Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 5660–5663, June 1995
Developmental Biology

Mitochondrial DNA replication but no nuclear DNA replication during development of *Dictyostelium*

GAD SHAULSKY AND WILLIAM F. LOOMIS*

Center for Molecular Genetics, Department of Biology, University of California at San Diego, La Jolla, CA 92093

Communicated by J. T. Bonner, Princeton University, Princeton, NJ, March 20, 1995

ABSTRACT ***Dictyostelium discoideum* cells initiate development when nutrients are depleted. DNA synthesis decreases rapidly thereafter but resumes during late aggregation, only in prespore cells. This observation has been previously interpreted as indicating progression of prespore cells through the cell cycle during development. We show that developmental DNA replication occurs only in mitochondria and not in nuclei. We also show that the prestalk morphogen known as differentiation-inducing factor 1 can inhibit mitochondrial respiration. A model is proposed for cell type divergence, based on competition to become prespores, that involves mitochondrial replication in prespore cells and reduction of mitochondrial activity in prestalk cells.**

During the cell cycle of *Dictyostelium discoideum* a brief period of chromosomal replication (S phase) immediately follows mitosis and cell division (1). This is followed by a variable period of growth (G_2) before the cells reenter mitosis. When development is initiated by removal of the food source, cells in mid–late G_2 are more likely to differentiate into prespore cells (2–4). Cells expressing either prespore- or prestalk-specific genes are found randomly dispersed throughout early aggregates but the prestalk cells soon sort out to the top, where they form an easily recognized tip (5). The tip elongates and leads the slug as it migrates until culmination is initiated by the formation of a tube surrounding prestalk cells at the front. As more and more prestalk cells enter the stalk tube, vacuolize, and die, the stalk extends down through the prespore cell mass to the base. Subsequent extension of the stalk lifts the ball of prespore cells, which start to encapsulate as they reach the top. Although there have been reports of cell divisions and increases in total cell number occurring after the aggregation stage, it is not clear whether these represent replicative divisions or cytokinesis of multinucleated cells to generate mononucleated cells (4, 6, 7). Studies with a mutant strain of *D. discoideum* that forms very small fruiting bodies allowed highly accurate counts of total cell number that showed no evidence for cell division following the initiation of development (8).

DNA synthesis during development has been measured by incorporation of [^{3}H]thymidine (7, 9). Clear evidence was found for a period of incorporation at about the time that cell type divergence occurs in aggregates. Moreover, autoradiography of slugs showed that most of the cells that had incorporated the label were found in the posterior and so were likely to be prespore cells (9). The cell type specificity of DNA synthesis has been confirmed by the use of antibodies specific to 5-bromo-2′-deoxyuridine (BrdUrd) incorporated into DNA (4). Not only were the labeled cells found exclusively in the posterior of slugs, but BrdUrd-labeled cells were also directly shown to have accumulated a prespore-specific antigen. These observations were interpreted as indicating that prespore cells replicate their chromosomes and divide at the slug stage (4). We have found that all of the incorporated BrdUrd is found in mitochondrial DNA (mtDNA) and that there is no significant incorporation into nuclear DNA in either of the cell types.

MATERIALS AND METHODS

Growth, Labeling, and Fractionation of Cells. Cell growth and development on filters were performed as described (10). BrdUrd (0.5 mM) was added to the growth medium or to the development buffer as indicated. Nuclei were prepared according to Kuspa *et al.* (11) and mitochondria were prepared according to Sussman and Rayner (12). The mitochondrial pellet was treated with DNase before preparation of mtDNA (S. Alexander and H. Alexander, personal communication).

