

The necessity of mitochondrial genome DNA for normal development of *Dictyostelium* cells

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Summary

Most unexpectedly, there is now increasing evidence that mitochondria have novel and crucial functions in the regulatory machinery of the growth/differentiation transition, cell-type determination, cellular movement and pattern formation. Here we created ρ^{Δ} cells with a reduced amount (about 1/4) of mitochondrial DNA (mtDNA) from *Dictyostelium discoideum* Ax-2 cells, by exposing Ax-2 cells to ca. 30 $\mu\text{g/ml}$ of ethidium bromide (EtBr) in axenic growth medium. Importantly, the ρ^{Δ} cells exhibited a series of fascinating behaviors: when they were starved, they showed a marked delay of differentiation and stopped their development at the slug stage, thus failing to construct fruiting bodies. Moreover, cell patterning and cell-type proportioning were found to be greatly modified in slugs (referred to as ρ^{Δ} slugs) derived from ρ^{Δ} cells. That is,

prestalk differentiation was significantly enhanced in ρ^{Δ} slugs, while prespore differentiation was markedly inhibited. In addition, the clear anterior prestalk/posterior prespore pattern was considerably disturbed in ρ^{Δ} slugs, presumably because of incomplete sorting between the two types of differentiated cells. After the assay of phototaxis, ρ^{Δ} slugs also exhibited highly disordered movement towards the light source. Taken together, these results suggest that mtDNA might have important multiple functions in a variety of cellular processes during *Dictyostelium* development.

Key words: Mitochondrial DNA, Rho-depleted cells, Growth, Differentiation, Pattern formation, Phototaxis, *Dictyostelium*

Introduction

Mitochondrial diseases and mitochondria-dependent sterility, as well as a close relationship between mitochondria and programmed cell death (apoptosis), have been generally recognized in a variety of cell lines. Increasing evidence also indicates that mitochondria have novel and crucial functions in the regulatory machinery of growth/differentiation transition, cell-type determination, cellular movement and pattern formation, particularly in the development of *Dictyostelium discoideum* (Maeda, 1971; Matsuyama and Maeda, 1995; Wilczynska et al., 1997; Matsuyama and Maeda, 1998; Inazu et al., 1999; van Es et al., 2001).

D. discoideum Ax-2 cells grow axenically as free-living cells and multiply by mitotic fission with a doubling time of about 7.5 hours, pinocytosing external nutrients. The differentiation phase is induced by nutritional deprivation, and starving cells gather together forming streams in response to pulsatile emissions of cAMP from the aggregation center (Konijin et al., 1967; Bonner, 1970). The cell aggregate assumes a hemispherical structure (mound). Subsequently, a tip arises at the apex of the mound, elongates and forms a migrating slug. At the slug stage, there is clear zonal differentiation along the long axis, with anterior prestalk cells (pst) and posterior prespore cells (psp). In differentiating prespore cells, there arise unique vacuoles called prespore-specific vacuoles (PSVs) (Maeda and Takeuchi, 1969; Hohl and Hamamoto, 1969). The PSV has been shown to be constructed from a mitochondrion with the help of the Golgi complex (Matsuyama and Maeda,

1998). During culmination, the prestalk cells of a slug differentiate into stalk cells, while the prespore cells differentiate into spores. The proportion of the two cell types (the number-ratio of pst/psp in the slug or stalk cells/spores in the fruiting body) is usually constant, with a 2.5-fold tolerance range (Rafols et al., 2000). The growth and differentiation phases in this organism are temporally separated from each other and easily controlled by nutritional conditions. In addition, the developmental system is relatively simple compared with that in other organisms. Moreover, a particular checkpoint (referred to as a PS-point) from growth to differentiation phase has been precisely specified in the mid-late G2 phase of the cell cycle (Maeda et al., 1989). This is the point from which cells initiate differentiation when placed under conditions of nutritional deprivation. Thus, *Dictyostelium* development offers us a particularly useful system for elucidating the cellular and molecular mechanisms of the growth/differentiation transition (GDT), pattern formation in cell masses and a variety of other problems of current interest in developmental biology.

As previously presented, the *dia3* gene, which encodes a mitochondrial protein cluster including ribosomal protein S4 (RPS4), is expressed specifically during the GDT of Ax-2 cells: its overexpression enhances the progress of cell differentiation, while its partial inactivation by means of homologous recombination greatly impairs differentiation and morphogenesis after starvation (Inazu et al., 1999). In differentiating prespore cells, the mitochondrion exerts a

remarkable structural transformation to form a sort of vacuole (M-vacuole), in which PSV, a cell-type-specific organelle (Maeda and Takeuchi, 1969; Hohl and Hamamoto, 1969), is constructed with the help of the Golgi complex (Matsuyama and Maeda, 1998). Mitochondrial cyanide (CN)-resistant respiration has been shown to be tightly involved in cell-type proportioning (e.g. the ratio of prestalk/prespore cells in a slug or stalk cells/spores in a sorocarp): application of specific inhibitors of CN-resistant respiration to starved *Dictyostelium* cells induces the formation of unique cell masses, in which almost all of the cells differentiate into prestalk cells and then stalk cells, thus being completely devoid of prespore and spore differentiation (Matsuyama and Maeda, 1995). The large subunit (mt-lrRNA) of *Dictyostelium* mitochondrial rRNA is also essential for phototaxis and thigmotaxis of the migrating slug (Wilczynska et al., 1997). Taken together, these data offer indications of the dynamic involvements of mitochondria and mitochondrial DNA in a variety of processes during *Dictyostelium* development.

The mitochondrial genome (mtDNA) of *D. discoideum* consists of 55,564 bp of nucleotides that encode two rRNAs, ten subunits of the NADH dehydrogenase complex (NAD 1, 2, 3, 4, 4L, 5, 6, 7, 9 and 11), apocytochrome b (cyt b), three subunits of the cytochrome oxidase (COX1/2 and 3), four subunits of the ATP synthase complex (APT1, 6, 8 and 9), 15 ribosomal proteins and five other open reading frames (ORFs), excluding intronic ORFs (essential peptides of enzymes for oxidative phosphorylation, three rRNAs and 18 tRNAs) (Gray et al., 1998; Ogawa et al., 2000). Mitochondria and mtDNA are believed to be closely associated with cell growth, because mitochondria are organelles that produce chemical energy in the form of ATP (Kotsifas et al., 2003; Zhu et al., 1997). Ethidium bromide (EtBr) is known to be a potent inhibitor of mtDNA replication and transcription in mammalian cultured cells (Nass, 1970; Leibowitz, 1971; Zylber et al., 1969) and avian cultured cells (Desjardins et al., 1985; Desjardins et al., 1989), but it does not substantially affect the synthesis of nuclear DNA (Radask et al., 1971; Nass, 1972; Leblond-Larouche et al., 1979). In cultures of *Dictyostelium* Ax-3 cells, EtBr actually inhibits any new synthesis of mtDNA (Firtel and Bonner, 1972; Kobilinsky and Beattie, 1977), and growth of Ax-3 cells stops after one or two generations in the presence of 10 µg/ml EtBr (Stuchell et al., 1973). The creation of cells completely lacking mtDNA, termed ρ^0 or ρ^0 cells, by repeated cell division in the presence of EtBr, has been achieved in both mammalian and avian cell cultures. In this study, in order to elucidate possible involvements of mtDNA in *Dictyostelium* development, we aimed to prepare ρ^0 cells from *D. discoideum* Ax-2 cells, by means of their exposure to EtBr in axenic growth medium. In *Dictyostelium* cells, it proved to be difficult to create complete ρ^0 cells, presumably because of the prerequisite of mtDNA for cell growth. However, we succeeded in preparing ρ^0 -like cells with about 1/4 of mtDNA compared with that of Ax-2 cells. In the remainder of this article we refer to these cells as ρ^Δ or rho-depleted cells. Fascinating phenotypes of these ρ^Δ cells are described, and possible roles of mtDNA in *Dictyostelium* development are discussed, with special emphasis on cell differentiation and pattern formation.

Materials and Methods

Cells and growth conditions

Vegetative cells of *D. discoideum*, axenic strain Ax-2 (clone 8A), were axenically grown in growth medium (PS medium) [1% Special Peptone (Oxoid: Lot. No. 333 56412), 0.7% Yeast extract (Oxoid), 1.5% D-glucose, 0.11% KH₂PO₄, 0.05% Na₂HPO₄·H₂O, 40 ng/ml vitamin B₁₂, 80 ng/ml folic acid]. Cells transformed with a vector (*pEcmAO-gal* or *pD19-gal*) bearing a bacterial β -galactosidase gene under the control of cell-type-specific promoters were also used and grown in PS medium containing 50 µg/ml of G418 (Geneticin, Life Technologies). The *ecmAO* promoter directed gene expression specifically in prestalk (pstAO) cells (Zhukovska et al., 1996), whereas the *D19* promoter allowed expression in prespore cells (Dingermann et al., 1989). The transformants were transferred to G418-free PS medium before use and shaken for 24-48 hours to remove the effects of G418 on growth and subsequent differentiation, as pointed out by Buhl and MacWilliams (Buhl and MacWilliams, 1991). Ten milliliters of the cell suspension were cultured in a 200 ml Erlenmeyer flask coated with Sigmacote (Sigma), at 22°C on a rotary shaker at 150 rpm.

