Tissue-specific G1-phase cell-cycle arrest prior to terminal differentiation in *Dictyostelium*

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Summary

The cell cycle status of developing *Dictyostelium* cells remains unresolved because previous studies have led to conflicting interpretations. We propose a new model of cell cycle events during development. We observe mitosis of about 50% of the cells between 12 and 18 hours of development. Cellular DNA content profiles obtained by flow cytometry and quantification of extra-chromosomal and chromosomal DNA suggest that the daughter cells have half the chromosomal DNA of vegetative cells. Furthermore, little chromosomal DNA synthesis occurs during development, indicating that no S phase occurs. The DNA content in cells sorted by fluorescent tissue-specific reporters indicates that prespore cells divide before prestalk cells and later encapsulate as G1-arrested spores. Consistent with this, germinating spores have one copy of their chromosomes, as judged by fluorescence in situ hybridization and they replicate their chromosomes before mitosis of the emergent amoebae. The DNA content of mature stalk cells suggests that they also attain a G1 state prior to terminal differentiation. As prestalk cells appear to be in G2 up to 22 hours of development, our data suggest that they divide just prior to stalk formation. Our results suggest tissue-specific regulation of G1 phase cell cycle arrest prior to terminal differentiation in *Dictyostelium*.

Key words: Flow cytometry, Bromo-deoxyuridine, G1 arrest, Differentiation

Introduction

In most organisms, cells undergoing terminal differentiation exit the cell cycle in G1 and enter a quiescent state known as G_0. Sporulating yeast cells and differentiating cells in metazoa often arrest their cell cycle in G1 (Chen-Kiang, 2003; Hsieh et al., 2000; Zavitz and Zipursky, 1997). The regulatory events governing cell cycle exit are becoming increasingly understood, as in the case of myocyte differentiation where inhibitors of cyclin-dependent kinases control the nature and pattern of muscle development in animals (Halevy et al., 1995; Zhang et al., 1999). It has been thought that *Dictyostelium* cells differ from most other organisms by carrying out terminal differentiation in G2 (Weeks and Weijer, 1994). However, the currently accepted model of cell cycle progression during *Dictyostelium* development has been challenged in recent years (Shaulsky and Loomis, 1995). Here, we re-examine the *Dictyostelium* cell cycle during development and our results suggest that both major cell types exit the cell cycle in G1 prior to terminal differentiation.

Dictyostelium cells must transition out of the growth phase cell cycle when they encounter starvation conditions and initiate multicellular development (Kessin, 2001). The relationship between cell fate determination and cell cycle regulation in *Dictyostelium* has been a topic of intense study over the past 30 years. It has been well established that the cell cycle status of growing cells impinges on cell fate decisions after development is initiated. Cells that are in middle, or late, G2 phase at the time of starvation preferentially become prespore cells, whereas cells in the S, M or early G2 phase preferentially become prestalk cells (Azhar et al., 2001; Gomer and Ammann, 1996; Gomer and Firtel, 1987; McDonald and Durston, 1984; Ohmori and Maeda, 1987; Weijer et al., 1984a; Zimmerman and Weijer, 1993). Maeda and colleagues have proposed that cells exit the cell cycle at a particular point late in the G2 phase called the 'putative shift' (PS) point and those cells that must traverse G2 under starvation conditions for the longest time in order to arrive at the PS point will have a propensity to differentiate as prestalk cells (Maeda et al., 1989; Maeda, 1993; Araki et al., 1997; Maeda, 1997). Alternatively, it has been suggested that cells either exit the cell cycle early in G2, biasing them to differentiate as prestalk cells, or cells exit the cell cycle late in G2, biasing them to differentiate as prespore cells (MacWilliams et al., 2001). There is general agreement that all cells pause in G2 during the first half of development.

