction precipitated by 33-66 % $(NH_4)_2SO_4$ was suspended in buffer A, dialyzed and applied to ConA-agarose column. ConA-bound fraction was eluted by 400 mM methyl α-D-mannopyranoside. ConA-unbound and ConA-bound fractions were assayed for the activities and compared to the activities of CK-8 CM and the fraction precipitated by 33-66% ammonium sulfate.

Table V The activities of cell fusion induction and growth inhibition in the fractions obtained

Fractions	cell fusion	growth inhibition	total activity	total protein	specific activity
CK-8 CM	59.5%	100.0%	400,000U	7,040μg	62.5U/μg
$(NH_4)_2SO_4$	66.3	183.3	265,785	3,712	71.6
ConA-bound	68.3	137.3	48,055	476	100.9
Superose 6	54.0	106.3	15,945	6	2,657.5
MONO Q	42.6	72.2	9,025	0.5	18,050.0

The activities cell fusion induction and growth inhibition in the fractions obtained by application of each column were summarized. The total activity and the specific activity were calculated from the activity of cell growth inhibition by using the following formula:

$$\text{total activity (U:unit)} = \text{growth inhibition(\%)} \times \frac{\text{volume of total fraction}}{\text{volume of sample used for assay}}$$

$$\text{specific activity(U/}\mu\text{g)} = \frac{\text{total activity(U)}}{\text{total protein(}\mu\text{g)}}$$

The activity of cell growth inhibition was assayed in the same volume of each fraction and the activity was indi-

cated as the relative cell growth inhibition to CK-8 CM. Experiments were repeated more than ten times, and similar results were attained.

Table VI Absorption of CK-8 CM neutralizing activity of antiserum by proteins separated on SDS-PAGE

expt 1		expt 2	
MW range (KDa)	CK-8 CM activity	MW range (KDa)	CK-8 CM activity
Control[a]	28.9%	Control[a]	14.5%
340-	68.4	360-	77.1
150-340	60.5	230-360	43.5
66-150	110.5	115-230	48.1
- 66	110.5	-115	95.4

The active ConA-bound fraction from CK-8 CM (100 ml) was lyophilized and applied to 3-12 % gradient running gel. The proteins isolated in the gel were transferred to a nitrocellulose membrane and cut into several pieces according to the molecular weight range shown in Table. Each piece of the membrane was incubated at room temperature with the antiserum which neutralize the cell fusion activity of CK-8 CM. After 1 hour incubation, the each antiserum was collected, coupled to Protein A-sepharose and incubated with CK-8 CM for 2 hour at 4^{o}C. The supernatant was then assayed for the cell growth inhibition activity.

[a] A filter piece with out protein blot.

FIGURES AND FIGURE LEGENDS

Figure 1. Western bolts of HM1 cells cultured under different conditions.

A: solid-dark, B: solid-light, C: liquid-dark, D: liquid-light HM1 cells. E: HM1 cells cultured in a liquid medium of the same composition as the SM-agar plate but without agar. The same number of cells were loaded on each lane. The arrow indicated the position of the gp70.

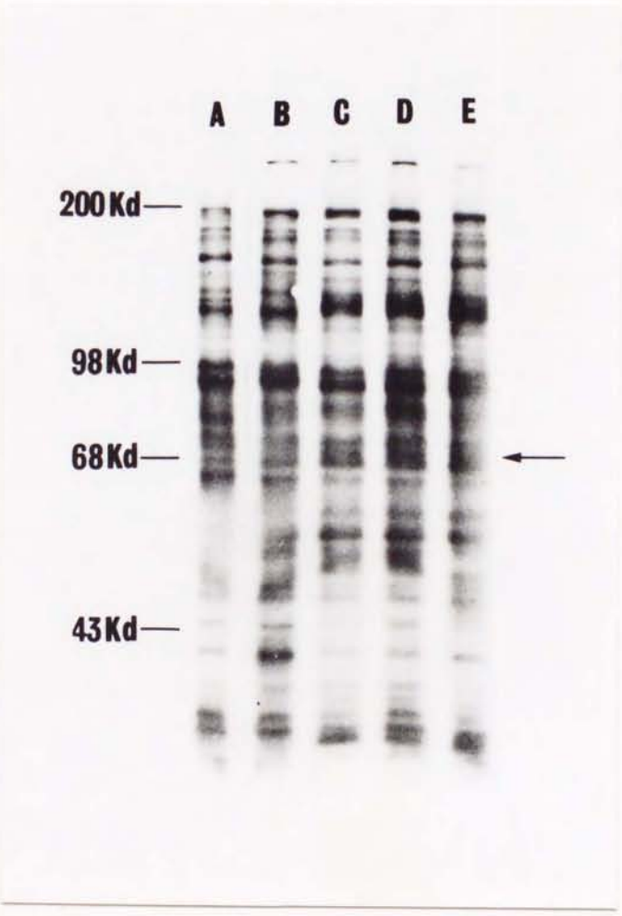
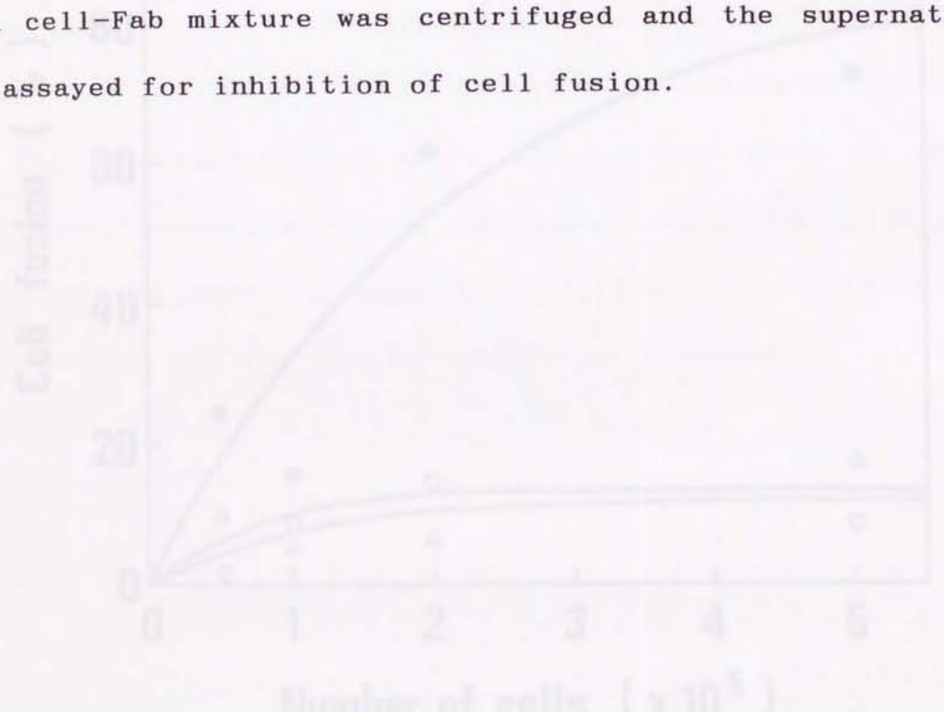

A
B
C
D
E
200Kd
98Kd
68Kd
43Kd

Figure 2. Absorption of inhibitory activity of Fab by intact HM1 cells.

Liquid-dark (●), liquid-light (o), solid-light (Δ) HM1 cells were incubated with 0.5 mg of Fab on ice for 1 hour with occasional swirling. At the end of incubation, each cell-Fab mixture was centrifuged and the supernatant was assayed for inhibition of cell fusion.