Preparation of ρ^Δ cells

Exponentially growing cells (2-5×10⁶ cells/ml) were transferred to fresh PS medium containing various concentrations of ethidium bromide (EtBr) at a density of 2-5×10⁵ cells/ml, followed by shake culture at 22°C in the dark. For this, culture flasks were covered with aluminum foil. Under this culture condition, ρ^Δ cells with a reduced amount of mtDNA were found to arise after about two times of cell division in PS medium containing 30 µg/ml of EtBr, as presented later.

Developmental conditions

To allow cells to differentiate, exponentially growing cells were harvested, washed twice in Bonner's salt solution (BSS; 0.6 g NaCl, 0.75 g KCl, 0.3 g CaCl₂, 1000 ml distilled deionized water) (Bonner, 1947) as starvation medium, and incubated either on agar at a density of 5×10⁶ cells/cm² or in a 24-well plate (Falcon, #3047) under submerged conditions at a density of 5×10⁵ cells/cm² (1 ml cell suspension/well) at 22°C. To obtain migrating slugs, starved cells were suspended in BSS at a density of about 2.5×10⁷ cells/ml and aliquots (2 ml) of the cell suspension were plated on 1.5% agar in 9 cm glass dishes and incubated at 22°C for 18-48 hours. Slugs that had migrated out of the original streaks were used for subsequent histochemical staining of β -gal activity and immunocytochemical detection of PSVs. In another experiments, starving cells were plated at a density of about 1.5×10⁶ cells/cm² on white Millipore filters (HAWP; Nihon Millipore) placed on 1.5% non-nutrient agar in 9 cm glass dishes and incubated at 22°C for 18-48 hours.

Immunocytochemical detection of prespore-specific vacuoles (PSVs)

Slugs migrating on 1.5% non-nutrient agar were collected by centrifugation and disaggregated by 1% pronase E (Merck, NJ) and 25 mM 2,3-dimercapto-1-propanol (BAL; Wako Chemical, Japan) dissolved in 50 mM Tris-HCl buffer (pH 7.0) for 15 minutes with intermittent pipetting (Takeuchi and Yabuno, 1970). The dissociated cells were washed three times in BSS, pre-fixed in ice-cold 50% methanol and fixed in absolute methanol for 10 minutes on an ice-bath. The fixed cells were dried on cleaned coverslips. They were dipped in PBS (140 mM NaCl, 3 mM KCl, 10 mM Na₂HPO₄, 2 mM K₂HPO₄, pH 7.2) for 5 minutes and stained with FITC-conjugated anti-*D. mucoroides* spore IgG in a moisture chamber for 45 minutes at room temperature, according to the method of Takeuchi (Takeuchi,

1963). The sample was washed three times in PBS (5 minutes for each) and mounted in PBS containing 20% glycerol. The percentage of cells (prespore cells) with stained PSVs to the total cells examined was determined under a fluorescence microscope.

Histochemical staining of β -galactosidase activity

Cell masses of the transformants (*pEcmAO-gal* or *pDI9-gal* cells) were fixed and stained for β -gal activity according to a modification of the method described previously (Dingermann et al., 1989). Filters on which slugs had been formed were floated on Z-buffer containing 0.1% glutaraldehyde (GA) and 0.1% TritonX-100 (TX-100) for 5 minutes and then submerged in the same solution for 10 minutes. After two washings in Z-buffer, the preparations were incubated at 37°C in the staining solution (5 mM of both $K_3[Fe(CN)_4]$ and $K_4[Fe(CN)_3]$; 1 mM X-gal; in Z buffer) for β -gal activity. The staining was stopped by washing in Z buffer and the stained slugs were photographed in color under a binocular microscope. To determine the cell types at a single-cell level, slugs were dissociated by pronase-BAL, washed three times in BSS and incubated on cleaned coverslips for 20 minutes at 22°C in a moisture. Cells adhering on the coverslip were fixed by two changes of Z-buffer containing 0.05% GA and 0.1% TX-100, and stained as described above. The cell type (PstAO cells or prespore cells) of dissociated slug cells was determined by counting the ratio of stained/nonstained cells under an optical microscope (Optiphot; Nikon & Co., Tokyo, Japan).

Staining of cells with a DNA-specific dye, DAPI

The vegetatively growing cells were washed twice with BSS by centrifugation at 400 g for 1 minute. Subsequently, ice-cold absolute methanol was poured into the cells suspended in BSS and centrifuged. The cells were resuspended in ice-cold absolute methanol. After centrifugation, the cells were suspended in 0.5 ml of ice-cold absolute methanol and fixed for 10 minutes on ice. Three to four drops of the cell suspension in methanol were put on a cleaned coverslip and dried. Staining of the fixed cells with DAPI was done as described previously (Leeman and Ruch, 1982; Maeda, 1986). The stained cells were observed under a fluorescence microscope.

Staining of cells with a mitochondrion-selective dye, MitoTracker Orange

The vegetatively growing cells were incubated in PS medium containing 0.5 μ M MitoTracker Orange CMTMRos (Molecular Probes) for 30 minutes at room temperature. The stained cells were washed twice with BSS. One drop of the cell suspension was put on a cleaned coverslip and cells were fixed with 3.7% formaldehyde in 20 mM Na/K phosphate buffer (pH 7.2) for 20 minutes. Subsequently, the cells were washed with PBS, basically according to the method of Morita et al. (Morita et al., 2002). The stained cells were observed under a fluorescence microscope.

Isolation of genomic DNAs and Southern blot analysis

Genomic DNAs were extracted by the method described by Nellen et al. (Nellen et al., 1987). Genomic Southern hybridization was performed using the Southern method (Southern, 1975) with a slight modification. Three to four micrograms of genomic DNA were extracted from the same number of EtBr (40 hours)-treated cells and nontreated cells, digested with appropriate restriction endonucleases and electrophoresed in 0.7% agarose gel. Before blotting, the gel was treated with 0.25 mM HCl for 10 minutes, and then with 0.5 mM NaOH and 1.5 mM NaCl for 30 minutes. The size-fractionated genomic DNA fragments were transferred to a nylon membrane and blotted for hybridization to the *rps4* cDNA probe or *Dd-TRAP1* cDNA probe that had been 32 P-labeled using a Megaprime™ DNA labeling

system (Amersham). The membrane was baked for 2 hours at 80°C to immobilize DNA and was stored at 4°C. Prehybridization was carried out at 65°C for 2 hours in a solution containing 5 \times Denhardt solution [0.02% Ficoll-400, 0.02% bovine serum albumin (BSA), and 0.02% polyvinylpyrrolidone], 5 \times SSPE (43.8 g NaCl, 6.9 g $NaH_2PO_4 \cdot 12H_2O$ and 1.85 g EDTA; pH 7.4) or 5 \times SSC (43.8 g NaCl and 22.05 g sodium citrate), 0.5% sodium dodecyl sulfate (SDS) and 20-100 μ g of denatured salmon sperm DNA per ml. Hybridization was carried out at 65°C for 20 hours in the same solution containing the 32 P-labeled cDNA probe by use of the Megaprime™ DNA labeling system. The membranes were washed twice with a washing solution (1 \times SSC and 0.1% SDS) at room temperature for 15 minutes and then twice with a solution (0.1 \times SSC and 0.1% SDS) at 65°C for 5-10 minutes. The membranes were exposed to X-ray films for 2 days at -80°C.

Electron microscopy

The vegetatively growing Ax-2 and ρ^A cells were fixed in 1% solution of osmium tetroxide (OsO_4) dissolved in BSS for 8 minutes at room temperature as described previously (Abe and Maeda, 1991). During subsequent dehydration in a graded series of ethanol (50%, 70%, 90%, 95% and 100%), specimens were prestained with uranyl acetate saturated in 50% ethanol for 2 hours at room temperature and were embedded in an epoxy resin, Epok-812 (Oken Shoji, Japan). After polymerization of the resin, ultrathin sections (60-80 nm thick) were cut with a Reichert-Nissei Ultracut S (VIM 535). The sections were stained with lead citrate according to Reynolds (Reynolds, 1963) and were observed with a Hitachi transmission electron microscope (H-8100).