It is important to understand the regulation of the cell cycle during development so that we can critically assess its role in cell type specification and terminal differentiation. The cell cycle status of developing cells after their initial pause in the G2 phase is unclear because data from different experiments have led to contradictory conclusions. Some cell division takes place in the first 6 hours, prior to aggregation, and additional

cell divisions occur between 12 and 20 hours, after multicellular structures are formed (Atryzek, 1976; Zada-Hames and Ashworth, 1978a). The currently accepted model posits that all cells that divide during development replicate their chromosomes after mitosis and undergo terminal differentiation as G2 cells (Weeks and Weijer, 1994). Flow cytometry studies have suggested that all the developmental cells have the same nuclear DNA content as G2 vegetative cells, implying that developmental cells are in the G2 phase (Durston et al., 1984; Weijer et al., 1984a). Some reports of DNA synthesis at the time of cell division appear to support these claims (Zada-Hames and Ashworth, 1978a; Zimmerman and Weijer, 1993). However, this model has been challenged by experiments showing that the bulk amount of DNA synthesis during development cannot account for complete replication of the chromosomes in more than a few percent of the cells. In fact, the majority of DNA synthesis during development appears to be accounted for by mitochondrial DNA (mtDNA) synthesis (Shaulsky and Loomis, 1995). Direct measurements of cellular DNA content also suggests that spores have about half the amount of DNA as vegetative (mainly G2 phase) cells (Sharpe and Watts, 1984). These results argue that developmental cells remain in G1 phase after mitosis.

In order to begin to explore the possible relationships between cell cycle regulation, cell-fate determination and terminal differentiation during *Dictyostelium* development, we need an accurate description of the cell cycle during development. We have revisited the question of cell cycle progression during development in an attempt to clarify basic issues of cell cycle timing in relation to cell differentiation events. We provide evidence for tissue-specific regulation that results in G1 phase cell cycle arrest in all cells prior to terminal differentiation.

Materials and methods

Growth and development of *Dictyostelium*

Strains were derived from the *Dictyostelium discoideum* strain AX4 by standard molecular genetic manipulations (Knecht et al., 1986; Kuspa et al., 1995; Mann et al., 1998). Cells were grown in HL-5 media or on bacterial plates (SM agar) and induced to undergo synchronous development on nitrocellulose filters as described previously (Sussman, 1987). Exponentially growing cells were counted, harvested, washed once in PDF buffer [22.2 mM potassium phosphate (pH 6.4), 20 mM KCl, 1.0 mM $CaCl_2$, 2.5 mM $MgSO_4$] and 5×10^7 cells were deposited on each filter on top of a cellulose pad saturated with PDF buffer. Filters were incubated within a 60 mm petri dishes at 22°C in a humid chamber.

Quantification of cells and nuclei

Two filters of developing cells were processed for each time-point as described previously (Zada-Hames and Ashworth, 1978a). The cellulose pad was removed and the filter was placed in its original petri dish and flooded with 3 ml 50 mM Tricine (pH 7.4) containing 1 mg/ml Pronase (Protease type VI, Sigma) and 0.25% 2,3-dimercaptopropanol (BAL). The cells were washed off the filter by repeated pipetting for about 3 minutes and the cell suspension was transferred to a 50 ml polypropylene tube. Additional BAL/pronase solution (3 ml) was used to wash the filter and dish again and the cell suspension and the filter were added to the tube. Finally, the Petri dish was washed with 10 ml 50 mM tricine buffer and this wash was added to the tube. The cell suspension and filter were vortexed vigorously in the tube for 30 seconds. The suspension was brought to 49.5 ml with saline, 0.5 ml of 0.5 M EDTA (pH 8.0) was added, and the tube was then shaken for 30 seconds and vortexed vigorously for an additional 60 seconds. Two determinations of 100-200 cells were made for each of four aliquots from each of two independently prepared tubes. The cells from two tubes were then sedimented (500 ***g***, 5 minutes), combined and resuspended in PDF containing 2% sucrose in saline solution.

To quantify nuclei in the cell samples, ethanol-fixed cells (5×10^5) cells were pelleted and resuspended in 100 μl 2% sucrose, 0.9% NaCl, in PDF. Cells were sedimented onto a microscope slide, 9 μl of Vectashield (H-1000, Vector Laboratories) containing DAPI was applied to the cells and the number of nuclei within individual cells was quantified by fluorescence microscopy.

Flow cytometry

Flow cytometry was carried out as described (Dien et al., 1994). Cells (1×10^7) were resuspended in 1.5 ml 0.9% NaCl, 2% sucrose, 5 mM EDTA, in PDF buffer and fixed by adding 5 ml 90% ice-cold ethanol dropwise, with gentle vortexing. The cells were then incubated for >30 minutes at 22°C and stored at 4°C. For staining with propidium iodide (PI) just prior to flow cytometry, cells (1×10^6) were pelleted from the fixation solution and resuspended in 1 ml of PI (50 μg/ml in phosphate-buffered saline, PBS). Thirty minutes before analysis, 1 μl of 100 mg/ml DNase-free RNase (Sigma, St Louis, MO) was added and the suspension incubated at 37°C. Samples were analyzed on a Beckman-Coulter Epics XL-MCL apparatus. The parameters were adjusted for the measurement of single cells using the forward and side scatter plots as guides.