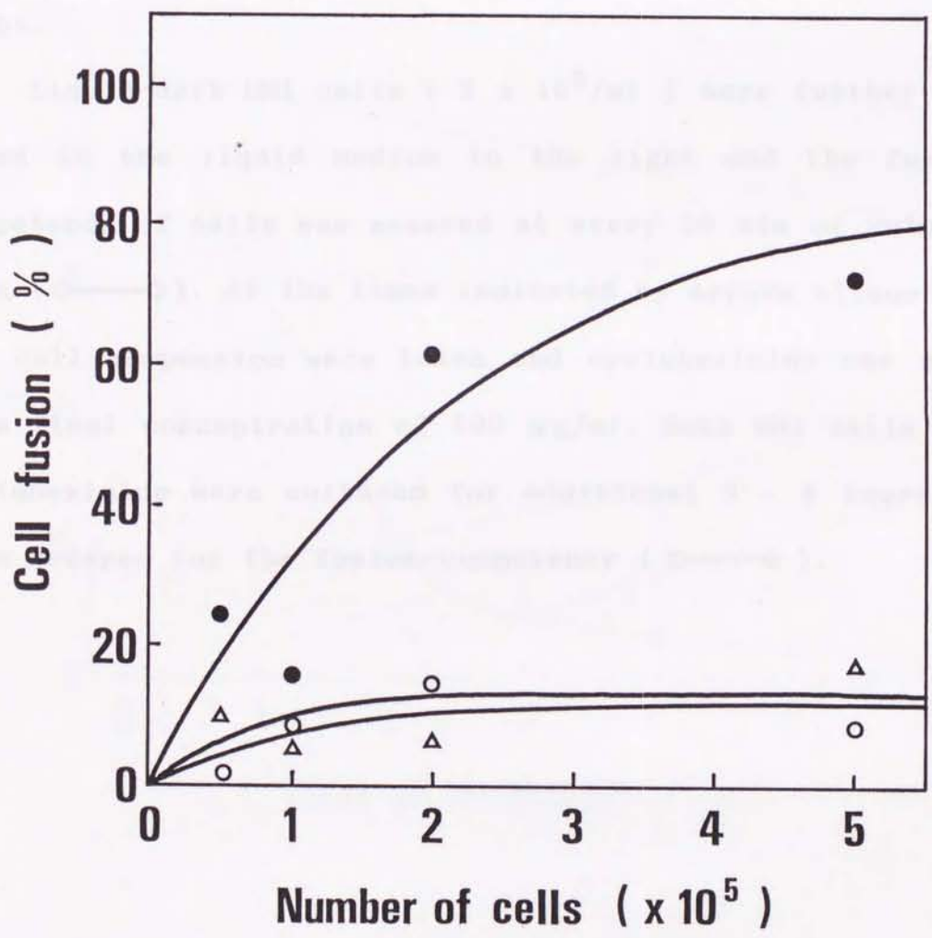

Cell fusion (%)
100
80
60
40
20
0
0
1
2
3
4
5
Number of cells (x 10^5)

Figure 3. Effect of cycloheximide on the loss of fusion-competency of HM1 cells during liquid incubation in the light.

Liquid-dark HM1 cells (3 x 10^6/ml) were further cultured in the liquid medium in the light and the fusion-competency of cells was assayed at every 30 min of cultivation (o——o). At the times indicated by arrows aliquots of the cell suspension were taken and cycloheximide was added to a final concentration of 100 μg/ml. Such HM1 cells with cycloheximide were cultured for additional 3 - 6 hours and then assayed for the fusion-competency (o-----●).

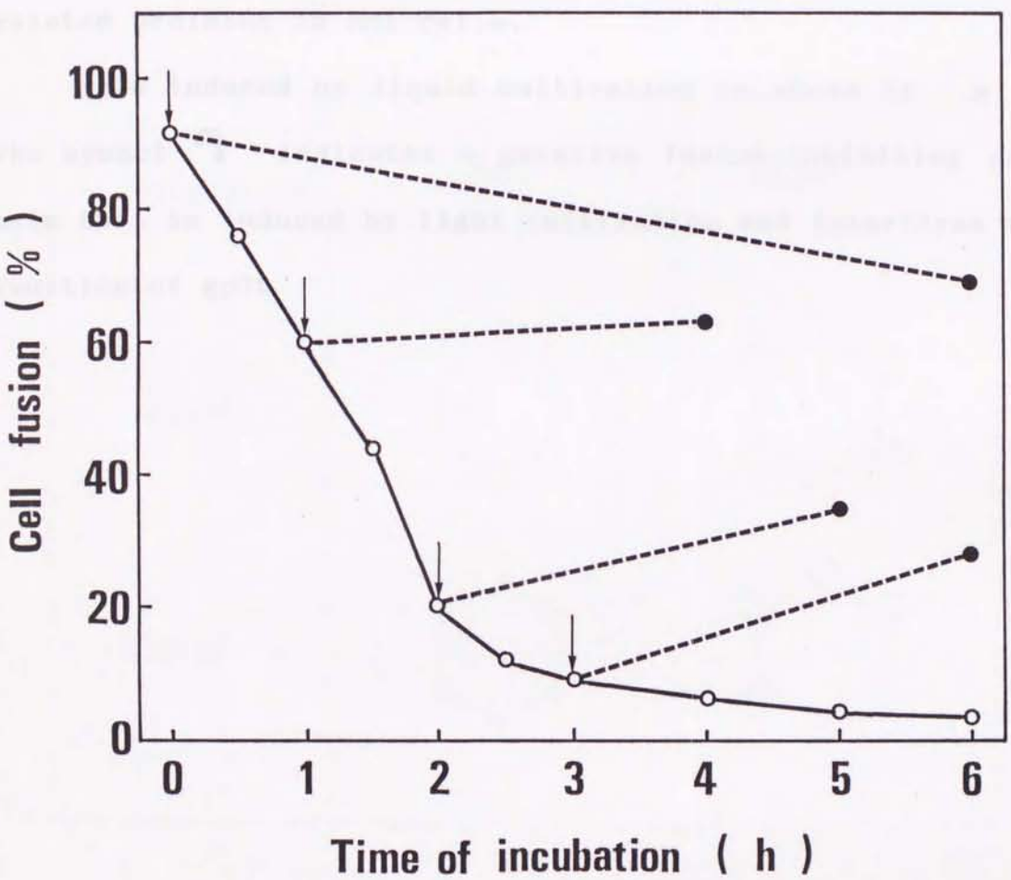

100
80
60
40
20
0
Cell fusion (%)
0
1
2
3
4
5
6
Time of incubation (h)

Figure 4. A model for environmental control of fusion-related proteins in HM1 cells.

gp70 induced by liquid cultivation is shown by ▲ . The symbol ⊓ indicates a putative fusion-inhibiting protein that is induced by light cultivation and interferes the function of gp70.

