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Unique Behavior and Function of the Mitochondrial Ribosomal Protein S4 (RPS4) in Early *Dictyostelium* Development

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ABSTRACT—Certain proteins encoded by mitochondrial DNA (mt-DNA), including mt-ribosomal protein S4 (*rps4*), appear to play important roles in the initiation of cell differentiation. Partial disruption of *rps4* in *Dictyostelium discoideum* Ax-2 cells by means of homologous recombination greatly impairs the progression of differentiation, while the the *rps4*^{OE} cells in which the *rps4* mRNA was overexpressed in the extra-mitochondrial cytoplasm exhibit enhanced differentiation (Inazu *et al*., 1999). We have prepared a specific anti-RPS4 antibody and generated transformants (*rps4*^{AS} cells) by antisense-mediated gene inactivation of *rps4*. Surprisingly, in the *rps4*^{AS} cells the progress of differentiation was found to be markedly inhibited, suggesting that the antisense *rps4* RNA synthesized in the extra-mitochondrial cytoplasm might be as effective as the partial disruption of *rps4* gene. Immunostaining of the *rps4*^{OE} cells with the anti-RPS4 antibody demonstrated that the RPS4 protein synthesized in the extra-mitochondrial cytoplasm is capable of moving to the nucleus, as predicted by PSORTII. Taken together with the results obtained using immunostained Ax-2 cells, we propose a possible pathway of RPS4 translocation coupled with differentiation.

Key words: mitochondria, RPS4, antisense RNA, differentiation, *Dictyostelium*

INTRODUCTION

In general, growth and differentiation are mutually exclusive and precisely regulated during development. Thus the mechanisms involved in the transition of cells from a proliferation to a differentiation state are of basic interest to developmental biologists and in the field of cancer research. *Dictyostelium discoideum* (strain Ax-2) cells grow and multiply by mitotic fission as long as nutrients are available. Upon exhaustion of nutrients, however, starving cells differentiate to acquire aggregation-competence and aggregate by means of chemotaxis to cAMP (Bonner *et al*., 1969) and EDTA-resistant cohesiveness (Gerisch, 1961). The cells in the aggregate then form into two major types in a migrating pseudoplasmodium (slug), anterior prestalk and posterior prespore cells. The slug eventually culminates to form a fruiting body consisting of a mass of spores and a supporting cellular stalk. The growth and differentiation phases are temporally separated from each other and easily controlled by nutritional conditions. A temperature-shift method for synchronizing cell-cycle phase of Ax-2 cells has been established (Maeda, 1986), and a particular checkpoint (referred to as the PS-point) from growth to differentiation phase has been specified in the mid-late G2 phase of the cell cycle (Maeda *et al*., 1989). This is the point from which cells initiate to differentiate 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).

We have identified several genes (*car1, caf1, quit3, dia1, dia2, dia3*) which are specifically or predominantly expressed in response to differentiation of starved Ax-2 cells from the PS-point and we have analyzed their functions (Abe and Maeda, 1994, 1995; Okafuji *et al*., 1997; Itoh *et al*., 1998; Chae and Maeda, 1998a, b; Chae *et al*., 1998; Inazu *et al*., 1999; Hirose *et al*., 2000). The cAMP receptor 1 (*car1*) gene is essential for differentiation (Sun *et al*., 1990; Sun and Devreotes, 1991), and CAR1-dependent events include receptor phosphorylation and influx of extracellular Ca^{2+} (Parent and Devreotes, 1996). The *car1* mRNA is specifically expressed in cells starved just before the PS-point, thus providing evidence of the involvement of this gene in the entry of cells into differentiation and also pointing to the specific existence of the PS-point in the cell cycle (Abe and Maeda, 1994). The *caf1* mRNA encodes a Ca^{2+}-binding protein with four EF-hand domains (Abe and Maeda, 1995), and its overexpression enhances differentiation in a Ca^{2+}-dependent manner (Itoh *et al*., 1998). Annexin VII, which is

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believed to be needed for Ca^{2+}-homeostasis in the cell, increases after starvation of Ax-2 cells (Bonfils *et al.*, 1994; Doring *et al.*, 1995). Since the *quit3* mRNA, which has no protein-coding region and contains the complementary sequence of annexin VII, is expressed more predominantly in the growth phase than in the differentiation phase, *quit3* may regulate annexin VII synthesis via a natural antisense transcript. This results in a striking increase of annexin VII production at the transition (GDT) of cells from growth to differentiation (Okafuji *et al.*, 1997). Taken together these data offer us indications of the importance of Ca^{2+} and its associated processes for the GDT of *Dictyostelium* cells. The *dia2* gene codes for a lysine- and leucine-rich novel protein (*Mr*,16.9 kDa), and its antisense-mediated gene inactivation greatly impairs the GDT (Chae *et al.*, 1998). In contrast, the overexpression of *dia1* (which codes for a novel 48.6 kDa protein) inhibits the progression of differentiation, while antisense RNA-mediated *dia1* inactivation enhances the initial step of cell differentiation (Hirose *et al.*, 2000). We have also demonstrated that the phosphorylation levels of 90 kDa and 101 kDa proteins are specifically reduced during early cellular differentiation from the PS-point (Akiyama and Maeda, 1992). The 90 kDa and 101 kDa phosphoproteins were identified as homologues of GRP94 (glucose-regulated protein 94; the endoplasmic reticulum HSP90) in *D. discoideum* (Dd-GRP94) (Morita *et al.*, 2000) and a *Dictyostelium* homologue of EF-2 (Watanabe *et al.*, 2003) respectively.

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 inativation by means of homologous recombination greatly impairs differentiation and morphogenesis after starvation (Inazu *et al.*, 1999). Interestingly, RPS4 has several nuclear localization signals after PSORTII and is predicted to be located in the nucleus with 95% or more probability, provided that this mitochondrial protein or *rps4* mRNA is capable of moving into the cytosol. The present work was undertaken to analyze the expression pattern of RPS4 protein and its intracellular localization during early *Dictyostelium* development, using a specific antibody raised against RPS4. It was also examined if the antisense *rps4* RNA expressed in the extra-mitochondrial cytoplasm is effective in the GDT.