The DNA in developing cells or spore DNA might be less accessible to PI using staining procedures developed for vegetative cells and this might distort the flow cytometry profiles. We therefore treated samples with 70% formamide at 85°C to denature the DNA prior to flow cytometry (Baerlocher et al., 2002). The flow cytometry profiles were very similar between these treated cells and untreated cells.

Separation of cell types to measure DNA content

Slug dissection

ecmA/*GFP*-marked AX4 cells were washed with DB buffer (5 mM Na_2HPO_4, 5 mM KH_2PO_4, 1 mM $CaCl_2$, 2 mM $MgCl_2$, pH 6.5) and resuspended in water to 10^9 cells/ml. Cells were streaked on a 6 cm line (~100 μl) on water agar plates made with 2% Noble agar (Difco) and allowed to develop in a dark chamber with unidirectional illumination. After 24-36 hours, slugs were dissected with a tungsten wire to harvest GFP-labeled slug anteriors and unlabeled slug posteriors. At least 50 anterior (or posterior) slug sections were put into 100 μl 2% Sucrose, 0.9% NaCl, in PDF. Pronase (1 mg/ml) and BAL (1 μl) were added to dissociate the cells by vortexing for 30 seconds. The cells were harvested by centrifugation, resuspended in 0.5 ml 2% sucrose in PDF, an equal volume of the fixation solution was added (4% paraformaldehyde, 30% picric acid, 10 mM PIPES, pH 6.5) and cells were mixed gently for 15 minutes. Cells were then harvested and resuspended in 0.2 ml of 2% sucrose in PDF and stored at 4°C. Stored cells were later prepared for flow cytometry, as described above.

DNA content of cell types during development

Strains AX4[*cotB/GFP*] and AX4[*ecmA/GFP*] were grown in HL-5 and prepared for development on filters. Cells from one-quarter of a filter (~1.25×10^7 cells) were harvested into a 15 ml conical tube and dissociated by vortexing in 4 ml of BAL/pronase, as described above. EDTA was added (0.2 ml, 0.5 M, pH 8) and the tubes were stored on ice prior to sorting with an Altra cell sorter (Beckman-Coulter) as described (Gerald et al., 2001). At least 5×10^5 sorted cells from each strain were collected in 3 ml and 300 μl of 20% sucrose, 9% NaCl, in PDF were added, followed by the addition of 10 ml of 95% ethanol

for fixation. Cells were stored at 4°C prior to measuring their DNA content by flow cytometry, as described above.

DNA synthesis

Bromo-deoxyuridine (BrdU) incorporation was carried out as follows. Cells pellets form growth cultures were washed at 22°C in PDF supplemented with 0.5 mM BrdU and resuspended in PDF with 0.5 mM BrdU at 1×10^8/ml. Cells (0.5×10^8) were spread on one filter with the underpad soaked with 1.5 ml 0.5 mM BrdU in PDF. After 36 hours of development, cells from one filter were harvested and used to make one 150 μl agarose block. Agarose blocks were processed to produce high molecular weight DNA as described, except that cellulase and hemicellulase were used to digest the cell walls of stalk cells and spores (Kuspa et al., 1992). Pulsed field gel electrophoresis and Southern transfer of the DNA to nylon filters was carried out as described (Shaulsky and Loomis, 1995; Vollrath and Davis, 1987). Detection of BrdU was carried out with anti-BrdU antibodies conjugated to peroxidase using an ECL detection kit (Amersham). Multiple exposures of each autoradiograph were used to assess linearity of the signals and quantification was carried out using standard image analysis software. Values for BrdU incorporation were normalized to the total DNA content in each lane using the Southern hybridization signal from a single-copy gene (*gdtB*) as an estimate the amount of chromosomal DNA on the blots. The *gdtB* hybridization signal also showed that <10% of the chromosomal DNA remained in the wells for any given sample. By comparison with the signal obtained from growing cells, we estimate that we could detect the labeling of >1% of any cellular DNA species.