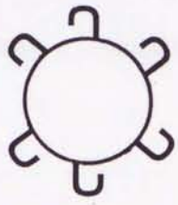
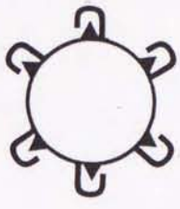
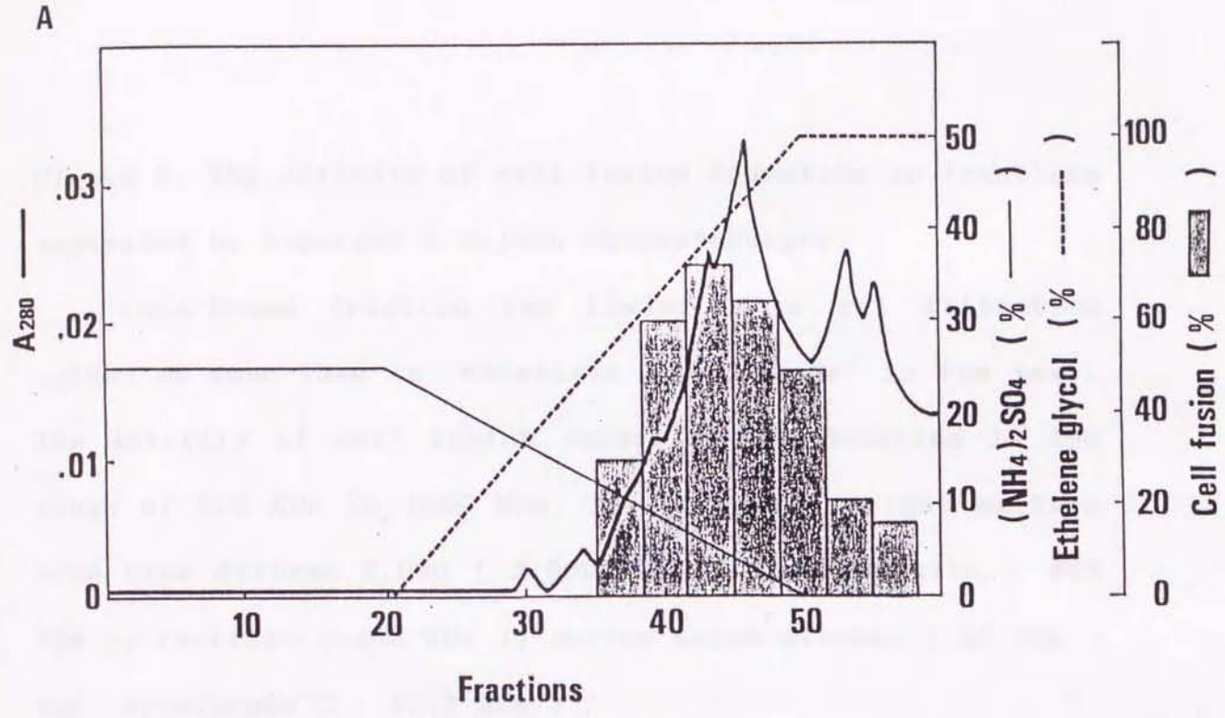
Culture conditions	Dark	Light
Solid		
Liquid		

Figure 5. The activity of cell fusion induction in fractions separated by hydrophobic column chromatography.

(A) Fraction precipitated by 33-66 % ammonium sulfate was collected and applied to a Phenyl-sepharose column. The activity of cell fusion induction in each fraction from the column was assayed. (B) The fraction was also subjected to a Phenyl-superose column on FPLC system. Results were similar to a as Phenyl-sepharose column.


A
A280 ——
.03
.02
.01
0
10
20
30
40
50
Fractions
50
40
30
20
10
0
(NH4)2SO4 (% ——)
Ethelene glycol (% -------)
100
80
60
40
20
0
Cell fusion (% ▨)

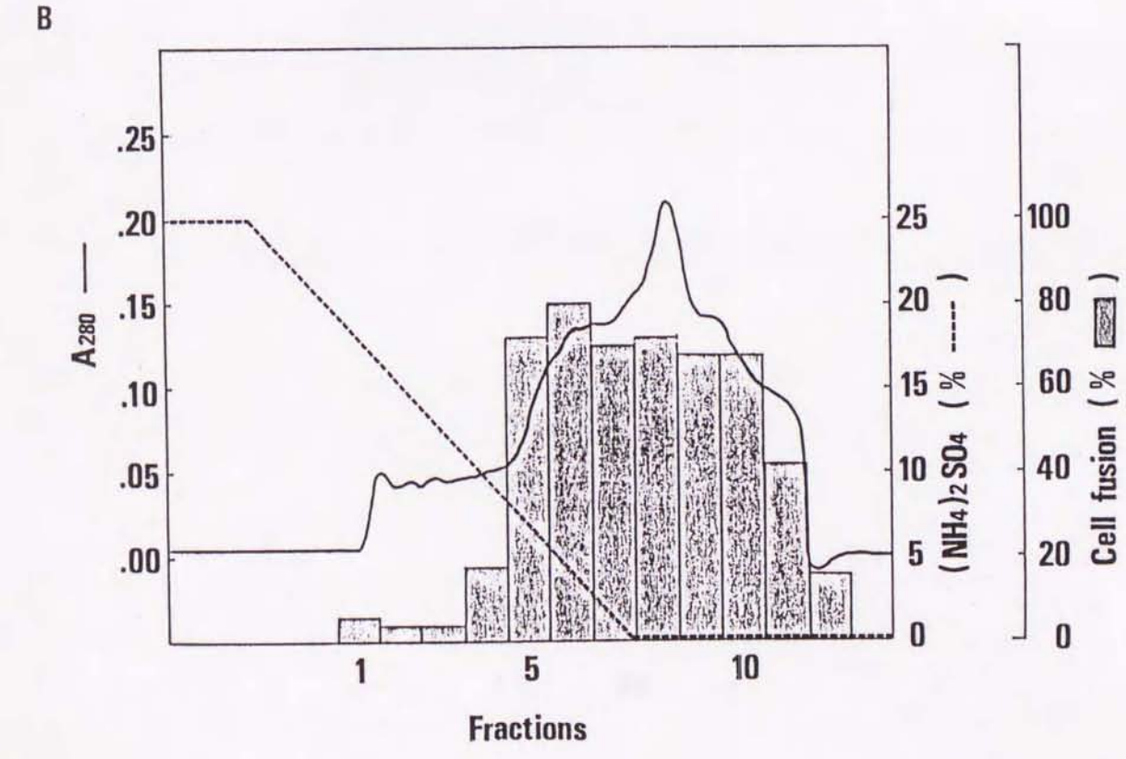

B
A280 ——
.25
.20
.15
.10
.05
.00
1
5
10
Fractions
25
20
15
10
5
0
(NH4)2SO4 (% ----)
100
80
60
40
20
0
Cell fusion (% ▨)

Figure 6. The activity of cell fusion induction in fractions separated by Superose 6 column chromatography.

ConA-bound fraction was loaded on a gel filtration column as described in "Materials and Methods" in the text. The activity of cell fusion induction was detected in the range of 500 KDa to 1000 KDa. The molecular weight markers were blue dextran 2,000 (2,000 KDa), thyroglobulin (669 KDa), ferritin (400 KDa), bovine serum albumin (67 KDa) and cytochrome C (12.3 KDa).