Dot Blot Analysis. DNA was treated with 3 M NaOH at 65°C for 1 hr, neutralized, and loaded on Magna NT nylon filters (Micron Separations, Westboro, MA) in triplicate. Dot blots were hybridized with the chromosomal DNA probe DIRS-1 (13) or with a 0.8-kb *Hin*dIII fragment cloned from mtDNA (14), kindly provided by S. Alexander and H. Alexander. Preparation of DNA and hybridization to blots were as described (10). For detection of BrdUrd, filters were blocked by incubation for 30 min with 1% bovine serum albumin in Tris-buffered saline/0.05% Tween 20, incubated with a 1:200 dilution of anti-BrdUrd antibody (Becton Dickinson) for 2 hr, washed, and incubated with an alkaline phosphatase-conjugated anti-mouse IgG antibody (Sigma; dilution, 1:2000) for 1 hr. The blots were washed and developed with 5-bromo-4-chloro-3-indolyl phosphate (50 μg/ml) and nitro blue tetrazolium (50 μg/ml) in 100 mM Tris·HCl, pH 9.5/100 mM NaCl/5 mM $MgCl_2$.

Pulsed-Field Electrophoresis. Cells were labeled and fractionated as above. Whole cells and purified organelles were washed, suspended in 100 mM EDTA, and embedded in low-melting-point agarose. Fragments of agarose-embedded organelles or cells were treated with *Sma* I restriction endonuclease and loaded onto a 1% agarose gel. Pulsed-field electrophoresis was performed with a CHEF-DR II apparatus (Bio-Rad) at 200 V, switch time from 8 to 18 sec over 20 hr at 17°C. Size markers were concatamers and *Hin*dIII-digested DNA of phage λ (c*I837S*$_{am}$*7*). Gels were stained with ethidium bromide and blotted to Magna NT nylon filter in alkaline buffer. Detection of BrdUrd and DNA probing were performed as described above.

Uncoupling by Differentiation-Inducing Factor 1 (DIF-1). Preparation of submitochondrial particles and measurement of the change in membrane potential were performed according to Yagi *et al.* (ref. 15 and references therein). Synthetic DIF-1 [1-(3,5-dichloro-2,6-dihydroxy-4-methoxyphenyl)-1-hexanone] was purchased from Molecular Probes.

RESULTS AND DISCUSSION

A haploid nucleus of *D. discoideum* contains 40 Mb of DNA carried in six chromosomes as well as another 1 Mb carried in

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Abbreviation: DIF-1, differentiation-inducing factor 1 [1-(3,5-dichloro-2,6-dihydroxy-4-methoxyphenyl)-1-hexanone].

*To whom reprint requests should be addressed.

about a hundred extrachromosomal copies of a 90-kb palindrome (rDNA) that includes the genes for ribosomal RNAs (12, 16, 17). mtDNA (50 kb) accounts for about 30% of the total cellular DNA (14). We grew cells for 5 hr in the presence of BrdUrd, fractionated them into nuclei and mitochondria, and extracted DNA from the respective fractions. Serial dilutions of whole cell DNA, nuclear DNA, and mtDNA were bound to nylon membranes and probed with a monoclonal antibody specific for BrdUrd as well as hybridized with the nuclear DNA probe DIRS-1 (13) and a fragment purified from a *Hin*dIII digest of the mtDNA (14). BrdUrd was incorporated into both nuclear and mitochondrial fractions during growth (Fig. 1*A*). The mitochondrial fraction was <1% contaminated with nuclear DNA, whereas the nuclear fraction contained about 4% mtDNA (Fig. 1 *B* and *C*) as judged by the relative levels of hybridization to the specific probes. The same levels of purification were found in the nuclear and mitochondrial fractions isolated from cells that had developed for 6 or 16 hr (Fig. 1). However, almost all of the BrdUrd present in DNA isolated from cells labeled between 6 and 16 hr of development was found in the mitochondrial fractions. What little was found in the nuclear fractions could be accounted for by the contamination with mtDNA. Moreover, the amount of BrdUrd incorporated into whole cell DNA during development could be completely accounted for as mtDNA (Fig. 1). These results indicate that there is a period of mtDNA synthesis during development but that there is little or no replication of nuclear DNA.