Estimating the proportions of DNA species

Cells collected from each developmental time point (5×10^7) were used to make two agarose blocks. The DNA in one block was digested with *Sma*I and the DNAs were resolved by pulsed-field gel electrophoresis. Gels were stained with ethidium bromide and multiple images were acquired at 325 nm at different light intensities and under-saturated images were used for quantification. The gel was shaken in water for at least 24 hours, and each side of the gel was exposed to UV light for 60 seconds to break DNA (Stratalinker UV lamp, Stratagene). DNA was transferred onto nylon membranes and hybridized with radiolabeled (^{32}P) probes (Vollrath and Davis, 1987). The mtDNA probe was a PCR-amplified product from nucleotides 9991 to 13579, which corresponds to a 3.6 kb *Eco*RI fragment of the mtDNA produced with primers Mito_5′ (agt tta gac act gct gg) and Mito_3′ (cta aaa cgc aca cct tct c) (Ogawa et al., 2000). The rDNA probe was a collection of 12 DNA fragments encompassing the rRNA-coding region (Sucgang et al., 2003). The probe for chromosomal DNA was derived from the DIRS element subclone pAK162 (Kuspa and Loomis, 1996). The ^{32}P signal was quantified by phosphoimaging using a Molecular Dynamics Storm system. The relative amount of rDNA or mtDNA in the gel (G_r or G_m, respectively) and in the wells (W_r and W_m) was determined for each sample. The images of ethidium bromide-stained gels were used to obtain the intensity of rDNA (I_r) and mitochondrial DNA (I_m) bands and the total DNA in each lane (including the DNA in the wells, I_t). To estimate the percentage of rDNA and mtDNA relative to total DNA, the fluorescence intensity of the rDNA and mtDNA bands in the gel were corrected by the proportion of those DNAs found in the wells by hybridization. Thus, the percentage of rDNA (P_r) in a sample was estimated as: $P_r=[I_r(1+W_r/G_r)]/I_t$, the percentage of mtDNA (P_m) was $P_m=[I_m(1+W_m/G_m)]/I_t$ and the percentage of chromosomal DNA (P_c) was estimated by the difference: $P_c=1–(P_r+P_m)$.

Predicting flow cytometry profiles

We predicted the DNA content expected of populations of developing cells using two models of cell cycle control. We assumed that all cells are in G2 at 6 hours of development, that the mean DNA content is determined by flow cytometry (F6) and that the proportion of the DNA species is defined as: $P_c6 + P_r6 + P_m6=1$. The chromosomal DNA content at 6 hours would then be: $F6\times P_c6$. The proportions of mtDNA and rDNA were taken from the experiments described above. The proportion of each DNA was also estimated at time ‘x’ as: $P_cx + P_rx + P_mx=1$. If all cells were in G2 at time x, the cellular chromosomal DNA in mononuclear cells should be the same as that of 6-hour cells ($F6\times P_c6=Fx\times P_cx$), and the relative DNA content would be predicted as: $Fx=(F6\times P_c6)/P_cx$. If all cells were in G1 phase at time ‘y’, the cellular chromosomal DNA in mononuclear cells should be one-half that of 6-hour-old cells, $F6\times P_c6=2\times Fy\times P_cy$. The relative DNA content would be predicted as: $Fy=(F6\times P_c6)/2P_cy$. If the population was a mixture of G1 and G2 cells at a time ‘n’, assuming all cells have the same proportion of DNA species, the DNA content of G1 and G2 population was estimated as $F1n=(F6\times P_c6)/2P1_cn$ for G1 cells and $F2n=(F6\times P_c6)/P2_cn$ for G2 cells. The average DNA content (Fx_{avg}) was then calculated according to the percentage of G1 and G2 cells in the population (a1 and a2) given that a1+a2=1. The average DNA content for the population was predicted to be $Fx_{avg}=a1[(F6\times P_c6)/2P1_cn] + a2[(F6\times P_c6)/P2_cn]$. The results reported are based on calculations made from the results described in Fig. 2C and estimates of the DNA content by extrapolation of the flow cytometry measurements in Fig. 2D.

DNA synthesis during spore germination

Cells were grown on bacterial lawns, allowed to develop, and the spores were allowed to mature for 1 day. Spores were harvested into 0.1% NP-40 in 10 mM potassium phosphate buffer, pH 6.4. A 10 ml syringe fitted with an 18-gauge needle was used to disperse the spores and disrupt non-spore cells. The spores were washed ten times, with the final wash carried out in buffer without detergent and were resuspended in buffer in glass tubes and incubated at 42°C for 30 minutes. Spores (1.5×10^7) were distributed into petri plates (150 mm) with 30 ml of HL-5 media containing 0.5 mM BrdU. Spores and amoebae were harvested and counted at various times. BrdU incorporation was determined as described above.