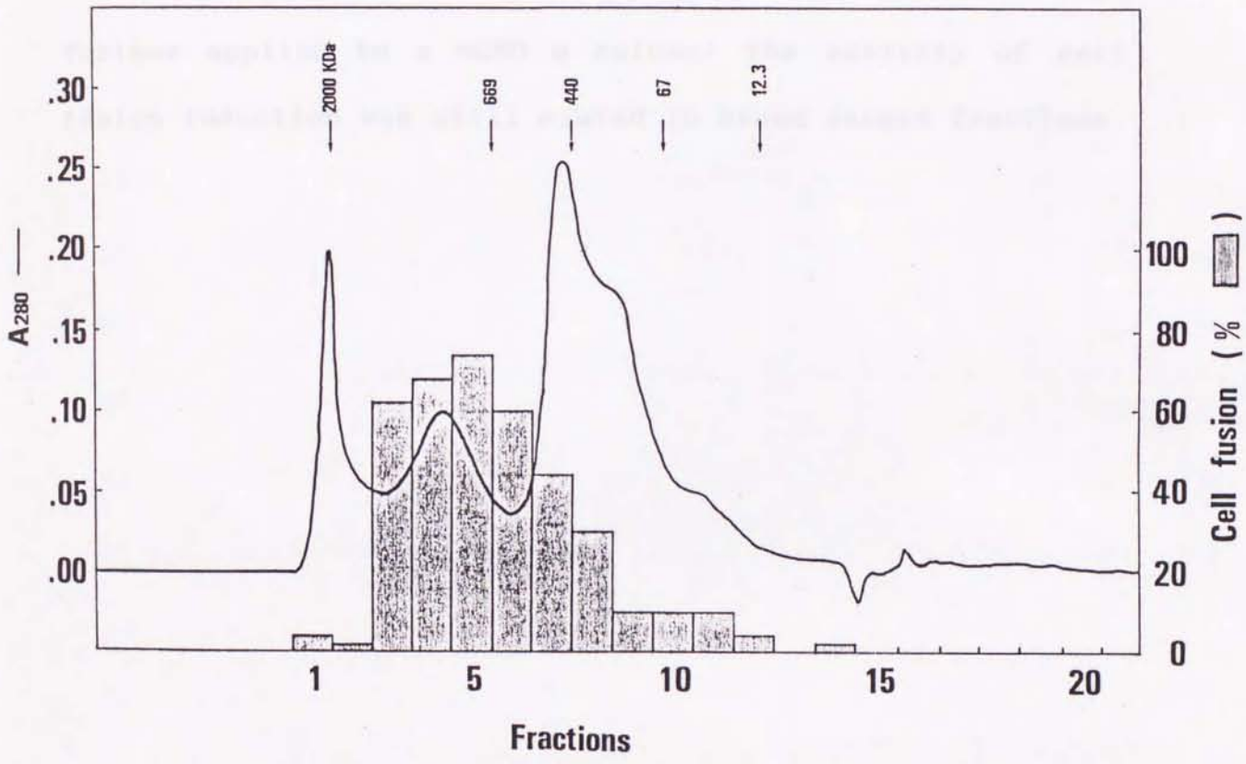

2000 KDa
669
440
67
12.3
A280
.30
.25
.20
.15
.10
.05
.00
100
80
60
40
20
0
Cell fusion (%)
1
5
10
15
20
Fractions

Figure 7. The activity of cell fusion induction in fractions separated by MONO Q column chromatography.

Active fractions separated by Superose 6 column were further applied to a MONO Q column. The activity of cell fusion induction was still eluted in broad ranged fractions.

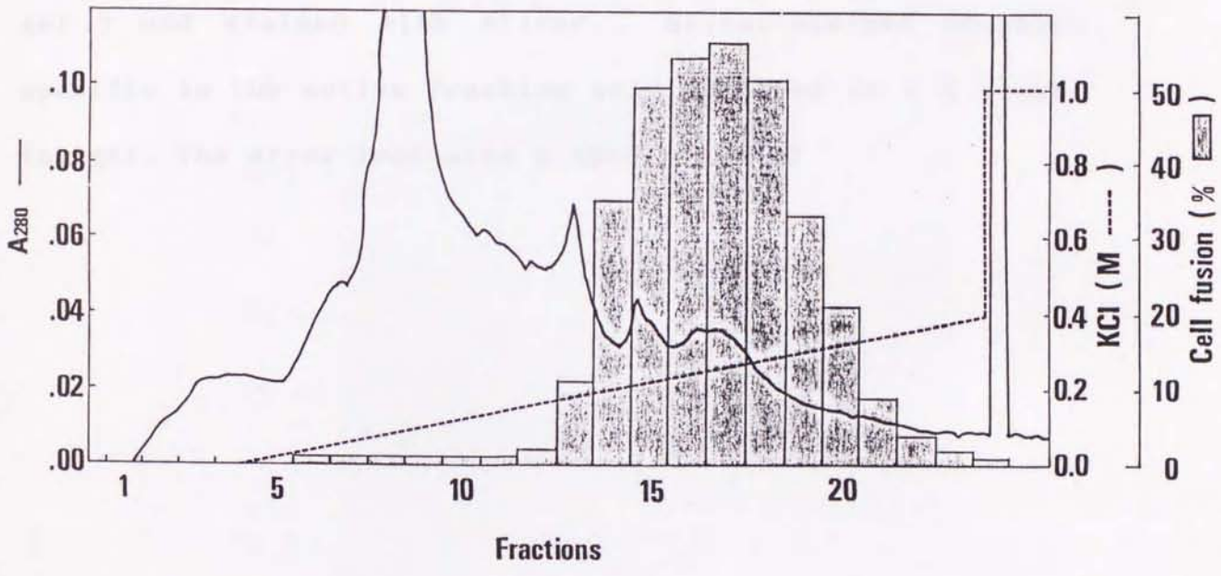

.10
.08
.06
.04
.02
.00
A280
1
5
10
15
20
Fractions
1.0
0.8
0.6
0.4
0.2
0.0
KCl (M -----)
50
40
30
20
10
0
Cell fusion (%)

Figure 8. SDS-PAGE analysis of fractions separated by Superose 6 column.

Each fraction obtained by Superose 6 column chromatography was applied to SDS-PAGE (7.5-12 % gradient running gel) and stained with silver. Silver-stained proteins specific in the active fraction only appeared in 4 % stacking gel. The arrow indicates a specific band.

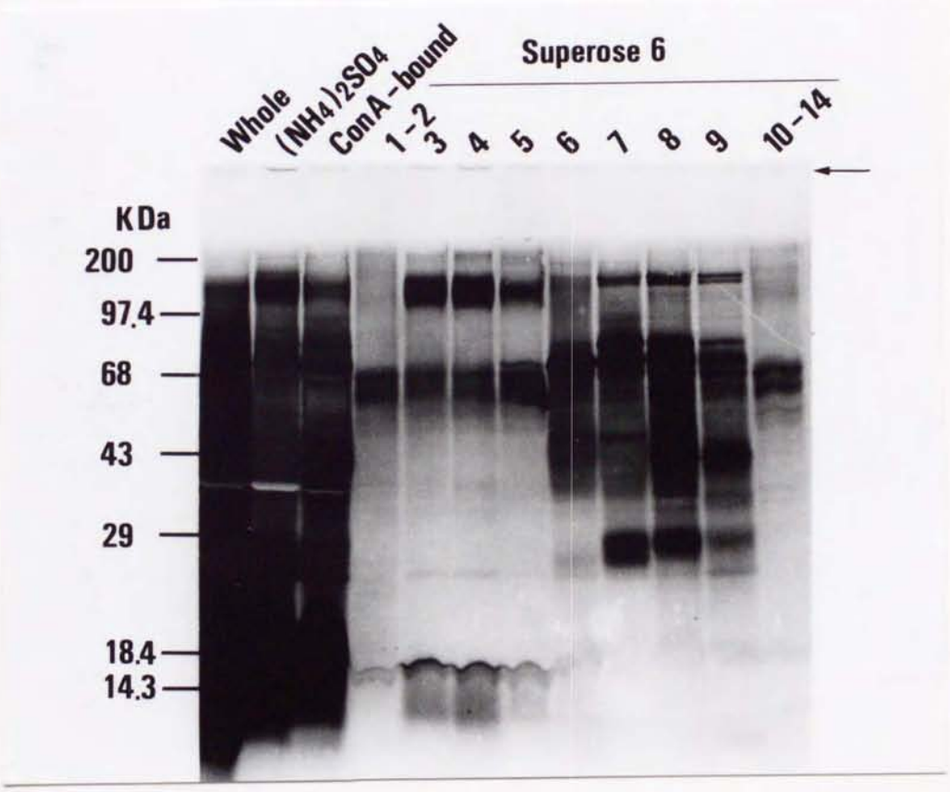
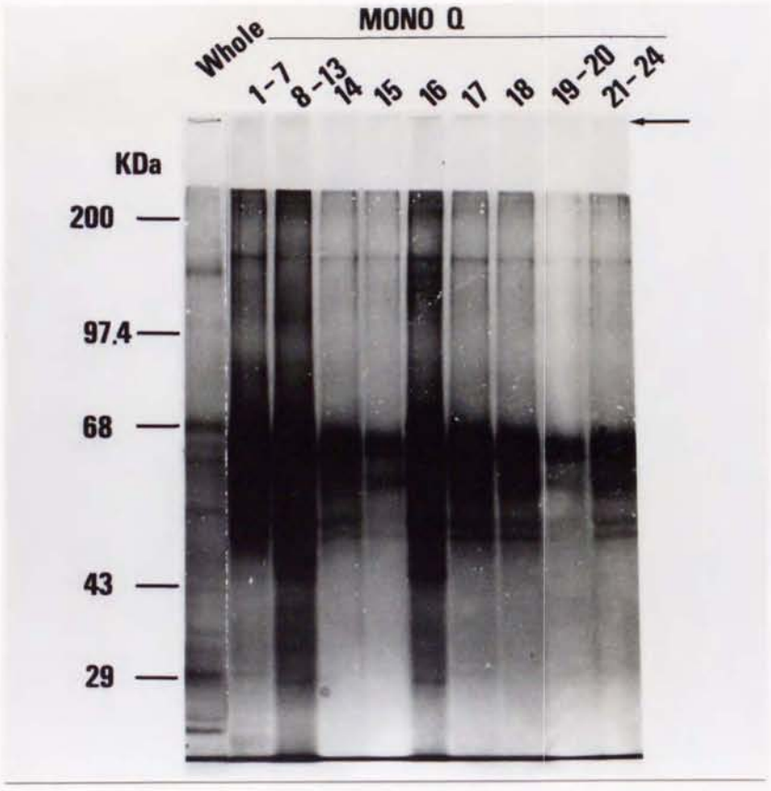