MATERIALS AND METHODS

Cell cultures and developmental conditions

Vegetative cells of *Dictyostelium discoideum* Ax-2 were grown axenically in PS-medium (1% Special Peptone (Oxoid: Lot. No. 333 56412), 0.7% Yeast extract (Oxoid), 1.5% D-glucose, 0.11% KH_2PO_4, 0.05% $Na_2HPO_4 \cdot 12H_2O$, 40 ng/ml of vitamin B_{12}, 80 ng/ml of folic acid, pH 6.4). *rps4*-underexpressing transformants ($rps4^{AS}$ cells) produced by antisense gene inactivation and *rps4*-overexpressing transformants ($rps4^{OE}$ cells) isolated previously by Inazu *et al.* (1999) were grown axenically in shaking cultures in PS-medium containing 50 μg/ml of G418. $rps4^{HR}$ cells, in which the *rps4* gene was disrupted in about half the mitochondrial genomes by means of homologous recombination (Inazu *et al.*, 1999), were also used for comparison. To allow cells to differentiate, cells were harvested at the exponential growth phase, washed twice in BSS (Bonner's salt solution; 10 mM NaCl, 10 mM KCl, 2.3 mM $CaCl_2$; Bonner, 1947) as starvation medium, and either shaken at a density of 1.5×10^7cells/ml or incubated in a 24-well titer plate (Falcon, #3047) at a density of 5×10^5 cells/well at 22°C.

Transformation of cells

pDNeo2 (Witke *et al.*, 1987) was used the original vector for preparation of antisence-mediated gene inactivation. This vector was cut by digestion with *Bam*HI and *Sal*I and then ligated overnight with the *rps4* gene that had been amplified by PCR using pBluescript II KS(+) with the mitochondrial DNA of *D. discoideum* as the template and then digested with *Bam*HI and *Xho*I. The ligates were inserted into XL1-blue competent cells. To produce cells underexpressing the *rps4* mRNA, Ax-2 cells were transformed with the antisense construct by electroporation, as described by Nellen *et al.* (Nellen *et al.*, 1987). Transformed cells were selected in 10 ml of PS-medium containing 10 μg/ml G418 in Petri dishes (9 cm diameter). Two days after the appearance of colonies of transformed cells, the colonies were cultured by shaking in PS-medium containing 20–50 μg/ml G418 for 2–3 days and then cloned in 96-well titer plates (Iwaki, Chiba, Japan).

Preparation of the anti-RPS4 antibody and western blot analysis

Chemically synthesized oligopeptide (EEPKLTAIKYPFTLQPEK; from the 368th to 385th amino acid of RPS4) with an additional cysteine residue at the C-terminus was conjugated with KLH (keyhole limpet hemocyanin) as a carrier protein by Research Genetics, Inc. (Huntsville, Alabama, USA). The KLH-conjugated oligopeptide was injected 4×1 ml s.c. into the foot pads of rabbits with complete Freund's adjuvant. The total amount of the antigen was 5 mg per animal. 5 weeks later, a total amount of 1 mg KLH-conjugated oligopeptide per animal with adjuv. was supplied s.c.. Samples of blood (about 50 ml) were collected 10 days after the final injection, and aliquoted serum containing the polyclonal anti-RPS4 antibody was stored at –80°C. The IgG fraction of the serum was absorbed by homogenates prepared from vegetatively growing Ax-2 cells that were almost devoid of RPS4 protein, and the absorbed antibody (referred to as the anti-RPS4 antibody) was used for western analysis and immunostaining.

Cells were harvested from shaken cultures and lysed in SDS sample buffer (2% SDS, 10% glycerol, 41.7 mM dithiothreitol, 0.01% bromophenol blue, and 62.5 mM Tris-HCl (pH 6.8)). Proteins were size fractionated on 10% SDS gels and blotted onto polyvinylidene difluoride (PVDF) membranes (Millipore). After blotting, the membranes were gently shaken in TBS-T (20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.5% Triton X-100) containing 5% BSA or 5% skim milk, overnight at 4°C. Subsequently, the membranes were incubated in the primary antibody solution (1/2,500 anti-RPS4 antibody in TBS-T with 5% BSA or 5% skin milk and 0.15% Tween 20), overnight at 4°C. After washing in TBS-T for 20 min, the membranes were incubated in the secondary antibody solution (1/30,000 HRP-conjugated anti-rabbit IgG, goat (Amersham Pharmacia Biotechnology) in TBS-T with 5% BSA for 1 hr, according to the suggestions made by ICN Biochemicals. The chemical enhanced chemiluminescence (ECL kit; Amersham Biosciences) was used for detection of the RPS4 protein.

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

Ax-2 cells were starved for 4 hr and then incubated in BSS containing 0.5 μM MitoTracker Orange CMTMRos (Molecular Probes) for 15 min. The stained cells were washed twice with

BSS, and observed under a scanning confocal fluorescence microscope.

Double staining of cells with the anti-RPS4 antibody and DAPI

After 0–4 hr of starvation, Ax-2 cells and *rps4* -overexpressing cells (*rps4*^{OE} cells) that had been allowed to adhere to coverslips were fixed with 100% methanol for 10 min. To prevent cell shrinkage during fixation, samples were pre-fixed with 50% and then 100% methanol (1 min for each). The fixed samples were incubated for 16 hr at 22°C in the primary antibody (50-times diluted anti-RPS4 antibody) solution. Following threefold washing (10 min for