Assay of phototaxis

For a phototaxis assay, a slight modification of the method of Fisher et al. (Fisher et al., 1981) was applied. Starved Ax-2 cells and EtBr-treated cells were incubated for 24-48 hours on 1.5% non-nutrient agar to allow slug formation. After incubation with a lateral light source at 22°C for 24-48 hours, slugs migrated over the agar surface leaving behind the usual trail of collapsed slime sheath. The plates were inspected and scored for normal or defective phototaxis described previously (Fisher et al., 1981). Trails of slugs were transferred to PVC discs, stained and digitized as described previously (Fisher et al., 1981), and then plotted from a common origin.

Results

Effect of EtBr on cell growth

The response of cells to a selective inhibitor of mtDNA replication, EtBr, is known to vary depending on its concentrations and the cell lines used (Desjardins et al., 1985; King and Attardi, 1989). Therefore, Ax-2 cells were grown in axenic growth medium containing various concentrations of EtBr to test its effect on growth. As shown in Fig. 1, the growth rate was dependent on the EtBr concentration: no growth occurred in the presence of more than 40 μ g/ml EtBr, and this was followed by cell death after 1-2 weeks of culture. At 30 μ g/ml EtBr, cells exhibited somewhat delayed growth and stopped dividing after 36 hours of shake culture, during which only two times of cell division took place (Fig. 1). The reduced growth rate and subsequent growth arrest were not recovered by the addition of uridine or pyruvate into growth medium (data not shown). Incidentally, lower concentrations (0.1-5 μ g/ml) of EtBr had little effect on cell growth.

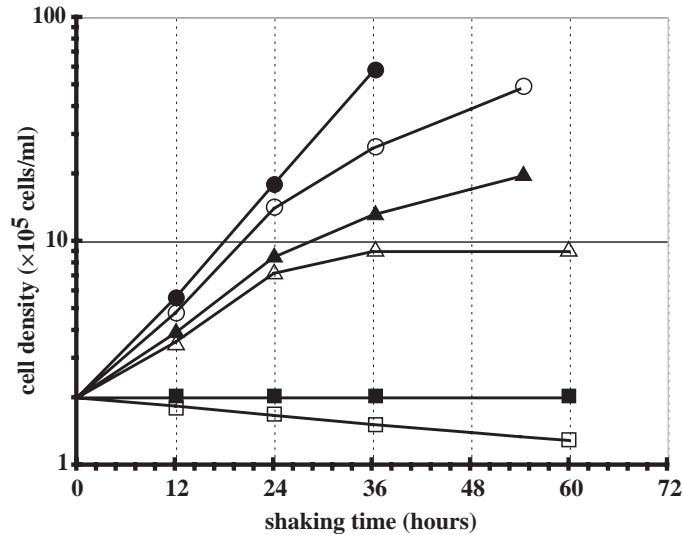


Fig. 1. Effect of EtBr on growth of *Dictyostelium discoideum* Ax-2 cells. Various concentrations of EtBr were added to exponentially growing cells (2×10^5 cells/ml) in axenic growth medium (PS medium), followed by cell counts under a haemocytometer. (●) control (non-EtBr-treated cells); (○) cells treated with 10 $\mu\text{g/ml}$ of EtBr, (▲) 20 $\mu\text{g/ml}$ of EtBr, (△) 30 $\mu\text{g/ml}$ of EtBr, (■) 40 $\mu\text{g/ml}$ of EtBr and (□) 50 $\mu\text{g/ml}$ of EtBr. Similar results were obtained by cell counts in three independent experiments.

Creation of ρ^A cells

To estimate approximately the amount of mtDNA in Ax-2 cells treated with various concentrations of EtBr, cells were stained with DAPI 40 hours after exposure to EtBr to measure mtDNA. As a result, cells exposed to lower (0.1–25 $\mu\text{g/ml}$) or higher concentrations (40–50 $\mu\text{g/ml}$) of EtBr were found to have DAPI-stained mitochondria, like those in nontreated cells. In the presence of 30 $\mu\text{g/ml}$ EtBr, however, cells that had divided for about two generations exhibited markedly reduced staining of mitochondria with DAPI in contrast to normal staining of

nuclei, thus indicating a selective decrease of mtDNA (Fig. 2C). There were no significant differences in the size of nucleus and the ratio of multinucleate cells to mononucleate cells between 30 $\mu\text{g/ml}$ EtBr-treated and nontreated Ax-2 cells.

The mitochondrial membrane potential has been shown to be transiently reduced in ρ^0 cells derived from several cell lines, as monitored by the staining of cells with MitoTracker Orange. To find out whether mitochondria in Ax-2 cells treated with 30 $\mu\text{g/ml}$ EtBr for 40 hours had any membrane potential, they were vitally stained with MitoTracker. The result showed that some of mitochondria contained in the EtBr-treated cells exhibited rather stronger staining (Fig. 2D) compared with that in nontreated Ax-2 cells (Fig. 2B), suggesting that in a limited number of mitochondria their membrane potential is increased by EtBr-treatment in *Dictyostelium* cells.

To confirm that the amount of mtDNA is selectively decreased in response to treatment of Ax-2 cells with 30 $\mu\text{g/ml}$ of EtBr, Southern blot analysis was carried out, using a nuclear DNA-specific probe (*Dd-trap1*; *Dictyostelium* homologue of *trap1*) and a mtDNA-specific probe (mitochondrial *rps4*). For this, total cellular DNAs were prepared from the same number of EtBr (40 hours)-treated cells and nontreated cells, digested with several restriction enzymes and compared after Southern blottings (Fig. 3). Here it is important to note that mtDNA is selectively reduced in the EtBr-treated cells, which is consistent with the result of DAPI staining shown in Fig. 2. The EtBr treatment seemed to have little effect on the amount of nuclear DNA. From densitometric measurements of the autoradiograms obtained, the amount of mtDNA in the EtBr-treated cells was estimated to be about 1/4 of that in nontreated Ax-2 cells. One can explain well the reduced value of mtDNA in the EtBr-treated cell, provided that the synthesis of mtDNA during two cell-doublings in the presence of 30 $\mu\text{g/ml}$ of EtBr is selectively and completely inhibited by EtBr. From another point of view, it is most likely that a certain amount of mtDNA (presumably more than 1/4 of original mtDNA) may be required to maintain cellular activities including growth. In other words, it might be impossible to create cells that have no mtDNA (ρ^0 cells) using *Dictyostelium* cells. Therefore, the

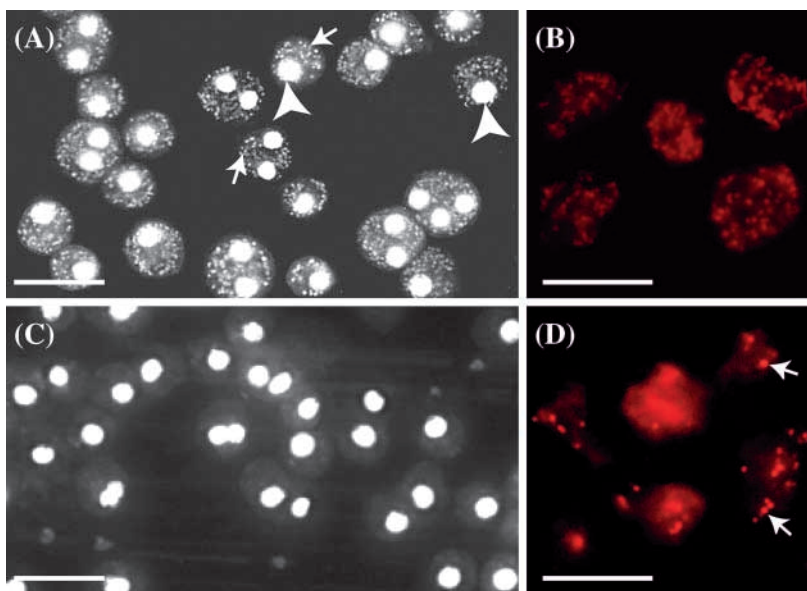


Fig. 2. Stainings of Ax-2 cells treated with 30 $\mu\text{g/ml}$ of EtBr for 40 hours and nontreated cells with DAPI (A,C) and MitoTracker Orange (B,D). In nontreated Ax-2 cells, DAPI stains are noticed in nuclei (A, arrowheads) and mitochondria as granular structures (A, arrows). In EtBr-treated cells, however, the DAPI-staining of mitochondria is almost vanished, although the staining of nuclei is retained (C). However, rather stronger staining by MitoTracker Orange is observed in a limited number of mitochondria (D, arrows) contained in EtBr-treated cells compared with nontreated cells (B) at the vegetative growth phase. Bars, 10 μm .