Whole
(NH4)2SO4
ConA-bound
Superose 6
1-2
3
4
5
6
7
8
9
10-14
KDa
200
97.4
68
43
29
18.4
14.3

Figure 9. SDS-PAGE analysis of each fraction separated by MONO Q column.

Fractions separated by a MONO Q column were subjected to SDS-PAGE using different percentage of gels; (A) 4 % stacking gel and 7.5 % non-gradient running gel, and (B) 3 % stacking gel and 3-12 % gradient running gel. The protein bands were stained with silver. The arrow in indicates a specific band for the active fractions.

A


MONO Q
Whole
1-7
8-13
14
15
16
17
18
19-20
21-24
KDa
200
97.4
68
43
29

B

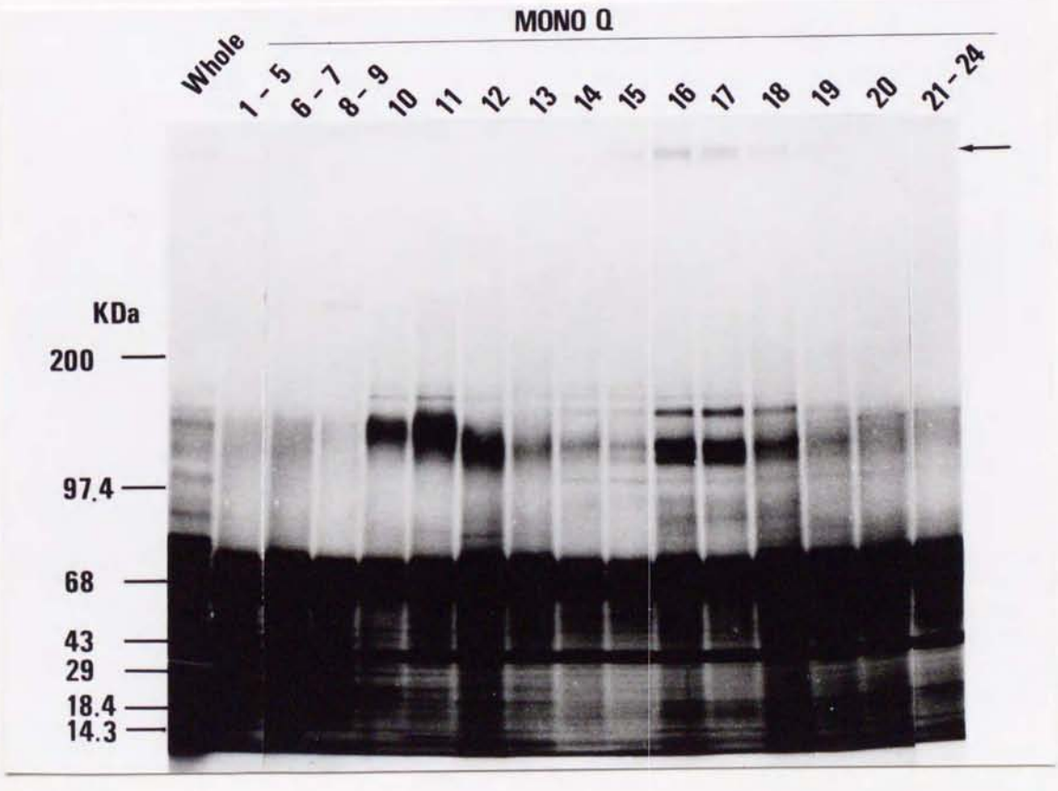

MONO Q
Whole
1-5
6-7
8-9
10
11
12
13
14
15
16
17
18
19
20
21-24
KDa
200
97.4
68
43
29
18.4
14.3

Figure 10. SDS-PAGE analysis of fractions obtained by each purification step.

CK-8 CM, 33-66 % $(NH_4)_2SO_4$ precipitate, ConA-bound fraction, active fractions (No. 3-6) separated by Superose 6, active fractions (No. 15-18) obtained by MONO Q were applied to SDS-PAGE using 3-12 % gradient running gel and dyed with silver-staining (A). (B) indicates immunoblotting pattern obtained by reaction with the CK-8 CM-neutralizing antiserum.

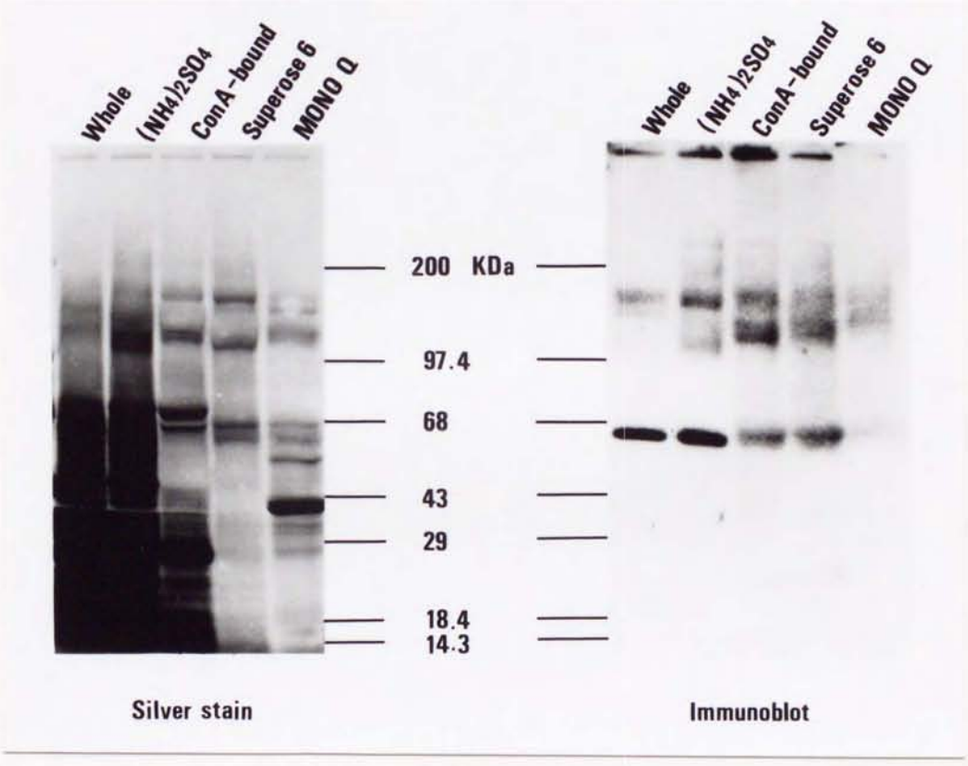

Whole
(NH4)2SO4
ConA-bound
Superose 6
MONO Q
200 KDa
97.4
68
43
29
18.4
14.3
Whole
(NH4)2SO4
ConA-bound
Superose 6
MONO Q
Silver stain
Immunoblot

Figure 11. Two dimensional gel electrophoresis of the active fraction separated by Superose 6 column.