We also separated nuclear DNA from mtDNA by pulsed-field electrophoresis following digestion of whole cell DNA with *Sma* I. This restriction enzyme cuts chromosomal DNA into fragments that average 800 kb but does not cut the 90-kb rDNA palindrome and cuts mtDNA only once, generating a linear 50-kb fragment (14). All three types of DNA were labeled with BrdUrd during growth, with no indication of preferential incorporation into one type or the other (Fig. 2). However, when cells were labeled with BrdUrd during development, mtDNA was strongly labeled, whereas neither rDNA nor chromosomal DNA showed any trace of labeling. By probing Southern blots of the gels with a chromosomal probe

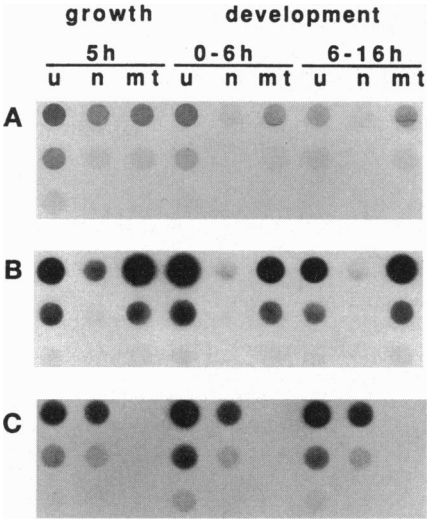

FIG. 1. DNA synthesized during growth and development. Cells were labeled with 0.5 mM BrdUrd for 5 hr during exponential growth or during development at the times indicated (0–6 hr or 6–16 hr). Nuclei and mitochondria were separated and their DNA was extracted. Approximately equal amounts of DNA from unfractionated cells (u), nuclei (n), or mitochondria (mt) were spotted in a series of 5-fold dilutions in each panel. (*A*) Anti-BrdUrd antibody was used to detect newly synthesized DNA. (*B*) mtDNA probe was hybridized to the blot. (*C*) Chromosomal DNA probe DIRS-1 was hybridized to the blot.

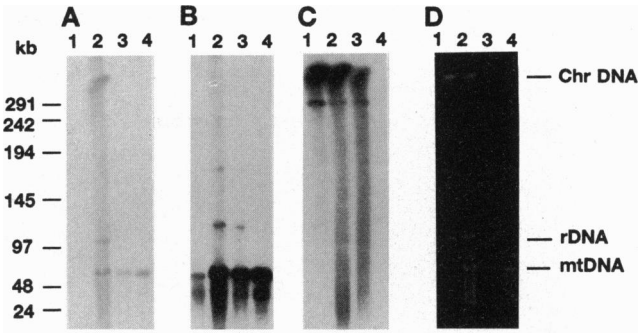

FIG. 2. Pulsed-field electrophoresis of DNA. Cells were labeled with BrdUrd during growth or between 6 and 16 hr of development. *Sma* I-digested DNA from nuclei of unlabeled cells (lanes 1), from unfractionated cells labeled with BrdUrd during growth (lanes 2), from unfractionated cells labeled with BrdUrd between 6 and 16 hr of development (lanes 3), or from purified mitochondria of cells labeled with BrdUrd between 6 and 16 hr of development (lanes 4) was resolved by pulsed-field electrophoresis, blotted, and identified with anti-BrdUrd antibody (*A*), mtDNA probe (*B*), chromosomal (Chr) DNA probe (*C*), and ethidium bromide (*D*).

or a mitochondrial fragment, we showed directly that there was no measurable cross-contamination of the bands (Fig. 2 *B* and *C*). We conclude that the prespore-specific DNA replication of developing *D. discoideum* cells (4, 9) is restricted to mtDNA and that there is no significant replication of nuclear DNA in any of the cell types.