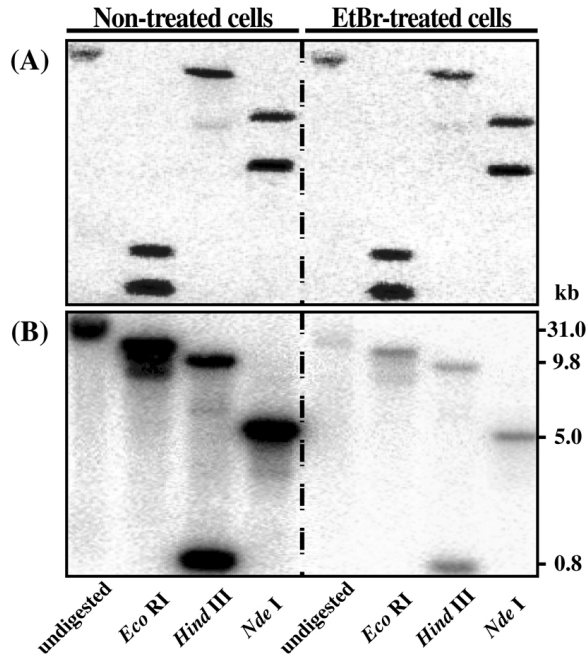


Fig. 3. Southern blot analysis of total DNAs extracted from Ax-2 cells and cells treated with 30 $\mu\text{g/ml}$ of EtBr for 40 hours. The DNAs were digested with the indicated restriction enzymes and electrophoresed. After transfer of the size-fractionated DNA fragments to nylon membranes, they were hybridized with the ^{32}P -labeled (A) nuclear DNA-specific probe *Dd-trap1* or (B) mtDNA-specific probe *rps4*, followed by autoradiography.

EtBr-treated cells with about 1/4 of mtDNA were used in further experiments, as ρ^{Δ} cells.

Ultrastructural features of ρ^{Δ} cells

Electron microscopic observations of ρ^{Δ} cells and vegetatively growing Ax-2 cells revealed some characteristic differences between them. As was expected, the most striking difference was the morphology of the mitochondria; in ρ^{Δ} cells many of the mitochondria exhibited marked structural transformation to form a sort of vacuole, engulfing the nearby cytoplasm (Fig. 4B,D). We previously reported that somewhat similar mitochondrial transformation occurred in differentiating prespore cells just before PSV formation (Matsuyama and Maeda, 1998). Nontreated Ax-2 cells contained normal-shaped mitochondria with reticular cristernae and an electron-opaque matrix, as shown in Fig. 4A,C. Incidentally, Kobilinsky and Beattie (Kobilinsky and Beattie, 1977) have reported the EtBr (10 $\mu\text{g/ml}$)-induced mitochondrial transformation in *D. discoideum* Ax-3 cells.

Decreased mtDNA causes delayed differentiation and abnormal morphogenesis

When Ax-2 cells were harvested at the exponential growth phase, washed and incubated on agar, they formed aggregation streams after 6 hours and mounds after 12 hours of incubation at 22°C (Fig. 5A). Subsequently, a tip was formed at the apex of each mound, which elongated and formed a migrating slug

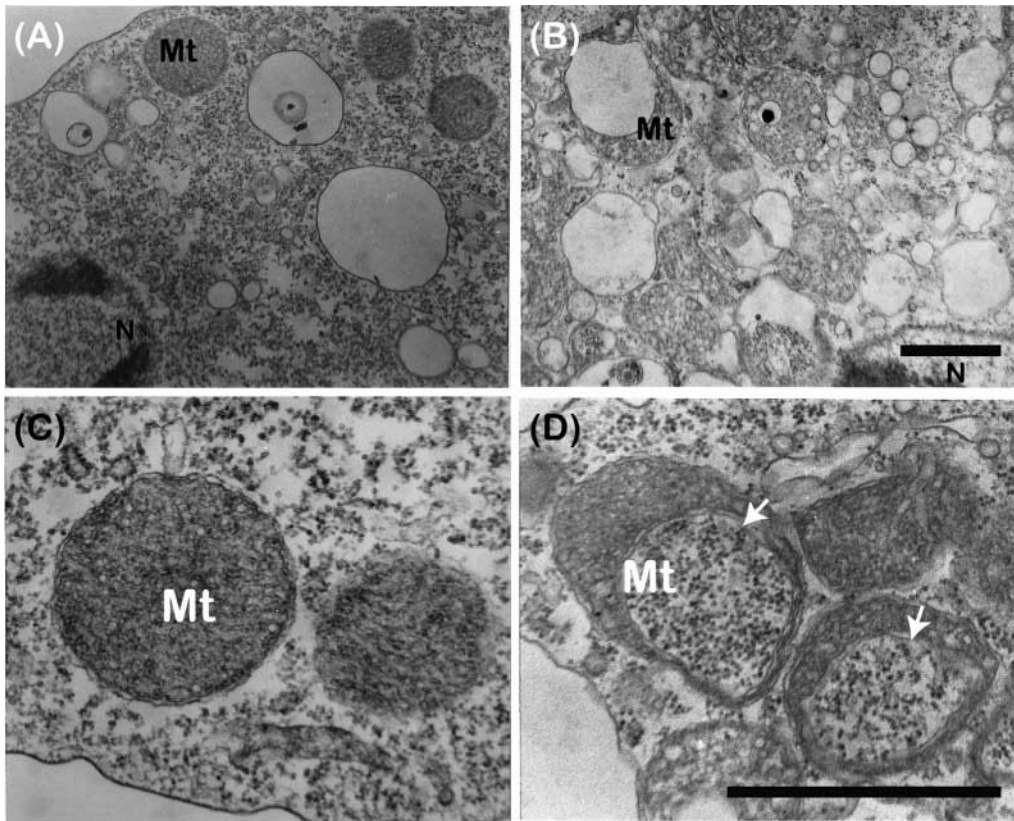


Fig. 4. Electron micrographs showing marked structural transformation of mitochondria in ρ^{Δ} cells. (A,C) Vegetatively growing Ax-2 cells have normal-shaped mitochondria, whereas (B,D) ρ^{Δ} cells have markedly transformed mitochondria having a sort of vacuoles (arrows), engulfing the nearby cytoplasm. Mt, mitochondria; N, nucleus. Bars, 1 μm .