In the first dimension, PAGE containing Softes 12 was performed. Gel obtained was then applied to SDS-PAGE as the second dimension. The proteins were detected by immunoblotting by the CK-8 CM-neutralizing antiserum. Arrowhead indicates the 65 KDa protein.

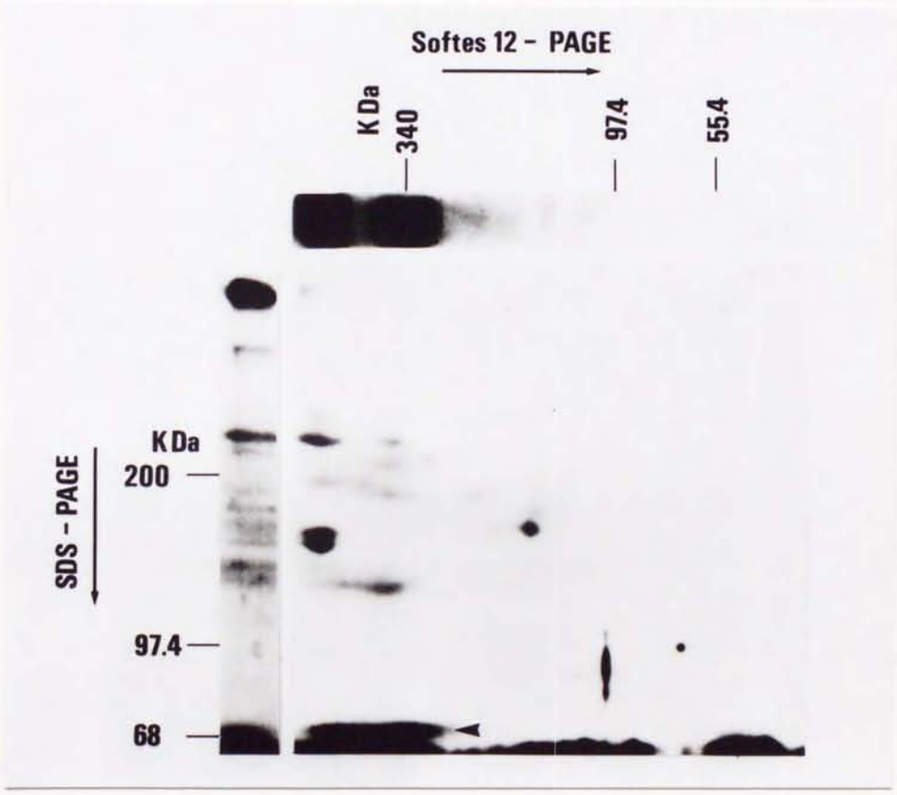

Softes 12 - PAGE
K Da
340
97.4
55.4
SDS - PAGE
K Da
200
97.4
68

Figure 12. Change of the number of multinuclear cells during cultivation with bacteria.

Fusion competent NC4 and HM1 cells were mixed in an equal number in BSS (containing 2.7 mM Ca^{2+}) following a diluted F2 treatment and 18 hours cultivation with bacteria in low Ca^{2+}BSS (containing 0.54 mM Ca^{2+}). Cell fusion was initiated immediately after the mix and completed within 60 min. Consequently a large number of multinuclear cells were produced. The cell suspension was successively cultivated with bacteria at 22°C on a shaker. During cultivation, the number of total cells were counted (●—●) and the ratio of the multinucleates to the total cells were estimated (o---o). Cells containing more than 3 nuclei were considered as multinucleates. Nm and Nw indicate the number of multinucleates and total cells in the suspension, respectively.

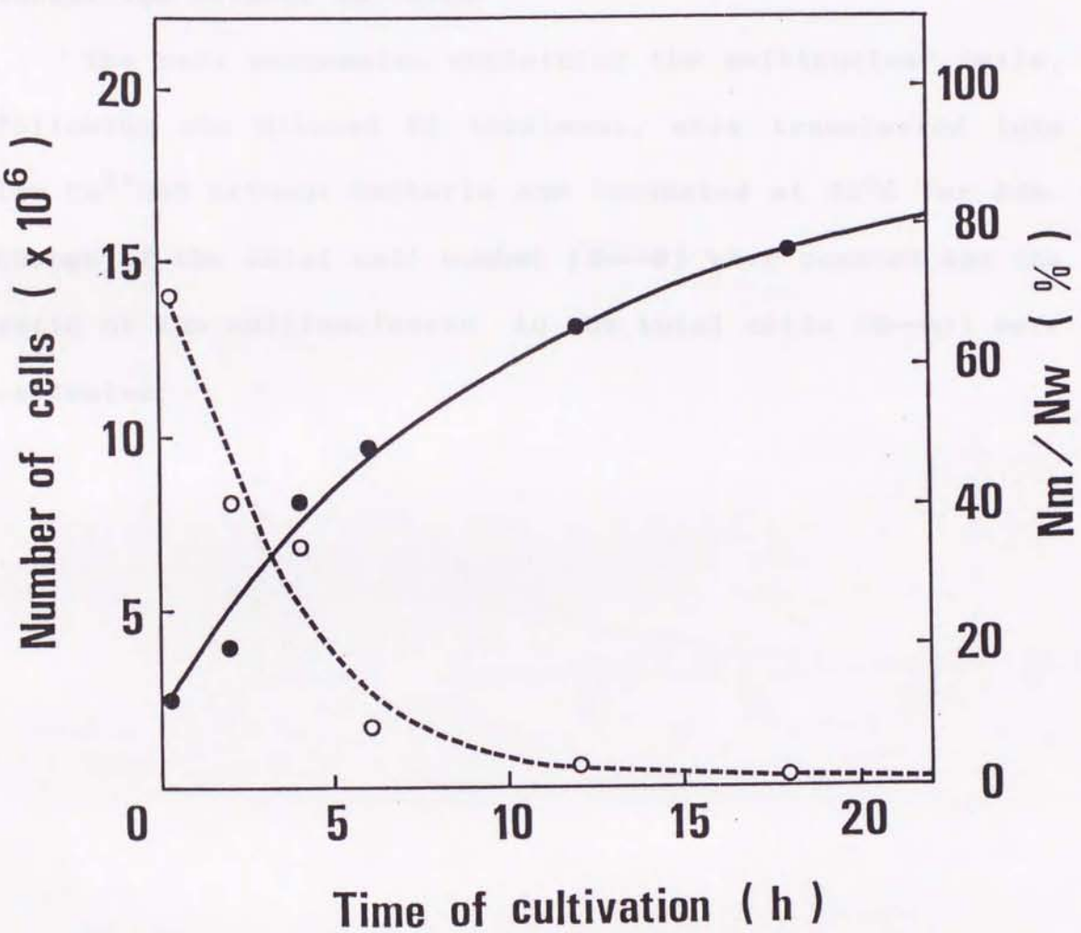

Number of cells (x 10^6)
Nm / Nw (%)
Time of cultivation (h)
0
5
10
15
20
0
20
40
60
80
100

Figure 13. Change of the number of multinuclear cells during incubation without bacteria.