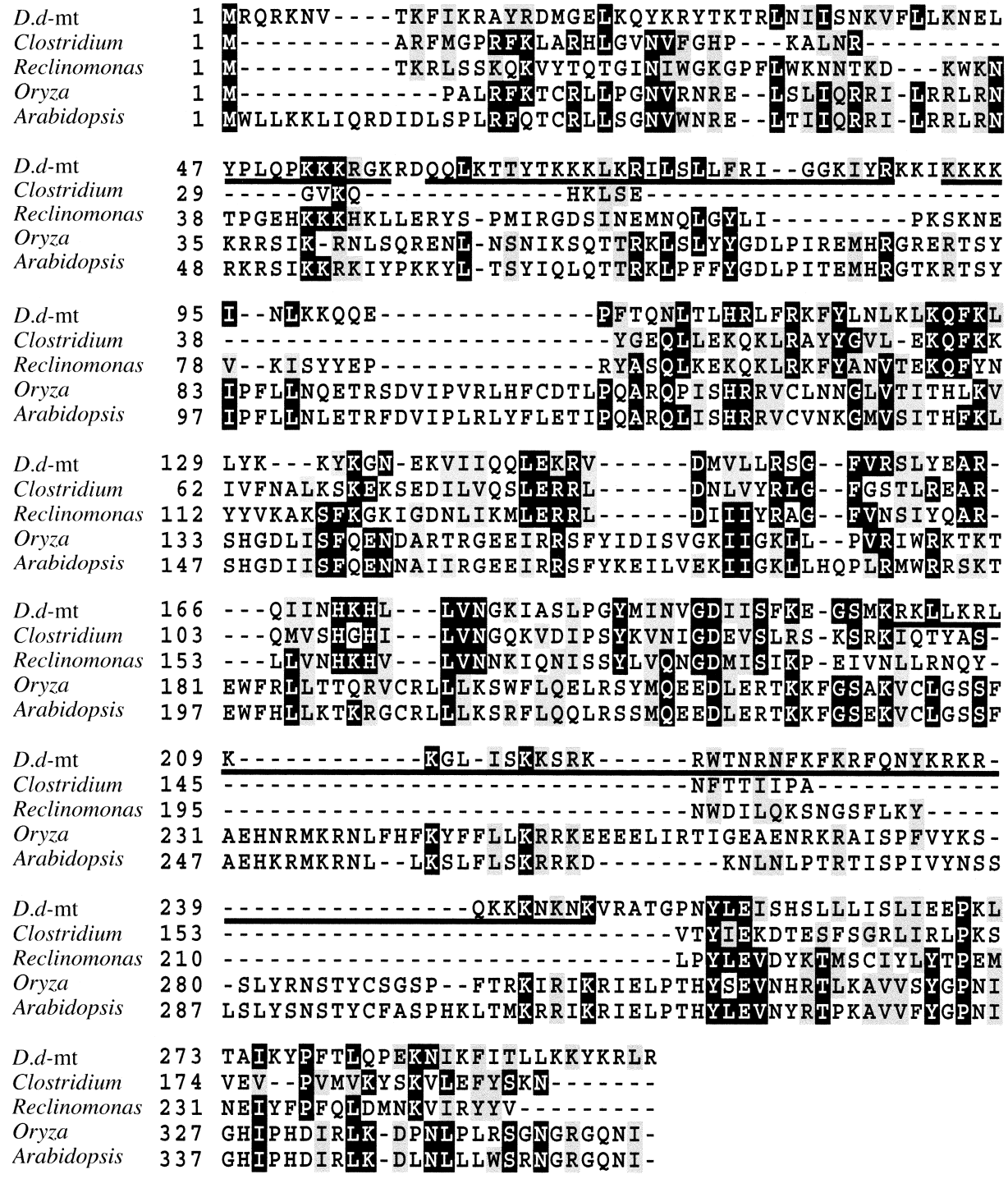

Fig. 1. Amino acid alignment of *Dictyostelium discoideum* mitochondrial RPS4 (*D.d*-mt), *Clostridium* RPS4 (*Clostridium*), *Reclinomonas* RPS4 (*Reclinomona*s), *Oryza* mitochondrial RPS4 (*Oryza*) and *Arabidopsis* mitochondrial RPS4 (*Arabidopsis*). Identical amino acids are blocked in black and similar amino acids are indicated with gray shading. In *D.d*-mt, part 4-type and bipartite-type nuclear localization signals predicted by PSORTII are underlined. The nuclear localization signals are specifically present in *D.d*-mt; such signals have not been reported in mt-RPS4 protein of other organisms, yet.

each) in phosphate-buffered saline (PBS; 0.14 M NaCl, 3 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.2), the samples were incubated with the secondary antibody solution (FITC-conjugated anti-rabbit IgG antibody, goat; 2,000 times-diluted with 1% BSA) for additional 3 hr at 22°C. After repeated washings with PBS, the samples were mounted in PBS containing 20% glycerol. They were visualized by a fluorescence microscope. In the case of double staining with the anti-RPS4 antibody and DAPI (4',6'-diamidino-2-phenylindole-dihydrochroride), cells adhering to coverslips were fixed with 2.0% formaldehyde solution containing 0.1% glutaraldehyde for 20 min, followed by treatment with 0.1% Triton X-100 solution to make cells permeable. The fixed samples were immunostained with the anti-RPS4 antibody as described above, and then stained with 0.5 μg/ml of DAPI for 1–4 hr at room temperature.

Subcellular fractionation

Vegetatively growing Ax-2 cells (t_0-cells) and t_4-cells starved for 4 hr were fractionated by a slight modification of the method of Ikeda and Takeuchi (1971). Vegetative and starving Ax-2 cells were separately harvested from shaken cultures, washed twice with BSS, and suspended in 5 mM Tris-HCl (pH 7.5) containing 0.25 M sucrose. The cells were homogenized and centrifuged at 400×g for 10 min to remove undisrupted cells. The supernatant was centrifuged twice at 900×g for 10 min to obtain the membrane fraction that mainly contained the cell membrane and nuclei as the pellet. The resulting supernatant was then centrifuged twice at 2,000×g for 20 min to the pelleted particulate fraction including mitochondria. The supernatant was centrifuged at 18,000×g for 60 min, and the resulting supernatant was collected as the cytosolic faction. The pellet fractions were washed with 5 mM Tris-HCl (pH, 7.5) containing 0.25 M sucrose, and then were dissolved in the SDS sample buffer for western blot analysis. The cytosol fraction was two-times diluted with 2×SDS sample buffer, followed by western blotting.

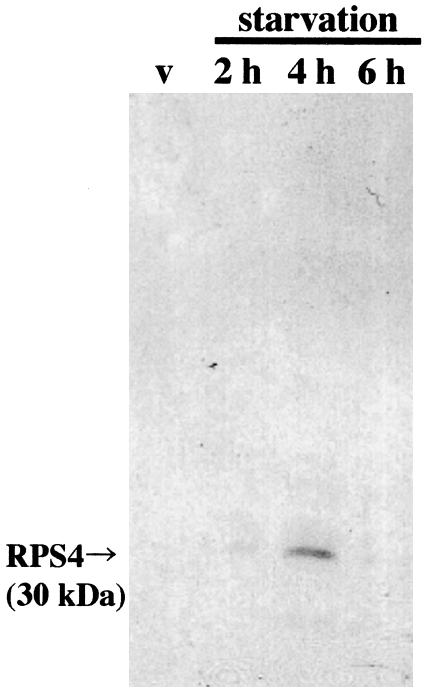

Fig. 2. Western blot analysis of the RPS4 protein in Ax-2 cells, using the absorbed anti-RPS4 antibody. Cells were harvested at the exponential growth phase (v), washed twice in BSS and shaken for the indicated times (hr) at 22°C. Western blottings were performed as described in Materials and Methods. It is clear that the antibody detects monospecifically the 30 kDa RPS4 protein, particularly in t_4-cells starved for 4 hr.