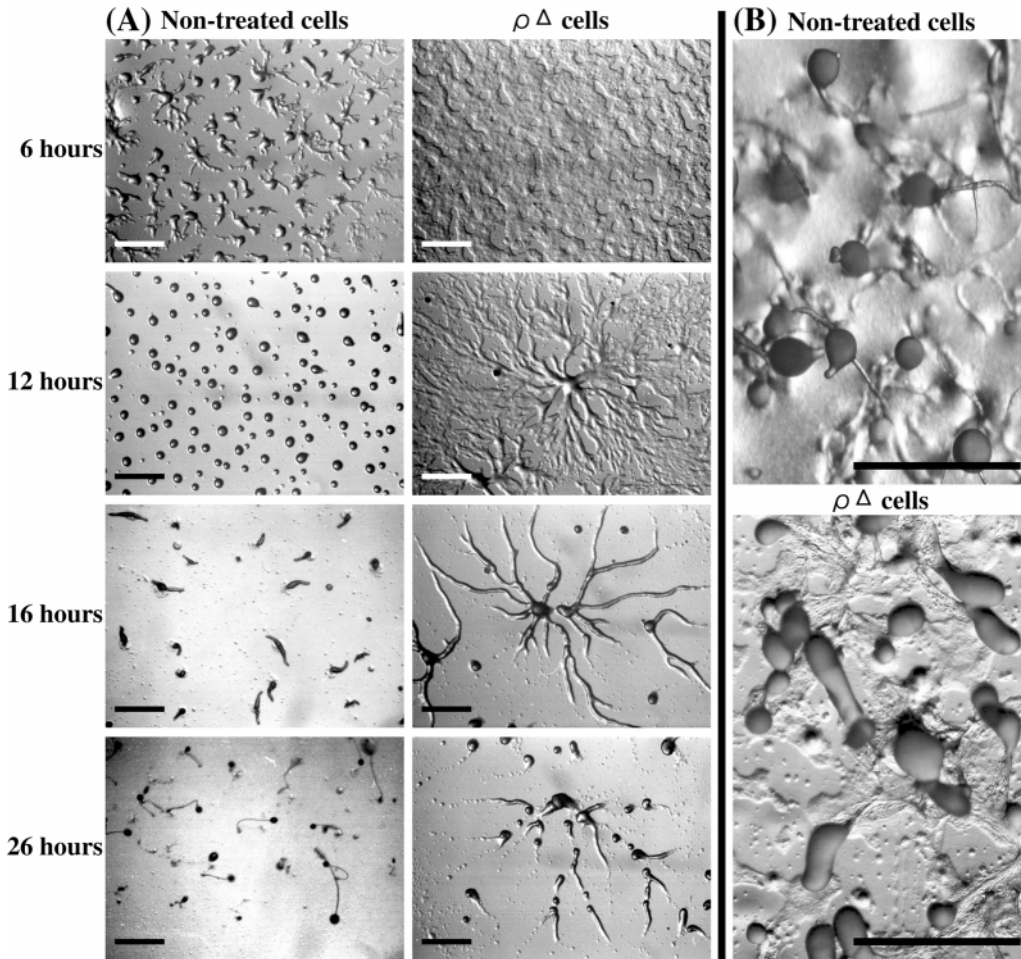


Fig. 5. Development of starved Ax-2 cells and ρ^{Δ} cells on agar. Ax-2 cells and ρ^{Δ} cells were washed twice in BSS and plated on 1.5% non-nutrient agar at a density of 5×10^6 cells/cm². This was followed by incubation for the indicated times at 22°C. (A) Nontreated Ax-2 cells formed aggregation streams after 6 hours and mounds after 12 hours of incubation. Subsequently a tip was formed at the apex of each mound, elongated and constructed a migrating slug after 16 hours of incubation. This was followed by formation of a fruiting body at about 26 hours of incubation. By contrast, ρ^{Δ} cells exhibited delayed and somewhat abnormal morphogenesis; large aggregation streams were formed after 16 hours of incubation, followed by their subdivision to smaller cell masses (A). (B) Gross morphology of final structures: fruiting bodies derived from nontreated cells and irregular-shaped slugs derived from ρ^{Δ} cells. The ρ^{Δ} cells stopped their development at the slug stage and never formed fruiting bodies. Bars, 1 mm.

after 16 hours of incubation. This was followed by the formation of a fruiting body at about 26 hours of incubation. By contrast, ρ^{Δ} cells exhibited delayed and somewhat abnormal morphogenesis; large aggregation streams were formed after 16 hours of incubation, followed by their subdivision to smaller cell masses (Fig. 5A). After a prolonged time (about 48 hours) of incubation, ρ^{Δ} cells formed irregular-shaped slugs, but failed to develop to fruiting bodies (Fig. 5B). Another experiment revealed that Ax-2 cells treated with lower concentrations (15, 20 or 25 μ g/ml) of EtBr for 40 hours at the growth phase had almost the same amount of mtDNA as that of nontreated Ax-2 cells, after DAPI staining, and that they exhibited normal development after starvation. Also, when Ax-2 cells were harvested and starved either on 1.5% non-nutrient agar or in BSS, both of which contained 30 μ g/ml of EtBr, they showed normal development without any loss of mtDNA.

More striking delay of differentiation was observed in ρ^{Δ} cells starving under submerged conditions. Most ρ^{Δ} cells showed no sign of cell aggregation and remained as round-shaped single cells even after 12 hours of incubation, while nontreated Ax-2 cells acquired aggregation-competence and began to aggregate at 7 hours of incubation, which was followed by the formation of tight mounds during 12–24 hours of incubation (Fig. 6). By contrast, ρ^{Δ} cells formed large aggregation streams at 24 hours, which were then subdivided

into smaller mounds during another 4 hours of incubation, principally as the case on agar (Fig. 6).

The genes *car1* (for cAMP receptor 1), *csA* (for contact site A) and *rps4* (for mitochondrial ribosomal protein subunit 4) have been shown to be specifically expressed after starvation, coupling with the initiation of cell differentiation. So, we examined expression patterns of these marker genes in EtBr-treated (ρ^{Δ}) cells and nontreated Ax-2 cells and compared them. As a result, expression of all of the genes was found to be significantly delayed and reduced in ρ^{Δ} cells, being consistent with the delay in their development after starvation (Fig. 7).

ρ^{Δ} cells exhibit almost normal respiration and chemotaxis to folic acid

In spite of greatly reduced mtDNA in ρ^{Δ} cells, measurements of O₂ consumption using the O₂ electrode revealed the respiration rate of ρ^{Δ} cells at the growth phase to be almost normal (about 90% of that of nontreated Ax-2) (data not shown). More importantly, ρ^{Δ} cells were found to exhibit completely normal chemotactic movement towards folic acid, a chemoattractant functioning at the growth phase (Pan et al., 1972). Therefore, the delay in developmental progression as observed in ρ^{Δ} cells seemed to be mainly due to the reduced

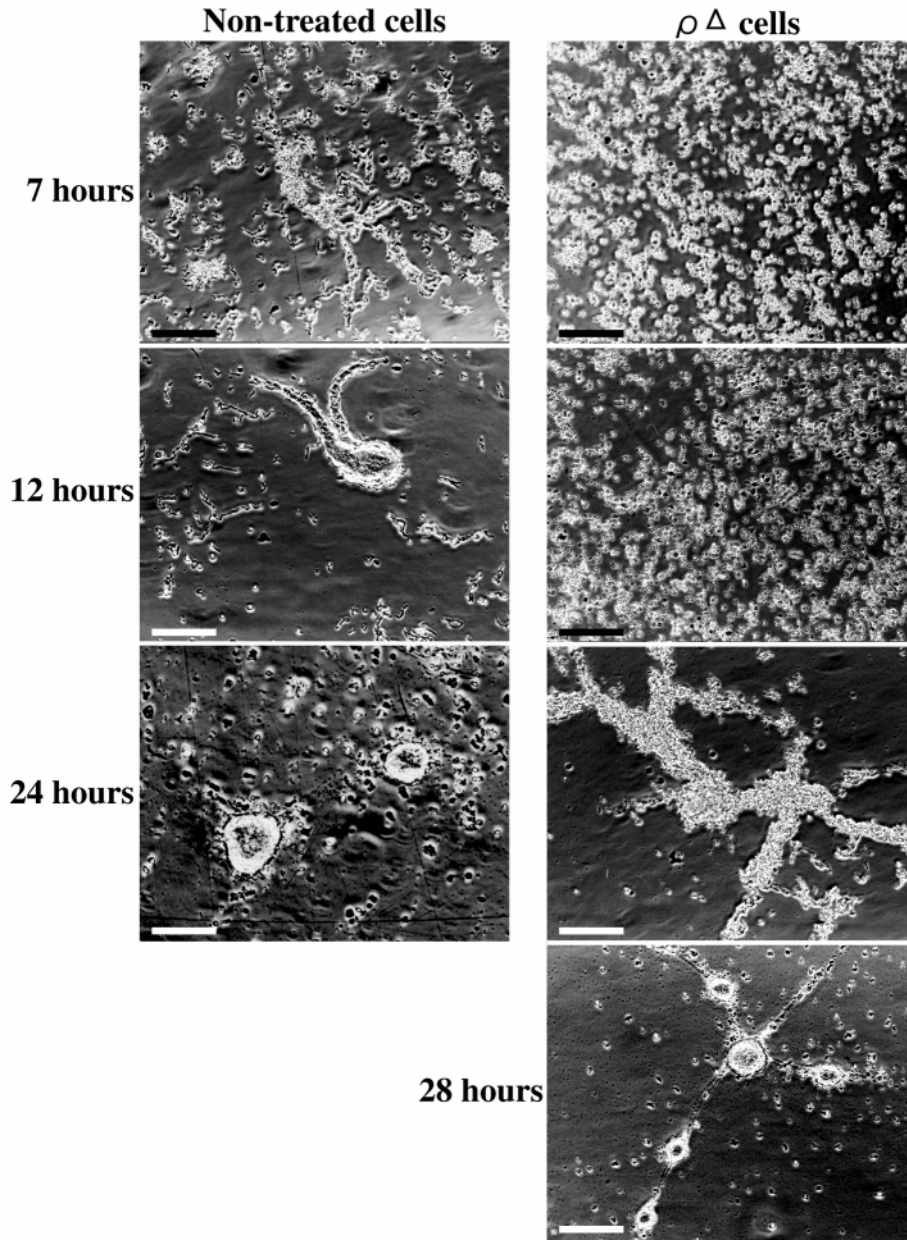


Fig. 6. Development of starved Ax-2 cells and ρ^{Δ} cells under submerged conditions. Ax-2 cells and ρ^{Δ} cells were washed twice in BSS and plated in a 24-well plate (1 ml cell suspension/well) at 5×10^5 cells/cm². This was followed by incubation for the indicated times at 22°C. Almost all the ρ^{Δ} cells showed no sign of cell aggregation and remained as round-shaped single cells even after 12 hours of incubation, while nontreated Ax-2 cells acquired aggregation-competence and began to aggregate at 7 hours of incubation, followed by formation of tight mounds during 12–24 hours of incubation. By contrast, ρ^{Δ} cells formed large aggregation streams at 24 hours, which were then subdivided into smaller mounds during another 4 hours of incubation. Bars, 200 μ m.

mtDNA, but not to general damage as realized by reduced intracellular ATP levels.