Fluorescence in situ hybridization (FISH)

FISH was used to assess chromosome copy number. The hybridization, DAPI staining and image analysis was carried out as described previously (Sucgang et al., 2003). A 7 kb genomic fragment of the *tagB* gene, that hybridizes to *tagB* and to two highly similar genes (*tagC* and *tagD*) that are tightly linked within a 20 kb region of chromosome 4 was used as the probe (Shaulsky et al., 1995). After hybridization and DAPI staining, preparations were visualized on a Delta Vision deconvolution microscope and images were processed with the SoftWoRx (version 2.5) software package (Applied Precision, Issaquah, WA). Nuclei were scored as having one or two hybridization signals by manually inspection while traversing through the three-dimensional images. Within each nucleus that was scored, fluorescent signals that were approximately fivefold lower in intensity than the more intense signal were not counted as a chromosomal locus. Over 100 cells from each of several preparations for each biological sample were scored and the results were averaged.

Results

Cytokinesis and mitosis during development

To clarify the cell cycle status of developing *Dictyostelium* cells we counted cells and nuclei while monitoring the cellular DNA content and DNA synthesis during development. We observed two increases in the cell number; a 25% increase during the first 6 hours and an additional 50% increase between 12 and 18 hours (Fig. 1A). Cell division was not scored after 20 hours because some of the cells had become integrated into the stalk.