Since prespore-specific DNA synthesis had been shown to start only after aggregation is well underway (4, 7), it was of interest to study the phenomenon in mutant strains defective in development. Cells carrying mutations in the *dagA* gene are unable to aggregate because of the loss of a cytosolic regulator of adenylyl cyclase necessary for relay of the cAMP signal (18). These mutants express neither prespore- nor prestalk-specific genes. Cells carrying mutations in the *lagC* gene aggregate but do not express cell type-specific genes (19). Cells carrying mutations in *tagB* aggregate and express cell type-specific genes but are blocked at the tight-aggregate stage (20). The three mutants were labeled with BrdUrd between 6 and 16 hr after initiation of development and DNA was separated by pulsed-field electrophoresis. Each of the strains incorporated the label into mtDNA (Fig. 3), indicating that mtDNA replication is not dependent on prior aggregation, cell type divergence, or slug formation. It is possible that replication of mtDNA is a primary step in the dependent sequence leading to prespore differentiation.

Prestalk cells can differentiate to become prespore cells if the prespore cells at the posterior are surgically removed (21). We have shown that prespore cells in intact slugs actively inhibit prestalk cells from becoming prespore cells (10). We used the plant gene encoding ricin A to block protein synthesis in prespore cells by fusing it to the regulatory region of a prespore gene so that it was expressed as soon as prespore cells differentiated. Cells carrying this construct died during development because the prespore cells were poisoned and the remaining prestalk cells redifferentiated into prespore cells and so expressed the ricin A gene as well. We proposed that all developing cells in *Dictyostelium* have a tendency to become prespore cells and that the proportions are maintained in slugs by a mechanism of lateral inhibition that prevents prestalk cells from becoming prespores. A natural morphogen of *D. discoideum*, DIF-1, represses prespore differentiation and induces prestalk differentiation (22). This dichlorinated alkyl phenone (23) accumulates during aggregation and is present throughout the slug (24). Whereas prestalk cells are sensitive to DIF-1, prespore cells appear to be relatively insensitive. Fig. 4 shows that DIF-1 is a potent uncoupler of mitochondrial respiration

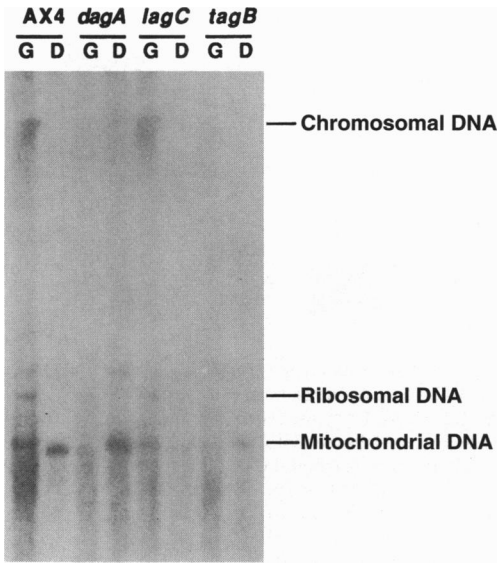

FIG. 3. DNA synthesis during growth and development of wild-type and mutant cells. Cells of strains AX4 (wild type), AK108 (*dagA*$^-$), AK127 (*lagC*$^-$), and AK523 (*tagB*$^-$) were labeled with BrdUrd during growth (G) or hours 6–16 of development (D) and their DNA was size-fractionated by pulsed-field electrophoresis and probed with anti-BrdUrd antibody.

as measured by its ability to reduce the membrane potential of submitochondrial particles (15). DIF-1 appears to be about 100 times more potent than the classic uncoupler 2,4-dinitrophenol and to be comparable to the potent uncoupler carbonyl cyanide *m*-chlorophenylhydrazone (26).