RESULTS

Uniqueness of *Dictyostelium* RPS4 protein

An alignment of the deduced amino acid sequence of *Dictyostelium discoideum* mitochondrial RPS4 (Dd-mtRPS4) with sequences of RPS4 homologues from other species show a moderate degree of homology (25–35%) (Fig. 1). Although the homology of Dd-mtRPS4 to human mt-RPS4 is not so high as a whole, the N-terminus of Dd-mtRPS4 which is supposed to be an RNA-binding region is well conserved. More significantly for this work, several putative nuclear localization signals are present in Dd-mtRPS4, but not in mt-RPS4 protein of other organisms (Fig. 1). Thus the Dd-mtRPS4 protein, if present in the cytosol, would be predicted to be transferred to the nucleus with 95% or more probability according to the PSORTII Search.

Expression pattern of RPS4 protein during early development

Western blottings using the anti-RPS4 antibody absorbed beforehand by homogenates of vegetative growth phase cells that were supposed to be almost devoid of RPS4 protein gave a single band at the predicted position of 30 kDa RPS4, as shown in Fig. 2. As was expected, the RPS4 protein was scarcely noticed in vegetatively growing Ax-2 cells, and began to increase transiently in response to starvation, reaching the maximum level after 4–6 hr (Fig. 3).

In *rps4*^{AS} cells expressing the antisense *rps4* RNA, the expression level of RPS4 protein was considerably reduced as a whole (Fig. 3A). In contrast, *rps4*^{OE} cells overexpressing the *rps4* mRNA in extra-mitochondrial space exhibited an augmented expression of RPS4 even during the vegetative growth phase (Fig. 3B). Like the *rps4*^{AS} cells, *rps4*^{HR} cells in which about a half of the mitochondrial copies of the *rps4* gene were disrupted by homologous recombination showed a reduced level of RPS4 expression (Fig. 3C). The time course of RPS4 expression in Ax-2 cells was found to vary fairly from experiment to experiment, the expression peak being 6 hr, 4 hr, and 2 hr in Fig. 3A, B, and C, respectively. In this connection, it has been recently revealed that the expression of *rps4* mRNA is moderately augmented depending on increased cell density during the vegetative growth phase: the *rps4* mRNA is scarcely expressed at low cell densities less than 1×10^6 cells/ml in growth medium, whereas the expression becomes detectable at $2–3\times10^6$ cells/ml and increases gradually coupled with increased cell densities in growth medium. This seems to indicate that the *rps4* mRNA expression is under control of prestarvation factor(s) (PSFs) which accumulates as a function of cell density in growth medium, as the case for certain proteins that were previously believed to be induced by starvation. Thus Ax-2 cells acquire differentiation-competence during the vegetative growth phase in a PSF-dependent manner, and cells

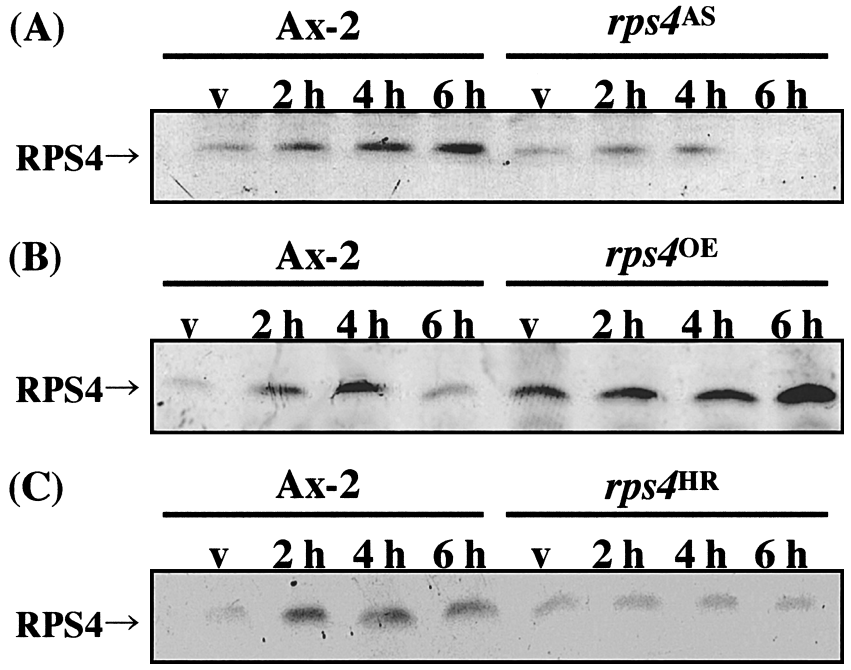

Fig. 3. Expression patterns of the RPS4 protein during the early development of Ax-2 cells and several transformed cells. Cells were harvested at the exponential growth phase (v), washed twice in BSS and shaken for the indicated times (hr) at 22°C, followed by western blot analysis using the anti-RPS4 antibody. The expression patterns in Ax-2 cells, (A) *rps4*^{AS} cells expressing the antisense *rps4* RNA, (B) *rps4*^{OE} cells overexpressing the *rps4* mRNA in the extra-mitochondrial cytoplasm and (C) *rps4*^{HR} cells in which the *rps4* gene was disrupted by homologous recombination in about half mitochondrial genomes in the cell. The relative amount of RPS4 protein at t_4- and t_6-cells of Ax-2 varies from experiment to experiment.

exposed to more PSF exhibit more rapid differentiation and morphogenesis when starved. Considering from these facts, it is most likely that the variation of RPS4-expression time course, as observed in Ax-2 cells, might be due to slight differences in cell density at the time-point of cell's starvation.