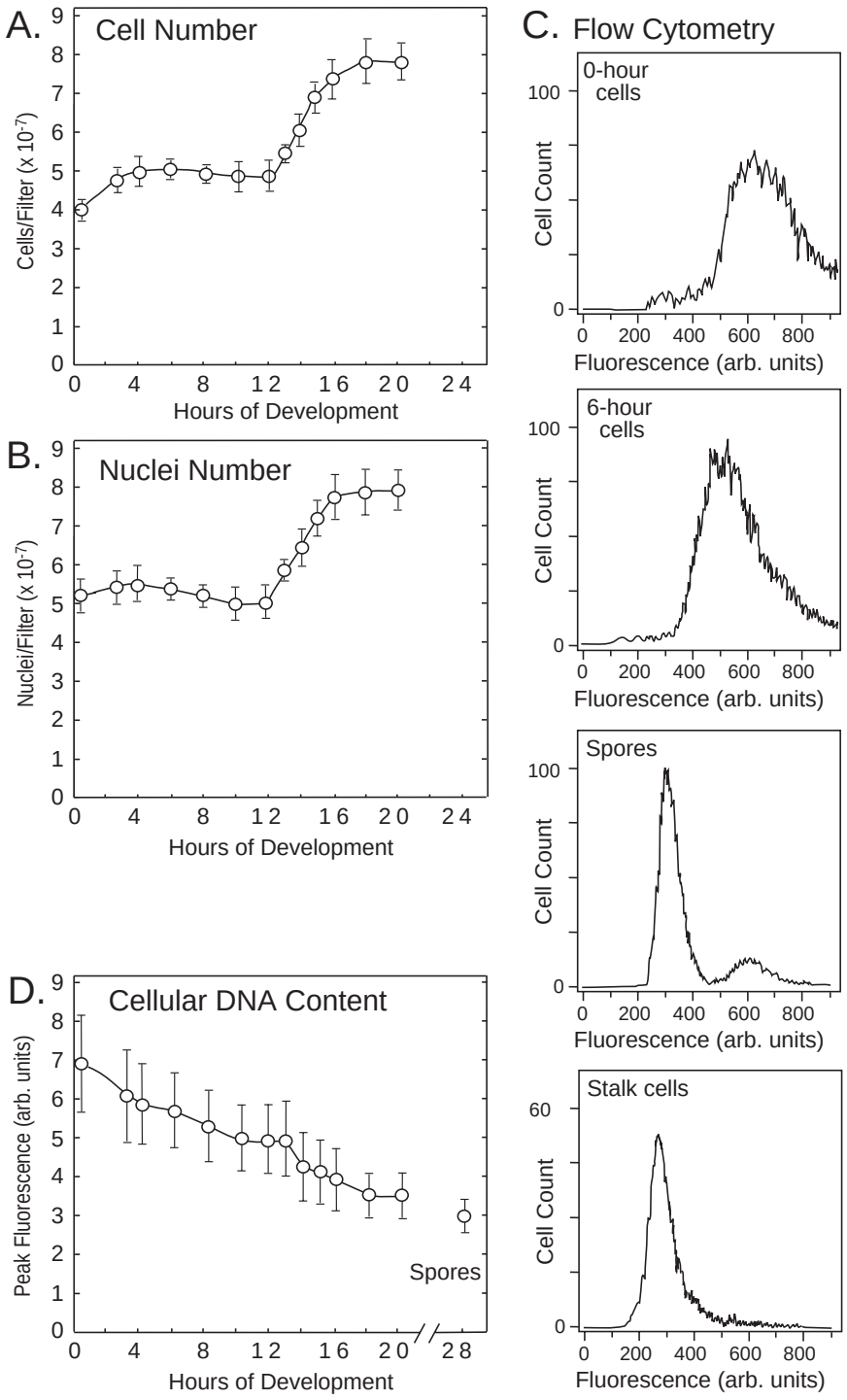

Fig. 1. Mitosis and cellular DNA content during development. (A) Wild-type AX4 cells were plated for development on filters, and counted at different times. (B) Mitosis was monitored in the same samples by counting nuclei in propidium iodide-stained cells. For cellular and nuclear counts, eight determinations were made for each sample of developing cells and the means (±s.e.m.) are reported. (C) Flow cytometry profiles of the DNA content in whole cells at various times of development. The profiles corresponding to the mononucleated cells are shown. Spores and stalk cells were purified from fruiting bodies after 36 hours of development as described in Materials and methods. (D) The cellular DNA content for each time of development is summarized by the peak values (±s.e.m.) of the mono-nucleated cell flow cytometry profiles. This graph summarizes multiple profile determinations from one experiment that is representative of four separate experiments.

Axenic strains of *Dictyostelium*, like the AX4 cells used in these studies, can become multinucleated when grown in shaken nutrient broth (Sharpe et al., 1984; Zada-Hames and Ashworth, 1978a; Zada-Hames and Ashworth, 1978b). Consistent with these earlier reports, we found that most of the early increase in cell number could be accounted for by cytokinesis of multinucleated cells. The average number of nuclei per cell decreased from 1.28 to 1.08 in the first 6 hours of development (compare Fig. 1A with 1B). Therefore, the cell division in the first six hours of development is mainly due to cytokinesis, while the cell division after 12 hours is due to mitosis. Cells grown on bacteria were mainly mononucleated and displayed only the later mitotic events during development (data not shown).

Cellular DNA content during development

The mitotic activity within aggregates means that some cells divide and either arrest in G1 phase or they undergo a round of DNA synthesis and return to G2 phase. If 50% of the cells divide and arrest in G1, a decrease in the cellular DNA content should be detectable between 12 and 18 hours of development. We used flow cytometry of whole cells to monitor changes in cellular DNA content during development. In principle, it would have been best to measure the DNA content by flow cytometry of nuclei, but we found this approach problematic. We carried out flow cytometry analyses on nuclei with several different nuclear purification and fixation protocols. We could reproduce published profiles, namely unimodal peaks that do not change during development, but we observed that nuclear fragility varies at different times in development for each of the procedures that we evaluated and this caused erratic and aberrant DNA content profiles (G.C., unpublished). The side-scatter and the forward-scatter profiles did not show a unique nuclear population using any preparation procedure. Instead, doublets and triplets of nuclei and cellular debris were the predominant particles and the samples invariably clogged the cytometer. As nuclear profiles were unreliable, we resorted to whole-cell flow cytometry and combined it with estimates of the changes in the extra-chromosomal DNAs to arrive at the likely cell cycle status of the cells.