The cell suspension containing the multinuclear cells, following the diluted F2 treatment, were transferred into low Ca^{2+}BSS without bacteria and incubated at 22°C for 24h. Change of the total cell number (●—●) were counted and the ratio of the multinucleates to the total cells (o---o) were estimated.

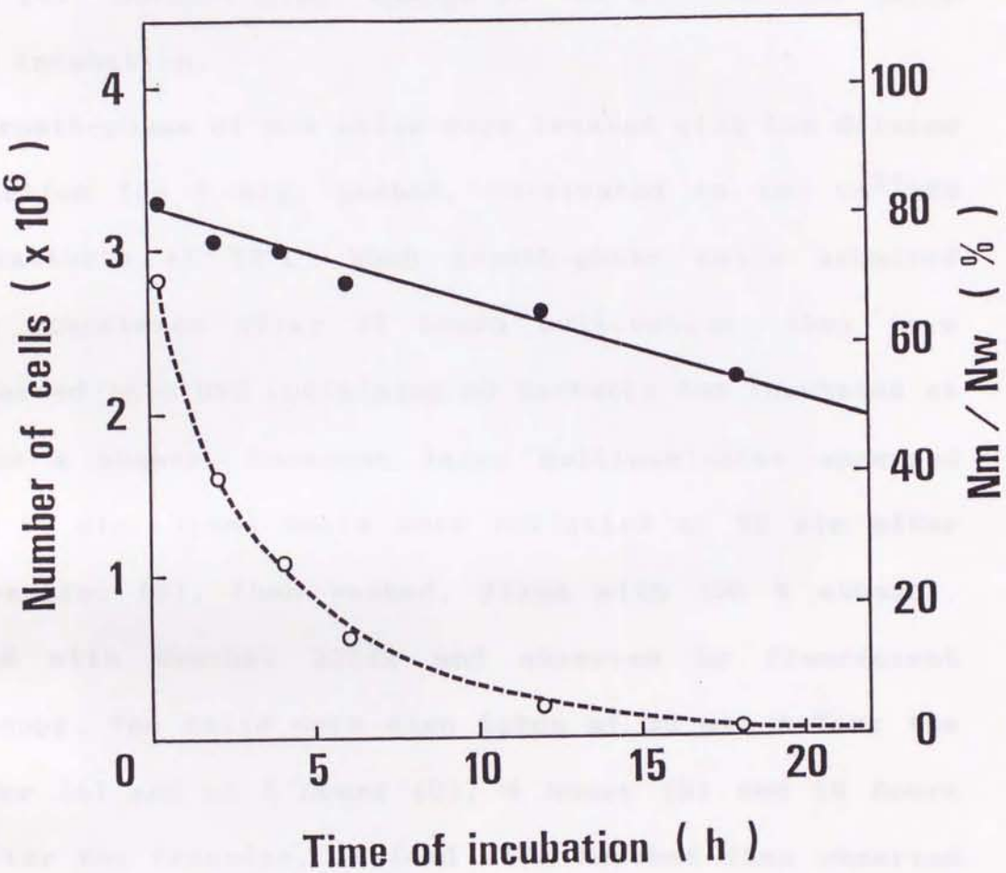

Number of cells (x 10^6)
Nm / Nw (%)
Time of incubation (h)
0
1
2
3
4
0
20
40
60
80
100
0
5
10
15
20

Figure 14. Morphological change of the multinuclear cells during incubation.

Growth-phase of NC4 cells were treated with the diluted F2 fraction for 3 min, washed, cultivated in low Ca^{2+}BSS with bacteria at 22°C. When growth-phase cells acquired fusion competence after 18 hours cultivation, they were transferred into BSS containing no bacteria and incubated at 22°C on a shaker. Numerous large multinucleates appeared within 60 min. These cells were collected at 30 min after the transfer (B), then washed, fixed with 100 % ethanol, stained with Heochst 33342 and observed by fluorescent microscopy. The cells were also taken at 30 min before the transfer (A) and at 2 hours (C), 6 hours (D) and 18 hours (E) after the transfer, stained with Hoechst then observed by fluorescent microscopy.