Relation of the RPS4 protein to the *car1* expression

Our previous findings suggested that in the regulatory cascade controlling early development, *rps4* expression at the onset of differentiation might be located upstream of the expression of cAMP receptor 1 (*car1*), because *car1* expression is markedly reduced in the *rps4*-inactivated cells like *rps4*^{HR} cells (Inazu *et al*., 1999). To investigate this further, the expression patterns of *rps4* in *car1*-null cells (JB4 cells) and parental Ax-3 cells were compared by western blot analysis. The result has demonstrated that the RPS4 protein exists in the *car1*-null cells, though its amount seems to be slightly reduced as compared to that in Ax-3 cells, particularly at the early stage of starvation (data not shown). Therefore, *car1* expression might have some effect on the synthesis of RPS4, but the cAMP receptor1 (CAR1) is not necessarily required for RPS4 formation.

Antisense *rps4* RNA expressed in the extra-mitochondrial cytoplasm greatly impairs the progression of cell differentiation

When cells of the transformed *rps*^{AS} strain and its parent Ax-2 cells were separately starved and incubated in BSS under submerged conditions at 5×10^5 cells/cm^2, most of *rps*^{AS} cells showed no sign of cell aggregation and remained as round-shaped single cells at 8 hr of incubation (Fig. 4A), while Ax-2 cells were elongated in shape, acquiring aggregation-competence, and some of them formed tiny aggregates (Fig. 4B). After a more prolonged time of incubation (12–16 hr), Ax-2 cells formed aggregation streams and then tight mounds (Fig. 4C, E). In contrast, some of *rps*^{AS} cells form aggregation streams, but many cells were still rounded in shape and remained as nonaggregated single cells (Fig. 4D, F).

As expected, starving *rps*^{AS} cells also exhibited marked delay of development on agar. At 10 hr of incubation, while most *rps*^{AS} cells showed no sign of cell aggregation, Ax-2 cells had already aggregated to form tight mounds. During further incubation, Ax-2 cells formed a tip on each aggregate, migrated as a slug, and eventually constructed a sorocarp after 24 hr of incubation. In contrast, a small population of *rps*^{AS} cells participated in sorocarp formation, but a considerable number of cells still remained as nonaggregated single cells.

Since *rps*^{AS} cells grew normally by binary fission in growth medium, with almost the same doubling time as parental Ax-2 cells, it was concluded that the observed effect of *rps4*-inactivation by the antisense RNA was limited to the process of cell differentiation. Here it is of interest to note that the developmental phenotype of *rps4*^{AS} cells is quite similar to that of *rps4*^{HR} cells in which about a half of