Altered patterning and cell-type proportioning in slugs formed from ρ^{Δ} cells

To examine the effect of reduced mtDNA on cell patterning in slugs, two types of transformants (*ecmAO-gal* and *D19-gal* cells) with the vectors bearing a bacterial β -galactosidase under the promoter of the prestalk-specific gene *ecmAO* and under the promoter of the prespore-specific gene *D19*, respectively, were used. The *ecmAO* gene was expressed in the anterior prestalk cells of migrating slugs and a small number of anterior-like cells (ALC) intermingled in the posterior region (Fig. 8A), as previously reported (Williams et al., 1989). By contrast, the *D19* gene was expressed in the posterior

prespore cells of migrating slugs (Fig. 8B) (Haberstroh et al., 1991). When vegetatively growing *ecmAO-gal* and *D19-gal* cells were separately treated with 30 μ g/ml EtBr for 40 hours and plated on Millipore filters 1.5% agar, they formed relatively normal-shaped slugs after 28 hours of incubation at 22°C (Fig. 8C,D), which was in slight contrast to the irregular-shaped slugs formed on 1.5% agar. A reduced amount of mtDNA in these transformants just after the EtBr-treatment was confirmed by DAPI-staining (Fig. 8C',D'). Moreover, the terminal development of slugs to fruiting bodies was also completely prohibited in these transformants even on Millipore filters, as the case for the slugs derived from ρ^{Δ} cells.

Here it is interesting to note that a considerable number of *ecmAO*-expressing cells (pstAO cells) are scattered in the posterior region (Fig. 8C), and that some *D19*-expressing cells (prespore cells) are also intermingled with the anterior prestalk

cells (Fig. 8D), thus giving an unclear prestalk/prespore pattern along the long axis of slugs.

The number-ratios of pstAO cells and prespore cells to the total number of cells in slugs were determined by histochemical detection of β -galactosidase activity (Table 1). Interestingly, the results of Table 1 show that the percentage (50.9%) of pstAO cells in slugs (referred to as ρ^{Δ} slugs) formed from ρ^{Δ} cells is about double of that (24.6%) in slugs (normal slugs) derived from nontreated transformants, whereas the percentage of prespore cells in ρ^{Δ} slugs is reduced from 75.3%

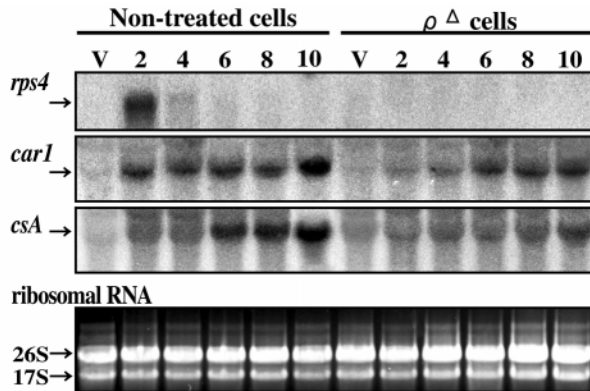
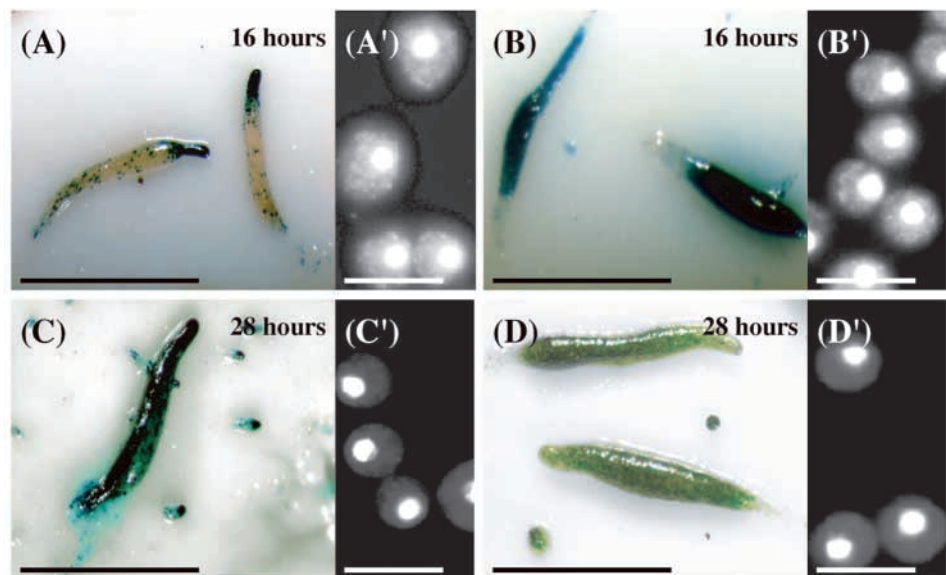


Fig. 7. Expression patterns of the *rps4*, *car1* and *csA* mRNAs during early development in Ax-2 cells and ρ^{Δ} cells. Cells were harvested at the growth phase (V), washed twice in BSS and shaken for the indicated times (hours) at 22°C. Total RNAs were prepared according to the method of Nellen et al. (Nellen et al., 1987). Northern hybridization was performed using the RI (Amersham), as previously described (Hirose et al., 2000). As probes for detection of the three kinds of mRNAs, PCR products obtained by amplification of the respective cDNA clones, using M13-20 and M13R primers, was used. The lower panel shows for each lane the amount of ribosomal 17S and 26S RNA stained with EtBr.

Fig. 8. Expression patterns of prestalk- and prespore-specific genes in slugs derived from transformed cells (*ecmA*O-gal cells and *D19*-gal cells) and their ρ^{Δ} cells. *ecmA*O-gal cells are shown in (A) and (C), while *D19*-gal cells are shown in (B) and (D). Nontreated transformed cells and their EtBr-treated cells (ρ^{Δ} cells) were separately washed twice in BSS and incubated for 16 or 28 hours on filters supported by 1.5% agar to obtain respective slugs. This was followed by histochemical staining of slug with X-gal to visualize the activity of β -galactosidase. In slugs derived from nontreated cells, the prestalk-specific *ecmA*O is expressed in the anterior prestalk region (A), whereas the prespore-specific *D19* is expressed in the posterior prespore region (B). In slugs derived from ρ^{Δ} cells, however, the boundary between the prestalk and prespore regions becomes unclear, presumably because of incomplete sorting between the two cell types (C,D). Images of respective DAPI-stained cells at the time-point of starvation are shown in (A'-D'). The DAPI staining of cytoplasmic granules (mitochondria) is observed in nontreated Ax-2 cells (A',B'), but not in ρ^{Δ} cells (C',D'). Bars, 0.4 mm (A-D); 10 μ m (A'-D').



to 47.8%. That is, almost all of cells in ρ^{Δ} slugs as well as in normal slugs are able to differentiate into either pstAO cells or prespore cells, although the ratio of pstAO cells/prespore cells is markedly increased, coupling with a reduced amount of mtDNA in ρ^{Δ} slugs.

Because the above results strongly suggested the induction of prestalk cells and the selective inhibition of prespore differentiation in ρ^{Δ} slugs, a more quantitative analysis, monitored by the prespore-specific vacuoles (PSVs), was carried out using FITC-conjugated anti-*D. mucoroides* spore IgG (Table 2). As expected, the ratio of prespore cells with PSVs were found to be decreased from a normal value of 71.9% to 49.5% in ρ^{Δ} slugs (Table 2), thus confirming the results of Table 1. Prespore cells usually had many strongly stained PSVs in the cytoplasm (Fig. 9A), as reported previously (Takeuchi, 1963). In ρ^{Δ} slugs, however, both the number of PSVs in each prespore cell and the strength of fluorescent FITC in each PSV were significantly lower compared with those in normally formed prespore cells (Fig. 9B). This was also confirmed using the electron microscope (Fig. 10).