Vegetative *Dictyostelium* cells displayed a broad peak of DNA content that is probably due to variable mtDNA content

and the presence of a small number of G1 cells (Fig. 1C). We also observed a smaller peak with twice the DNA content of the first peak, which we confirmed to be binucleated cells by direct microscopic examination (data not shown). These binucleated cells were no longer detectable as a second peak after 6 hours. For simplicity, we summarize the DNA content profiles using the average DNA fluorescence of mononucleated cells (Fig. 1D). In the first 10 hours of development there was a steady decrease in the average cellular DNA content. As we did not observe mitosis during this time, the DNA decrease is probably due to a decrease in the proportion of extra-chromosomal DNA (see below). The average cellular DNA content appeared to plateau from 10-13 hours of development and then decreased between 13 and 18 hours of development. The final spore and stalk cell DNA content is about 50% that of 6 hour cells (Fig. 1C,D). We consistently observed this pattern of cellular DNA loss during development in over a dozen similar experiments.

The decrease in cellular DNA content after 13 hours might be caused either by a shift of most cells from G2 into G1, or by the loss of extra-chromosomal DNA with chromosomal DNA maintained at constant levels by new synthesis. To distinguish between these possibilities, we estimated changes in the proportions of the three major cellular DNAs at different stages of development. We isolated high molecular weight DNA from cells at different times, separated the rDNA, mtDNA and chromosomal DNA by pulsed-field gel electrophoresis and directly quantified the DNAs based on their ethidium bromide staining (Fig. 2A). We then used Southern analyses to determine the extent of DNA trapping in the sample well for each DNA species, in order to correct these estimates (e.g. Fig. 2B and data not shown). We found that the proportion of rDNA relative to total cellular DNA does not change significantly during development, whereas the proportion of mtDNA decreases by about 50% during the first 13 hours of development (Fig. 2C). Significantly, the ratios of the three cellular DNAs did not change during the time of mitosis in the second half of development (Fig. 2C).

The DNA ratios in Fig. 2C and the cell number increase described in Fig. 1A were used to reconstruct the cellular DNA content of cell populations at each time point during development using two different assumptions of chromosome copy number after 12 hours of development. We assumed that all cells are in G2 at 6 hours of development and that after cells divide they either arrest in G1 phase, or they undergo S phase and arrest in G2 phase (see Materials and methods). Our measurements agree with the model that assumes a G1 arrest of post mitotic cells after 12 hours of development (Fig. 2D).