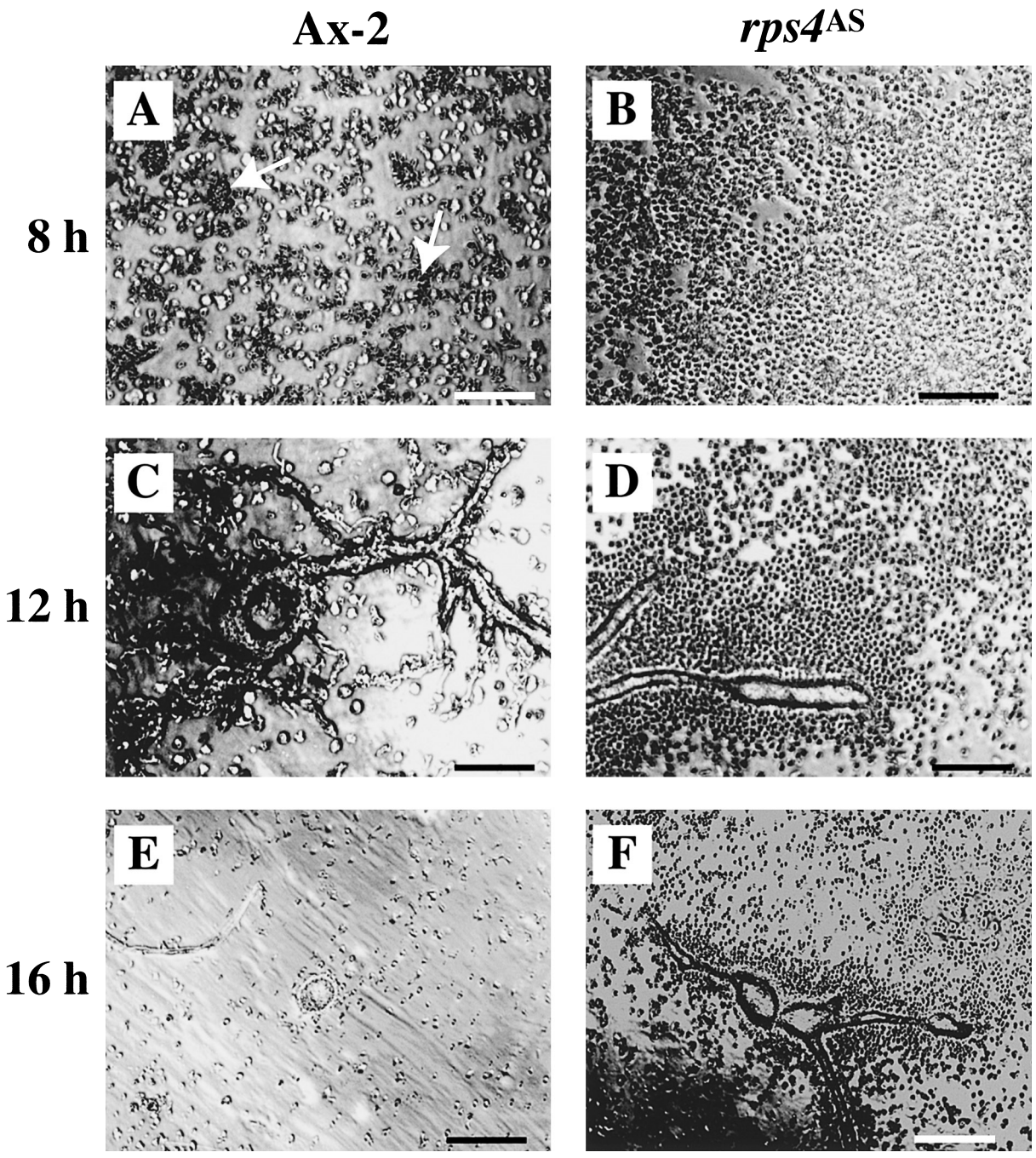

Fig. 4. Development of *rps4*^{AS} cells (B, D, F) and parental Ax-2 cells (A, C, E) under submerged conditions. *rps4*^{AS} cells and parental Ax-2 cells were harvested during the exponential growth phase, washed twice in BSS and plated in a 24-well titer plate at a density of 5×10^5 cells/ml (1 ml of cell suspension/well). This was followed by incubation at 22°C. At 8 hr of incubation, (A) Ax-2 cells acquire aggregation-competence and form small cell clumps (arrows), while (B) *rps4*^{AS} cells show no sign of cell aggregation and remain as round-shaped single cells. At 12–16 hr, (C, E) Ax-2 cells form aggregation streams and tight aggregates, but (D, F) many of *rps4*^{AS} cells still remain as round-shaped single cells, though a small number of cells participate in formation of aggregation streams. Bar, 200 μm.

the mitochondrial *rps4* genes have been disrupted by homologous recombination. During a prolonged time of axenic culture in growth medium, the phenotype of transformants such as *rps4*^{HR} cells has been shown to change moderately even in the presence of G418, eventually resulting in a return into the parental Ax-2-like phenotype. In the phenotypic revertants, we previously demonstrated that the *dia3* RNA including the *rps4* mRNA was recovered at almost the same level as that in Ax-2 cells (Inazu *et al.*, 1999). In the *rps4*^{AS} cells, however, the developmental phenotype was retained stably during a prolonged time of successive axenic culture in growth medium.

When starved *rps4*^{AS} cells and Ax-2 cells (vitally stained with MitoTracker) were mixed in various number-

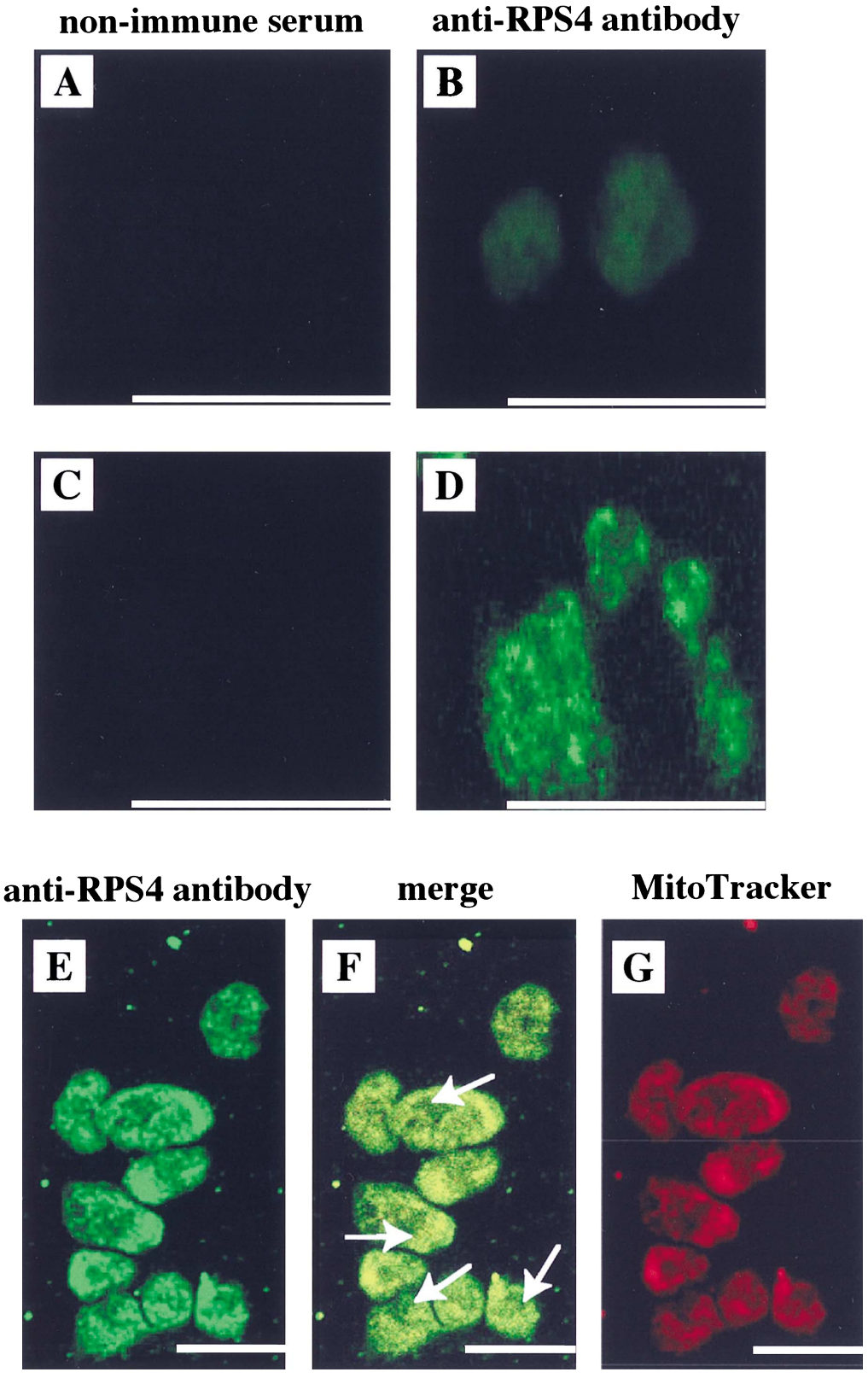

Fig. 5. Localization of RPS4 in Ax-2 cells just before starvation (B) and after 4 hr of starvation (D). Cells were harvested at the exponential growth phase, washed twice in BSS and fixed either immediately or after 4 hr of starvation at 22°C, followed by staining with the anti-RPS4 antibody or non-immune serum. At the vegetative growth phase (B), granular structures like mitochondria are weakly stained. In starving t_4-cells (D), the staining of mitochondria becomes highly marked. (E–G) Starving t_4-cells were double-stained with the anti-RPS4 antibody (E) and MitoTrtacker Orange (G), as described in Materials and Methods. Although both the stains are mostly colocalized, the extra-mitochondrial cytoplasm is very weekly stained only with the anti-RPS4 antibody (F, arrows). Bar; (A–D) 30 μm, (E–G) 15 μm.

ratios and incubated in BSS under submerged conditions at a density of 5×10^5 cells/cm^2, no synergism was observed between the two. In a mixed culture of *rps4*^{AS} and Ax-2 (10:1), they completely sorted out after aggregation: a few number of small aggregates, consisting of Ax-2 cells were formed in a non-aggregated sheet composed of *rps4*^{AS} cells. The inability of Ax-2 cells to compensate for the development of *rps4*^{AS} cells indicates that the phenotype of *rps4*^{AS} cells is cell-autonomous.