Although our data do not show it directly, it is possible that some of the observed effects of DIF-1 on cell type differentiation are mediated through effects on mitochondrial respiration and that high levels of mitochondrial activity are necessary for prespore differentiation. This suggestion is supported by the *in situ* staining studies of Takeuchi (27), who showed that the activity of two mitochondrial enzymes, succinate dehydrogenase and cytochrome-*c* oxidase, are higher in the posterior of slugs, where prespore cells are localized.

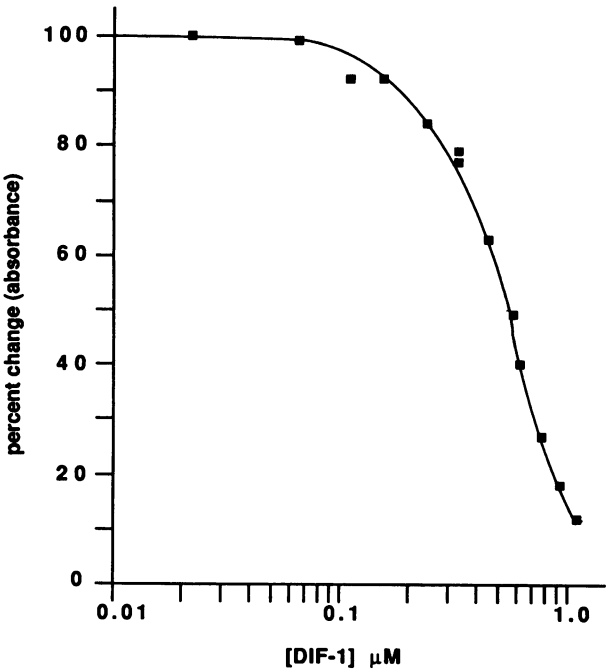

FIG. 4. Effect of DIF-1 on mitochondrial membrane potential. The percent change in absorbance of oxonol VI indicates membrane potential (25). DIF-1 was added to submitochondrial particles and found to uncouple mitochondrial respiration at concentrations above 100 nM. Half-maximal effect was found at 540 nM.

These results lead us to a model in which energy storage and energy metabolism determine the outcome of the competition to become prespores. When a homogeneous growing population of cells is induced to develop by removal of the food source, some cells will be about to divide or have just divided while others will be in mid–late G_2. Most of the prespore cells will be recruited from the mid–late G_2 cells, which are by definition bigger than newly divided cells and so contain more mitochondria and energy stores. If the competition to become prespore cells is biased by mixing cells with different nutritional histories and allowing them to develop together, it is known that those with higher energy stores will preferentially form prespore cells and override the cell cycle bias (28–30). It appears that cells which enter the competitive process of differentiation with higher potential energy win and become the initial prespore cells.

Even after the proportions of cell types are established in slugs, prestalk cells retain the ability to differentiate into prespore cells but are inhibited by the presence of previously differentiated prespore cells (10, 21). Prespore cells have higher mitochondrial activity than prestalk cells (27) and, as shown here, replicate their mitochondrial genomes during development. They also appear to be relatively insensitive to the natural morphogen DIF-1, which we have shown is not only an inhibitor of prespore differentiation but also a potent inhibitor of mitochondrial respiration. It is worth considering that energy metabolism at the mitochondrial level may be a primary means of establishing and maintaining cell type proportions in *D. discoideum* and other multicellular developing systems.

We are grateful to Drs. Akemi Matsuno-Yagi and Youssef Hatefi for help with the mitochondrial uncoupling assays. We thank Drs. Hannah and Steve Alexander for mtDNA probes, Dawn Maghakian for assistance with pulsed-field electrophoresis, and Dr. Adam Kuspa for useful discussions. This work was supported by the National Science Foundation (DCB 9017782). G.S. was supported by a fellowship (S-4-94) from the California Division of the American Cancer Society.