Impaired phototaxis of slugs derived from ρ^{Δ} cells

Wilczynska et al. (Wilczynska et al., 1997) have reported that in many heteroplasmic mitochondrial gene disruptants of *Dictyostelium* the phototaxis of migrating slugs as well as vegetative growth of cells are greatly impaired, particularly in mitochondrial large rRNA (mt-lrRNA)-deficient mutants. This raised the possibility that slugs derived from ρ^{Δ} cells might also exhibit impaired phototaxis. This possibility was tested by examining the accuracy of phototactic orientation in ρ^{Δ} slugs, comparing it with that in normal slugs derived from the parental Ax-2 cells. As was expected the result revealed that the normal slugs migrated almost directly towards the light source, but that the ρ^{Δ} slugs were highly disoriented (Fig. 11),

Table 1. Altered cell-type proportioning in slugs derived from ρ^{Δ} cells

Slugs derived from	Percentages of β -galactosidase-positive cells (mean \pm s.d.)
Nontreated	
<i>EcmAO</i> -gal cells	24.6 \pm 1.2% (% of prestalk AO cells to the total cells)
<i>D19</i> -gal cells	75.3 \pm 4.4% (% of prespore cells to the total cells)
30 μ g/ml EtBr-treated	
<i>EcmAO</i> -gal cells	50.9 \pm 5.1% (% of prestalk AO cells to the total cells)
<i>D19</i> -gal cells	47.8 \pm 2.8% (% of prespore cells to the total cells)

Exponentially growing transformed cells (*EcmAO*-gal cells and *D19*-gal cells) were treated with 30 μ g/ml of EtBr for 40 hours to create ρ^{Δ} cells. As controls, the transformed cells were not treated with EtBr. Subsequently, they were washed twice in BSS for starvation, plated on 1.5% non-nutrient agar and incubated for about 48 hours at 22°C to obtain slugs. Resulting slugs were chemically dissociated and stained with X-gal for detection of β -galactosidase activity, as described in Materials and Methods. At least 1000 cells were counted under an optical microscope for each sample in three independent experiments.

Table 2. Reduced ratio of prespore cells in slugs derived from ρ^{Δ} cells

Slugs derived from	Percentage of PSV-containing prespore cells (mean \pm s.d.)
Non-treated Ax-2 cells	71.9 \pm 8.8%
ρ^{Δ} cells	49.5 \pm 3.0%

Slugs derived from either starved Ax-2 cells or ρ^{Δ} cells obtained by 40 hours of treatment with 30 μ g/ml of EtBr were chemically dissociated, fixed and stained with FITC-conjugated anti-*D. mucoroides* spore IgG, as noted in Materials and Methods. Cells with three or more PSVs in the cytoplasm were scored as positive cells (prespore cells). At least 1000 cells were counted under a fluorescence microscope for each sample in three independent experiments.

suggesting the prerequisite of mtDNA for phototaxis. Alternatively, it is also possible that the phototactic defect as observed in ρ^{Δ} slugs may be a consequence of the incomplete sorting of prestalk and prespore cells in the slug, because only the anterior-most prestalk region of a migrating slug is sensitive to light during normal phototaxis (Poff and Loomis, 1973).

Discussion

Mitochondria are believed to be fully integrated into the eukaryotic cell's signaling systems as well as its metabolism, as exemplified well in the process of apoptosis. The mitochondrial heteroplasmy with mutated mtDNA as subpopulations is involved in the aging process and is characteristic of mitochondrial diseases that primarily affect central nervous system, heart and muscle tissues (Shoffner and Wallace, 1990; Schapira, 1993; Lawen et al., 1994). In *Dictyostelium discoideum* Ax-2, the *rps4* gene encoding for mitochondrial ribosomal protein S4 (RPS4) has been shown to be specifically expressed during the transition (GDT) of Ax-2 cells from growth to differentiation; *rps4*^{HR} cells in which an about half of *rps4* gene is disrupted by means of homologous recombination exhibit greatly delayed differentiation after starvation, whereas *rps4*^{OE} cells overexpressing the *rps4* mRNA in the extra-mitochondrial cytoplasm perform

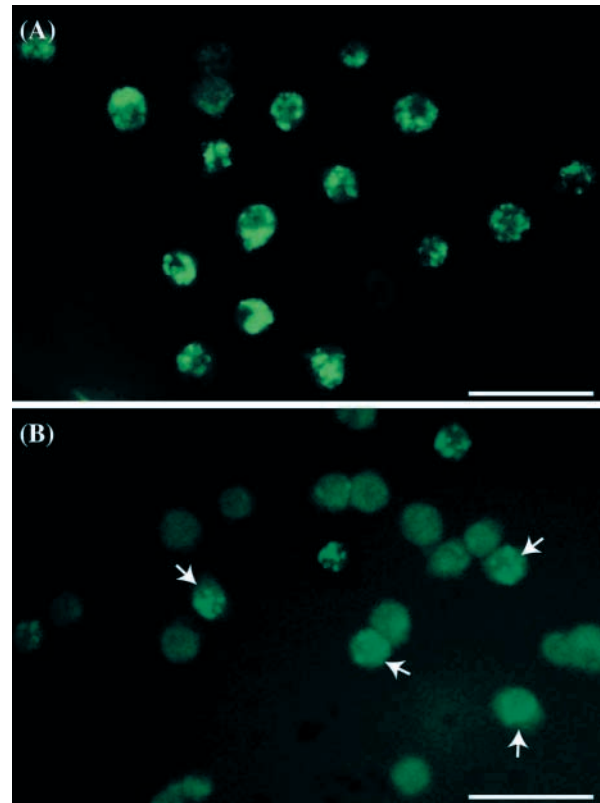


Fig. 9. Impaired prespore differentiation in slugs derived from ρ^{Δ} cells. Non-treated Ax-2 cells and ρ^{Δ} cells were separately washed twice in BSS and incubated for 16 or 28 hours on 1.5% agar to obtain respective slugs. This was followed by immunostaining of dissociated slug cells with FITC-conjugated anti-*D. mucoroides* spore IgG, as described in Materials and Methods. (A) Slug cells derived from nontreated Ax-2 cells. Prespore cells have many strongly stained granules (PSVs) in the cytoplasm. (B) In slug cells derived from ρ^{Δ} cells, however, it is clear that the number-ratio of prespore cells with PSVs is considerably decreased. In addition, both the number of PSVs in each prespore cell and the strength of fluorescent FITC in each PSV are lower (arrows) compared with those in slug cells derived from nontreated Ax-2 cells. Bars, 20 μ m.

enhanced differentiation through *car1* induction (Inazu et al., 1999). Disruption of the large subunit rRNA (mt-lrRNA) gene in a subpopulation of mitochondria impairs photosensory and themosensory signal transduction in the migrating slug stage of *D. discoideum* (Wilczynska et al., 1997). Also, the germ cell line is determined by the mt-lrRNA in *Drosophila* (Kobayashi and Okada, 1989; Iida and Kobayashi, 1998). Taken together, these data offer us indications as to the importance of mtDNA itself in a variety of cellular events during development.

The depletion or elimination of mtDNA with EtBr have been used to examine the genetic function of the mitochondrial genome and to create cells that are defective for ATP synthesis through mitochondrial respiration. Until now, ρ^0 cells devoid of mtDNA have been constructed from yeast (Goldring et al., 1970), algae (Heilporn and Limbosch, 1971), avian cell lines (Morais et al., 1988) and human cultured cells (King and Attardi, 1989) by means of EtBr-treatment.

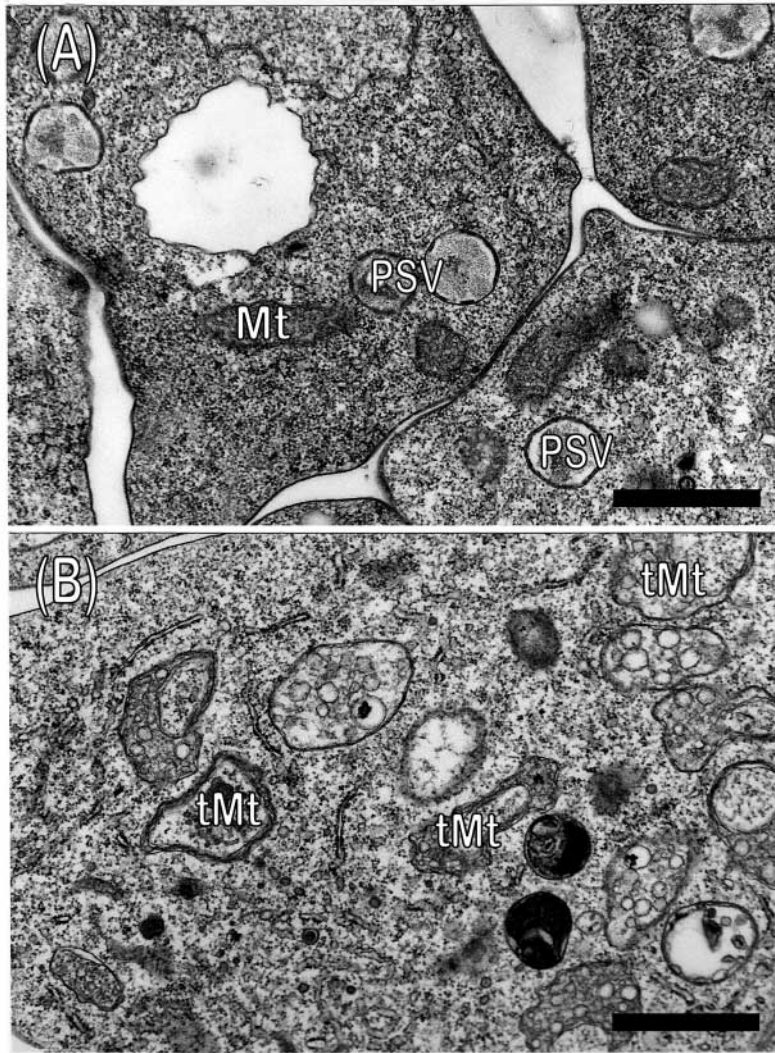


Fig. 10. Electron micrographs showing (A) mature PSVs in the prespore cells of a normal slug formed without EtBr-treatment, and (B) marked mitochondrial transformation and a scarce number of PSVs in the posterior region of a slug (ρ^{Δ} slug) formed from ρ^{Δ} cells. Markedly transformed mitochondria (tMt) as observed in ρ^{Δ} cells are still retained in ρ^{Δ} slugs (B). Mt, mitochondria; PSV, prespore-specific vacuole. Bars, 1 μm .