Is RPS4 protein synthesized in the cytoplasm capable of moving actively to the nucleus?

Immunostaining of Ax-2 cells by the anti-PRS4 antibody has revealed that RPS4 is only slightly detected in granular structures (presumably mitochondria) of vegetatively growing cells (Fig. 5B). In cells (t_4-cells) starved for 4 hr, their cytoplasmic granules were strongly stained, as shown in Fig. 5D. The granules were confirmed to be mitochondria by double-stainings of the cells with DAPI or MitoTracker Orange. Here it is of interest to note that the extra-mitochondrial region of t_4-cells is only slightly stained, and that only a weak nuclear staining is sometimes recognized in some of t_4-cells. In this connection, the presence of a small amount of RPS4 in the cytosolic fraction of t_4-cells was detected by subcellular fractionation of cell homogenates and subsequent immuno-blottings using the anti-RPS4 antibody. Although these observations seemed to suggest that a trace of RPS4 protein might be released from mitochondria to the cytosol and then to the nucleus, this possibility remains to be tested using a more sensitive detection method, because the staining, if present in the cytosol and nucleus, is quite weak.

When *rps4*^{OE} cells overexpressing the RPS4 protein were immunostained with the anti-RPS4 antibody and DAPI, they were strongly stained all over at the vegetative growth phase (Fig. 6A). In the t_4-cells, however, their nuclei in addi-

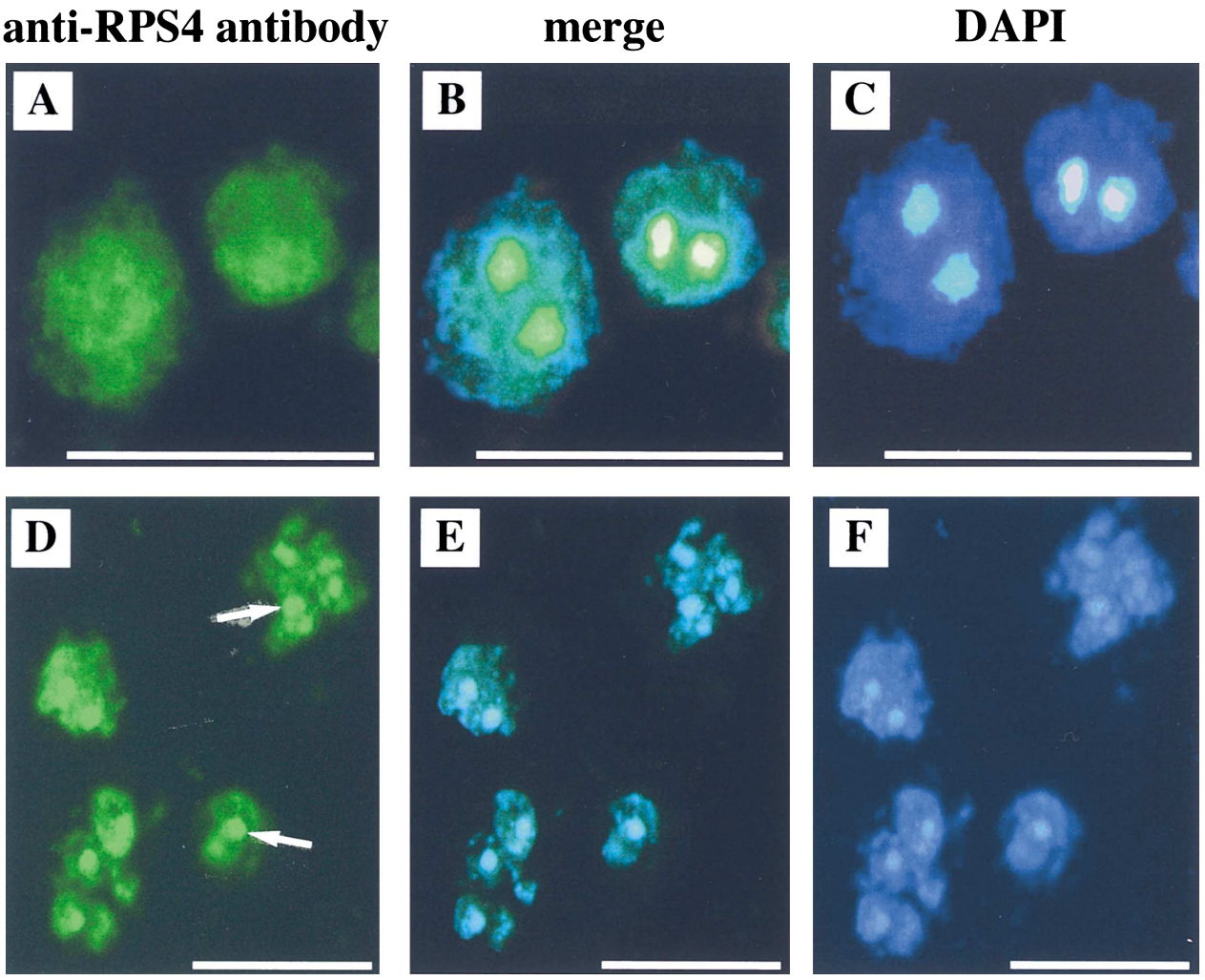

Fig. 6. Localization of RPS4 in *rps4*-overexpressing (*rps4*^{OE} cells) just before starvation (A) and after 4 hr of starvation (D). Cells were harvested at the exponential growth phase, washed twice in BSS and fixed either immediately or after 4 hr of starvation at 22°C, followed by double-staining with the anti-RPS4 antibody and DAPI. At the vegetative growth phase (A), the cells were strongly stained all over (A). In the t_4-cells, however, it is clear that their nuclei are strongly stained (D, arrows). Bar; (A–C) 20 μm, (D-F) 30 μm.

tion to the cytoplasm were strongly stained (Fig. 6D), thus at least indicating that the RPS4 protein synthesized in the cytoplasm may be preferentially transferred into the nucleus.