1. Weijer, C. J., Duschl, G. & David, C. N. (1984) *J. Cell Sci.* **70,** 111–131.
2. McDonald, S. A. & Durston, A. J. (1984) *J. Cell Sci.* **66,** 195–204.
3. Gomer, R. H. & Firtel, R. A. (1987) *Science* **237,** 758–762.
4. Zimmerman, W. & Weijer, C. J. (1993) *Dev. Biol.* **160,** 178–185.
5. Williams, J. G., Duffy, K. T., Lane, D. P., McRobbie, S. J., Harwood, A. J., Traynor, D., Kay, R. R. & Jermyn, K. A. (1989) *Cell* **59,** 1157–1163.
6. Bonner, J. T. & Frascella, E. B. (1952) *J. Exp. Zool.* **121,** 561–571.
7. Zada-Hames, I. M. & Ashworth, J. M. (1978) *Dev. Biol.* **63,** 307–320.
8. Sussman, R. R. & Sussman, M. (1960) *J. Gen. Microbiol.* **23,** 287–293.
9. Durston, A. J. & Vork, F. (1978) *Exp. Cell Res.* **115,** 454–457.
10. Shaulsky, G. & Loomis, W. F. (1993) *Dev. Biol.* **160,** 85–98.
11. Kuspa, A., Maghakian, D., Bergesh, P. & Loomis, W. F. (1992) *Genomics* **13,** 49–61.
12. Sussman, R. & Rayner, E. P. (1971) *Arch. Biochem. Biophys.* **144,** 127–137.
13. Zuker, C., Cappello, J., Lodish, H., George, P. & Chung, S. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 2660–2664.
14. Fukuhara, H. (1982) *Biol. Cell.* **46,** 321–324.
15. Yagi, T., Matsuno-Yagi, A., Vik, S. B. & Hatefi, Y. (1984) *Biochemistry* **23,** 1029–1036.
16. Firtel, R. A. & Bonner, J. (1972) *J. Mol. Biol.* **66,** 339–361.
17. Cockburn, A. F., Taylor, W. C. & Firtel, R. A. (1978) *Chromosoma* **70,** 19–29.
18. Insall, R., Kuspa, A., Lilly, P. J., Shaulsky, G., Levin, L. R.,

Loomis, W. F. & Devreotes, P. (1994) *J. Cell Biol.* **126,** 1537–1545.
19. Dynes, J., Clark, A. M., Shaulsky, G., Kuspa, A., Loomis, W. F. & Firtel, R. A. (1994) *Genes Dev.* **8,** 948–958.
20. Loomis, W. F., Kuspa, A. & Shaulsky, G. (1994) in *Genetic Engineering Principles and Methods*, ed. Setlow, J. K. (Plenum, Upton, NY), pp. 49–64.
21. Raper, K. B. (1940) *J. Elisha Mitchell Sci. Soc.* **59,** 241–282.
22. Berks, M. & Kay, R. R. (1990) *Development (Cambridge, U.K.)* **110,** 977–984.
23. Morris, H. R., Taylor, G. H., Masento, M. S., Jermyn, K. A. & Kay, R. R. (1987) *Nature (London)* **328,** 811–814.
24. Brookman, J. J., Jermyn, K. A. & Kay, R. R. (1987) *Development (Cambridge, U.K.)* **100,** 119–124.
25. Bashford, C. L. (1981) *Biosci. Rep.* **1,** 183–196.
26. Heytler, P. G. & Prichard, W. W. (1962) *Biochem. Biophys. Res. Commun.* **7,** 272–275.
27. Takeuchi, I. (1960) *Dev. Biol.* **2,** 343–366.
28. Blaschke, A., Weijer, C. & McWilliams, H. (1986) *Differentiation* **32,** 1–9.
29. Leach, C. K., Ashworth, J. M. & Garrod, D. R. (1973) *J. Embryol. Exp. Morphol.* **29,** 647–661.
30. Tasaka, M. & Takeuchi, I. (1981) *Differentiation* **18,** 191–196.