However, creation of ρ^0 cells is not exactly easy in many cell lines including human cultured cells, presumably because of deficiency in mitochondrial energy generation. Recently, it has been shown in *Caenorhabditis elegans* that mtDNA amplification is a necessary component of the normal developmental program, and that its blockage by EtBr results in an arrest at the L3 larval stage (Tsang and Lemire, 2002). In general, a high concentration of EtBr is known to induce cell apoptosis, reducing the mitochondrial membrane potential as monitored by MitoTracker staining. Unexpectedly, however, *Dictyostelium* cells never showed any characteristics of apoptosis even in the presence of relatively high concentrations of EtBr, and the mitochondrial membrane potential seemed to be rather augmented in a limited number of mitochondria in EtBr-induced ρ^{Δ} cells (Fig. 2). In this connection, similar observations have been reported in a cybrid cell line NARP3-2 by cytoplasmic transfer of mitochondria from fibroblasts of a patient with Leigh's disease into the ρ^0 206 osteosarcoma cell line (Tanaka et al., 2002). Two transfected cells were established; one had a higher percentage of mutant mtDNA (approximately 98%; termed NARP3-1) and the other had a lower percentage of mutant mtDNA (approximately 60%; termed NARP3-2). The

transfected NARP3-1 cybrids were similar to the ρ^0 206 cells; namely, they had a low mitochondrial membrane potential due to a deficiency of the wild-type mtDNA, but the mitochondrial membrane potential of NARP3-2 cybrids was increased. Therefore, it seems to be possible that in ρ^{Δ} cells with a reduced mtDNA copy number the mitochondrial membrane potential might be rather increased. In *Dictyostelium* cells, however, it has been claimed that there is a lot of variation between preparations in the intensity of fluorescent staining with dyes such as MitoTracker Orange (P. R. Fisher, personal communication).

As presented in this study, starved ρ^{Δ} cells exhibited greatly delayed differentiation and abnormal morphogenesis: they still remained as nonaggregated single cells even after a prolonged time of incubation, and failed to form fruiting bodies. Importantly, this developmental phenotype is similar to that of the transformant in which about a half of mitochondrial *rps4* gene was disrupted by means of homologous recombination (Inazu et al., 1999). The failure of ρ^{Δ} slugs to exert normal phototaxis is also consistent with the finding that disruption of the mt-lrRNA in a subpopulation of mitochondria impairs photosensory signal transduction at the slug stage (Wilczynska et al., 1997).

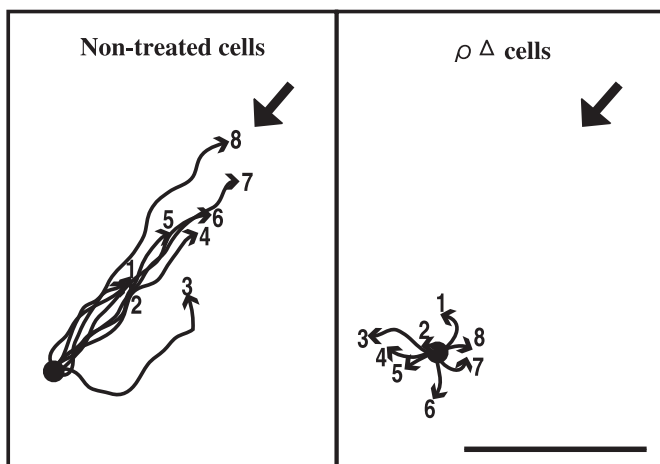


Fig. 11. A phototaxis-deficient phenotype of migrating slugs derived from ρ^{Δ} cells. The light source is shown by the large arrows. Whereas slugs derived from non-EtBr-treated Ax-2 cells migrated almost directly towards the light source, slugs (ρ^{Δ} slug) derived from ρ^{Δ} cells were highly disoriented. Phototactic behaviors of totally about 100 of respective slugs were traced, and the representative results of the slug no. 1-8 are shown in this figure. Bar, 1 cm.

In differentiating prespore cells, mitochondria exhibit a drastic structural transformation to form a sort of vacuole (M-vacuole), in which PSVs are constructed with the help of the Golgi complex (Maeda, 1971; Matsuyama and Maeda, 1998). Interestingly, during the formation of ρ^{Δ} cells mitochondria exhibit a marked morphological change and form unique vacuoles similar to the M-vacuole (see Fig. 4.) As shown in Table 2 and Fig. 8, however, formation of PSVs was rather suppressed in slugs (ρ^{Δ} slugs) formed from ρ^{Δ} cells. This seems to indicate that a reduced amount of mtDNA may be closely related to the induction of M-vacuole by mitochondria, but not to PSV maturation.

Interestingly, cell patterning and cell-type proportioning were found to be greatly altered in ρ^{Δ} slugs: ρ^{Δ} slug cells exhibited enhanced prestalk differentiation, whereas prespore differentiation was markedly suppressed. Somewhat similar phenomena are observed when starved Ax-2 cells were treated with nocodazole or calyculin A to prevent cell division at the early mound stage (Araki and Maeda, 1998). It has been shown that the cell division and subsequent nuclear DNA replication are resumed only in the cells just before prespore differentiation around the early mound stage (Zimmermann and Weijer, 1993; Araki and Maeda, 1998). Moreover, Shaulsky and Loomis (Shaulsky and Loomis, 1995) have claimed that mtDNA replication is resumed only in the cells destined to differentiate into prespore cells during late aggregation, and that it is a primary step in the developmental program leading to prespore differentiation. In this connection, our preliminary experiments using developing ρ^{Δ} cells have shown that cell division and mitochondrial DNA synthesis scarcely occur around the early mound stage in Ax-2 cells treated with 30 $\mu\text{g/ml}$ of EtBr for 40 hours at the vegetative growth phase. This seems to indicate that the cell-cycle progression including nuclear and/or mitochondrial DNA replication is essential for prespore differentiation, whereas a reduced amount of mtDNA, as realized in ρ^{Δ} slugs, is favorable to prestalk differentiation. In ρ^{Δ} slugs derived from *ecmA0*-gal and *D19*-gal transformants the boundary between the anterior prestalk and posterior prespore regions became unclear. This might be mainly because of imperfect prespore differentiation, resulting in incomplete sorting between the two cell types. Mitochondrial cyanide (CN)-resistant respiration has been shown to be tightly involved in cell-type proportioning (e.g. the ratio of prestalk/prespore cells in a slug or stalk cells/spores in a fruiting body): application of specific inhibitors of CN-resistant respiration to starved *Dictyostelium* cells induces the formation of unique cell masses, in which almost all of the cells differentiate into prestalk cells and then stalk cells, thus being completely devoid of prespore and spore differentiation (Matsuyama and Maeda, 1995). Thus, it is important to note that the inhibition of CN-resistant respiration and the enforced reduction of mtDNA have a somewhat similar effect on cell-type proportioning in that both of them preferentially prevent prespore differentiation.

In *Dictyostelium* cells, it is most likely that at least 1/4 of the mtDNA might be required for maintenance of cell growth, because Ax-2 cells (ρ^{Δ} cells) treated with 30 $\mu\text{g/ml}$ EtBr stopped dividing after two doublings in growth medium. If that is the case, it is impossible to create complete mtDNA-less cells (ρ^0 cells). In spite of such a situation, however, further

efforts will be needed to create ρ^0 cells that completely lack mtDNA using a suitable method. For this, we are now attempting to prepare ρ^0 cells by transforming Ax-2 cells with the *EcoRI* or *SmaI* gene fused to a mitochondrial targeting signal sequence, whose expression is under control of the tetracycline on/off system. More importantly, the establishment of an appropriate experimental system by which one can disrupt or overexpress specifically a certain mitochondrial gene will be promising to elucidate precisely the function of each mitochondrial gene.

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