DISCUSSION

We previously reported that the *rps4* gene encoding mitochondrial ribosomal protein S4 (RPS4) was specifically expressed during the transition of *D. discoideum* Ax-2 cells from growth to differentiation; *rps4*^{HR} cells in which the *rps4* gene is disrupted in about a half of the cell's mitochondrial genomes by homologous recombination exhibited greatly delayed differentiation after starvation, while *rps4*^{OE} cells overexpressing the *rps4* mRNA in the extra-mitochondrial cytoplasm perform enhanced differentiation through *car1* induction (Inazu *et al*., 1999). The present work has revealed that *rps4*^{AS} cells expressing the antisense *rps4* RNA in the extra-mitochondrial cytoplasm exert markedly impaired differentiation after starvation, as was the case for the *rps4*^{HR} cells . Again, these results indicate that both the partial disruption of *rps4* gene in mitochondria and the antisense-mediated partial inactivation of *rps4* in the extra-mitochondrial cytoplasm are likewise effective in suppressing the progression of cell differentiation, while the enforced expression of *rps4* mRNA in the extra-mitochondrial cytoplasm leads to enhanced differentiation. This seems to indicate that a trace of the *rps4* mRNA and RPS4 protein, both of which were synthesized in mitochondria, might be released to the extra-mitochondrial cytoplasm. Alternatively, it is also possible that the antisense *rps4* RNA might enter mitochondria to inactivate the *rps4* mRNA and inhibit RPS4 formation. After PSORTII Prediction, the RPS4 protein released into the cytosol is predicted to move preferentially to the nucleus. This was confirmed by the present immunohistochemical observation that in the *rps4*^{OE} cells the RPS4 protein in the cytoplasm is capable of moving actively to the nucleus (see Fig. 6D). Although the fact that only the RPS4 protein of *Dictyostelium* cells has several nuclear localization signals is quite mysterious, at least a part of the RPS4 protein seems to work in the nucleus to regulate cell differentiation. In general, it is difficult for proteins located in the mitochondrial matrix to go out to the cytosol, because mitochondria are partitioned by two (outer and inner) membranes. Recently, however, several mitochondrial proteins like AIF (apoptosis-inducing factor; Daugas *et al*., 2000a, b), endonuclease G (Ohsato *et al*., 2002) and HSP70 (heat shock protein 70; Susin *et al*., 1999) have been shown to move to the nucleus in response to apoptosis or heat shock. All of these proteins are coded by the nuclear genome DNA, followed by translocation to the mitochondrion and then again to the nucleus. In this connection, the behavior of the *Dictyostelium* RPS4 is unique in that it is coded by the mitochondrial DNA.

The *car1* gene encoding the cAMP receptor 1 (CAR1) is specifically expressed in Ax-2 cells that were starved just before the growth/differentiation checkpoint (PS-point) and now in the initial phase of cell differentiation (Abe and Maeda, 1994). As revealed by several workers, CAR1 is essential for differentiation (Sun *et al*., 1990; Sun and Devreotes, 1991). We previously demonstrsted that *car1* expression was markedly reduced in the *rps4*^{HR} cells, while the *rps4*^{OE} cells exhibited the precocious and pronounced *car1* induction, thus suggesting that the *rps4* expression might be located in the upstream of the *car1* expression in the pathway controlling early differentiation (Inazu *et al*., 1999). This is also supported by the fact that the RPS4 protein is expressed albeit slightly reduced levels in the *car1*-null cells (JB4 cells) as well as in parental Ax-3 cells.

The phosphorylation level of a 101 kDa protein has been shown to be specifically reduced at the initiation of differentiation from the PS-point (Akiyama and Maeda, 1992). Recently, we have revealed that the 101 kDa protein is a *Dictyostelium* homologue of elongation factor 2 (EF-2) (Watanabe *et al*., 2003). In this connection, it has been demonstrated in animal cells that the activity of EF-2 in translation is regulated by its phosphorylation levels, and that the dephosphorylated state is generally the active form (Ryazanov *et al*., 1988). A 32 kDa phosphoprotein (recently identified by Nakao *et al*. as ribosomal protein S6; RPS6) is known to be completely dephosphorylated under conditions of nutritional deprivation: Blockage of the dephosphorylation by phosphatase inhibitors such as okadaic acid and calyculin A completely inhibits differentiation of starving Ax-2 cells from the PS-point so that they advance instead through M- and S-phases to a particular position of the cell cycle (Akiyama and Maeda, 1992). On the other hand, Tapparo *et al*. (1998) have reported that ribosomal protein S4 (RPS4; not mitochondrial RPS4) and S10 (RPS10), both of which are coded by nuclear genome DNA, are expressed at the vegetative growth phase, followed by decrease in response to starvation. Taken together these data offer us indications as to the importance of differential operation of the machinery used for mitochondrial protein synthesis during the transition of cells from growth to differentiation.

Although it may be surprising, there is increasing evidence that mitochondria have novel and crucial functions as the regulatory machinery of growth/differentiation transition, cell-type determination, cellular movement and pattern formation. As shown here, the expression of *rps4* is necessary for differentiation of *Dictyostelium* cells from the PS-point. Recently, a novel mitochondrial protein (Tortoise) was found to be required for directional responses of *Dictyostelium* cells in chemotactic gradients (van Es *et al*., 2001). In addition, a *Dictyostelium* homologue (Dd-TRAP1) of TRAP-1, a mitochodrial HSP90, was isolated from *D. discoideum*, and its marked translocation between the mitochondria and cell cortex during early development has been demonstrated (Morita *et al*., 2002). The origin of the mitochondrion is believed to be an aerobic bacteria which once established a symbiosis with a host cell such as an archaebacterion and has been handing over parts of its own genome to the nuclear DNA of the host cell during evolution. The result is

that mitochondria depend on the nuclear genome for their normal biogenesis and function. In turn, they provide respiratory energy, in the form of ATP and reduced nucleotides to the host cell. What is surprising, however, is that mitochondria evidently have critical and somewhat unexpected roles in a variety of cellular events including differentiation and pattern formation.

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