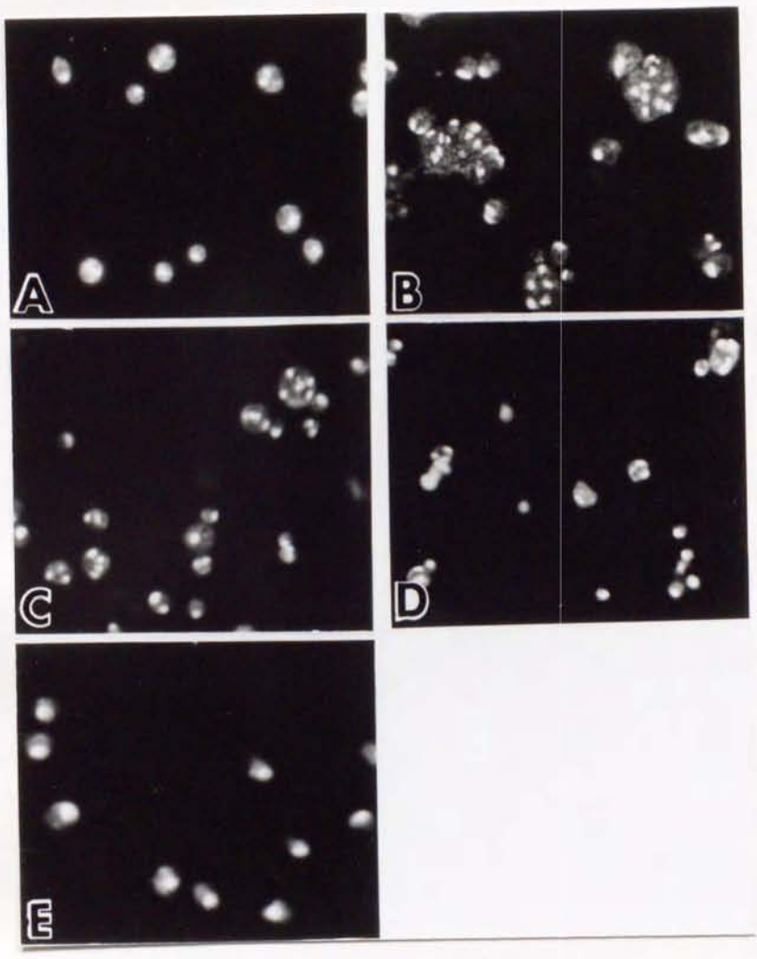
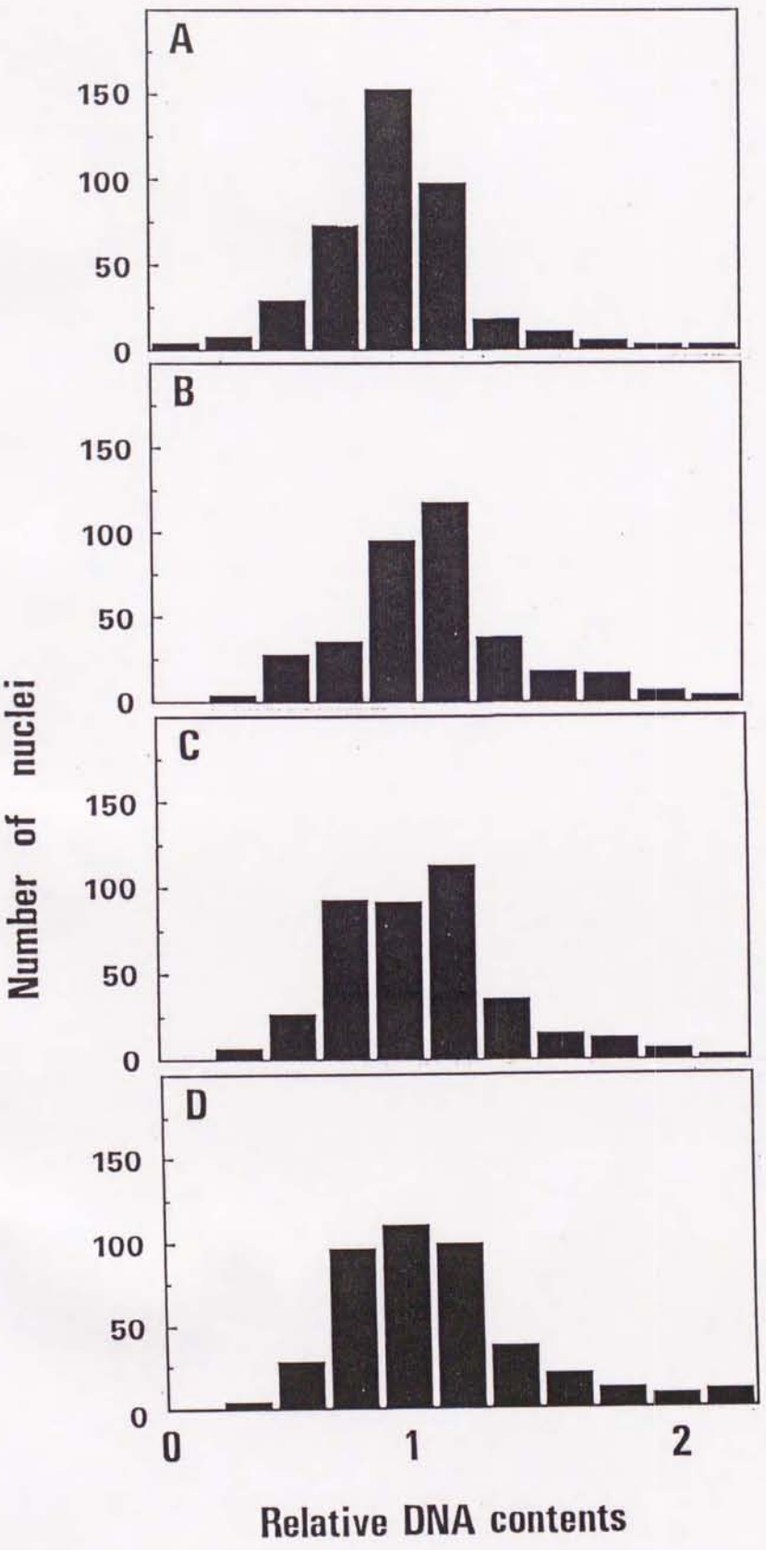
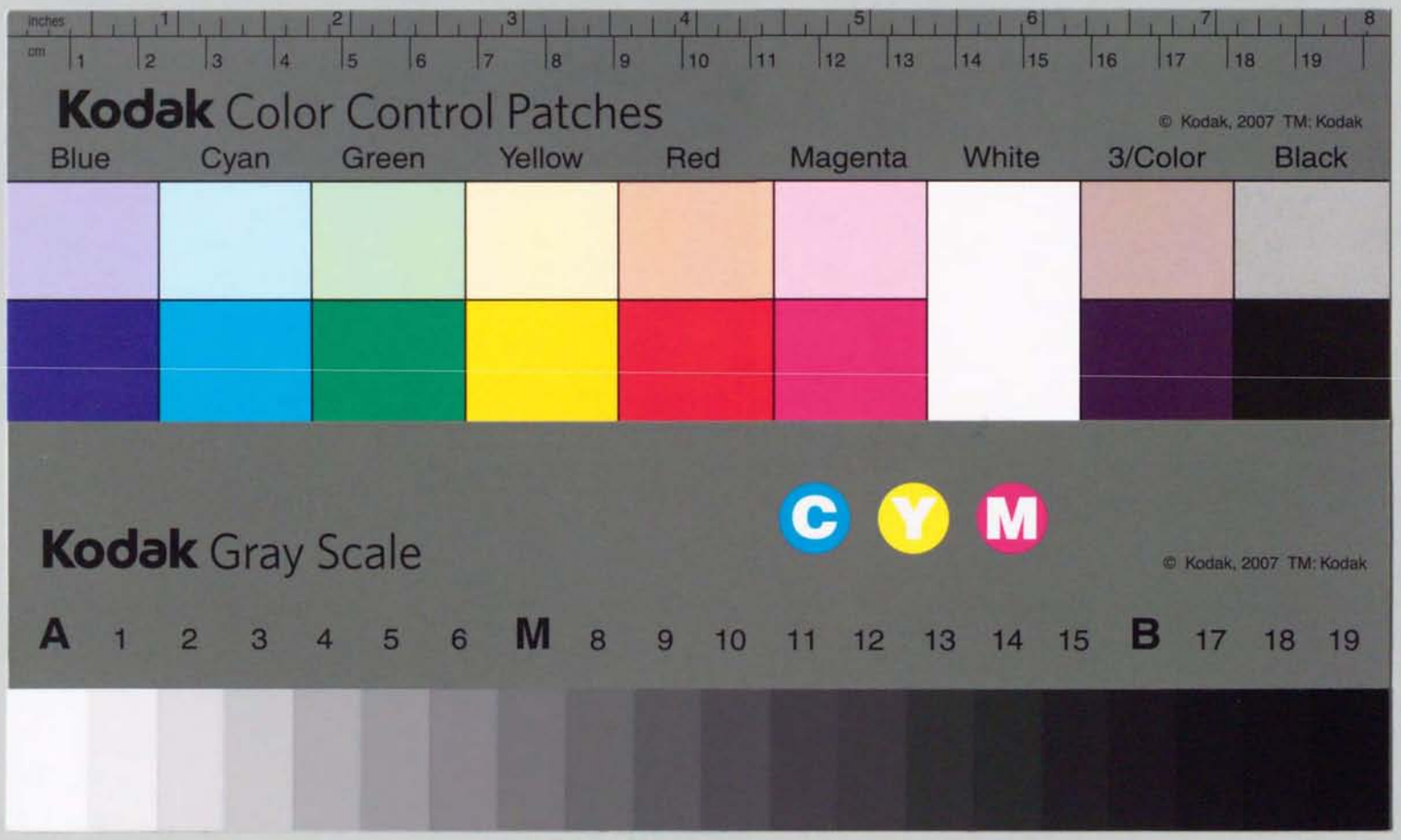

A
B
C
D
E

Figure 15. Change of DNA content of each nucleus during incubation.

A cell suspension containing the multinuclear cells, produced from NC4 and HM1 cells following the diluted F2 treatment, were incubated at 22^{o}C without bacteria. After 0 hour and 20 hours incubation, cells were taken and resuspended in NP-40 lysis buffer. Separated nuclei were fixed with 70 % ethanol and stained with DAPI. Relative DNA content of cells was measured by the method described in Materials and Methods. (A) shows a distribution of DNA contents of nuclei from NC4 growth-phase cells as a control. (B),(C) and (D) indicate the distributions of DNA contents of 0 hour, 20 hours cultivated cells with bacteria and 20 hours incubated ones without bacteria, respectively.


A
B
C
D
150
100
50
0
0
1
2
Number of nuclei
Relative DNA contents


inches
cm
Kodak Color Control Patches
© Kodak, 2007 TM: Kodak
Blue
Cyan
Green
Yellow
Red
Magenta
White
3/Color
Black
Kodak Gray Scale
C
Y
M
© Kodak, 2007 TM: Kodak
A 1 2 3 4 5 6 M 8 9 10 11 12 13 14 15 B 17 18 19