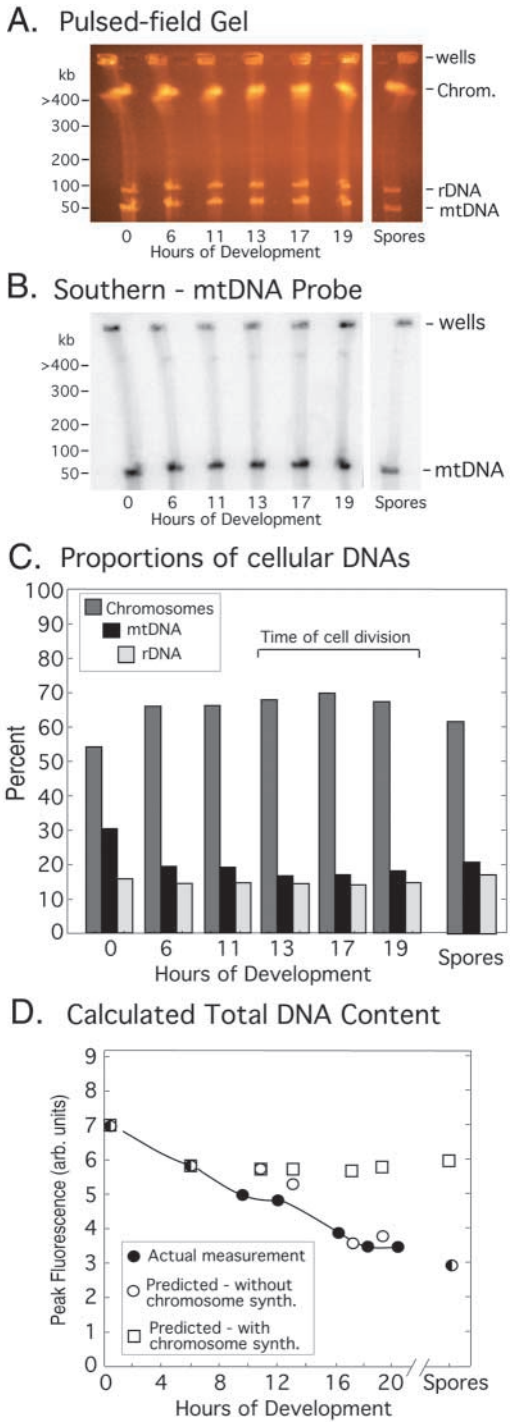

Fig. 2. Proportions of cellular DNAs during development. Changes in the relative proportions of the chromosomal DNA, mitochondrial DNA (mtDNA) and the extrachromosomal ribosomal DNA (rDNA) were estimated by quantification on pulsed field gels. (A) High molecular weight DNAs were separated by pulsed field gel electrophoresis and stained with ethidium bromide. The three DNA species were quantified by digital image analysis using several different exposures. Phosphoimager analyses of Southern hybridizations were used to correct for the proportion of each DNA trapped in the wells. The signal obtained for mtDNA is shown as an example (B). (C) Changes in the proportions of mtDNA, rDNA and chromosomal DNA are shown for a representative experiment. Note that their proportions do not change during the time cells undergo mitosis (indicated by the bracket). (D) The measured peak values for flow cytometry DNA profiles of mononucleated cells are plotted for the same population of cells described in C (filled circles). The expected peak values were calculated from the proportions of the different DNA species in C and assuming that all cells at 6 hours are in G2 and that all cells that divided later remained in G1 (open circles), or progressed through an S phase and arrested in G2 (open squares).

Quantification of DNA synthesis during development

We monitored the synthesis of chromosomal DNA, rDNA and mtDNA by BrdU incorporation. Cells were deposited on filters supplemented with BrdU and high molecular weight DNA was prepared after 28 hours of development. Stalk cells, spores and non-stalk cells (all cells remaining after stalks cells were removed, including spores) were also purified after BrdU labeling. We observed little incorporation of BrdU into the chromosomal DNA or rDNA of developing cells, but there was substantial incorporation into the mtDNA of spores (Fig. 3). We estimate that our limit of BrdU detection corresponds to the synthesis of about 1% of the chromosomal DNA loaded on the gels, based on direct comparisons with the BrdU incorporated into the DNA of growing cells during one cell doubling (Fig. 3A). The small amount of chromosomal BrdU incorporation observed in the 'non-stalk cell' sample which contains spores (6.3±2% of the growth control) compared with the much lower amount in the purified spore sample (2.2±0.6%) suggests that most of the chromosomal DNA synthesis occurs in cells that do not undergo terminal differentiation into spores or stalk cells. The small amount of BrdU incorporation into the chromosomes of spores or stalk cells indicates that the major terminally differentiated cell types do not undergo S phase at anytime during development.

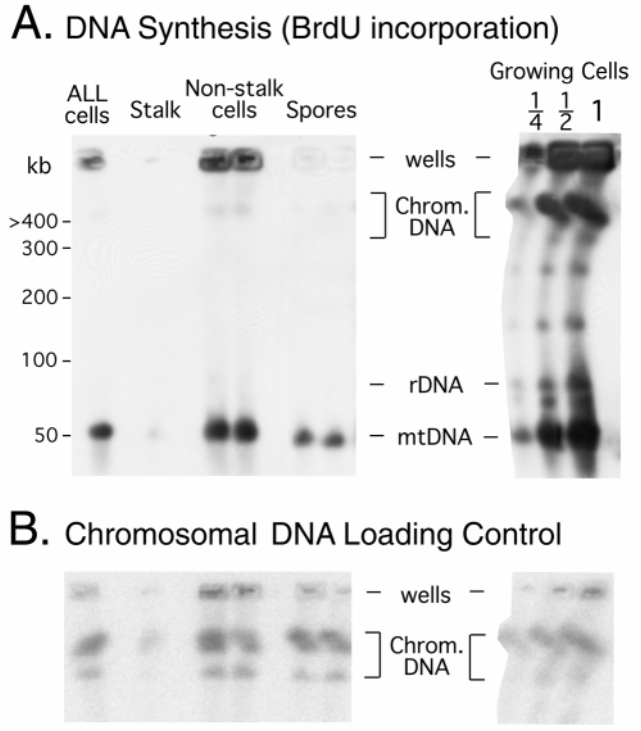

Fig. 3. DNA synthesis during development. DNA synthesis during development was monitored by BrdU incorporation. (A) High molecular DNA was separated on standard pulsed-field gels and BrdU was detected by immunostaining after Southern transfer of the DNA. The amount of BrdU incorporation into chromosomes was compared to level of labeling observed in growing cells during one cell cycle and normalized to the amount of chromosomal DNA on the blot, as detected with a chromosomal probe (B). See text for details.

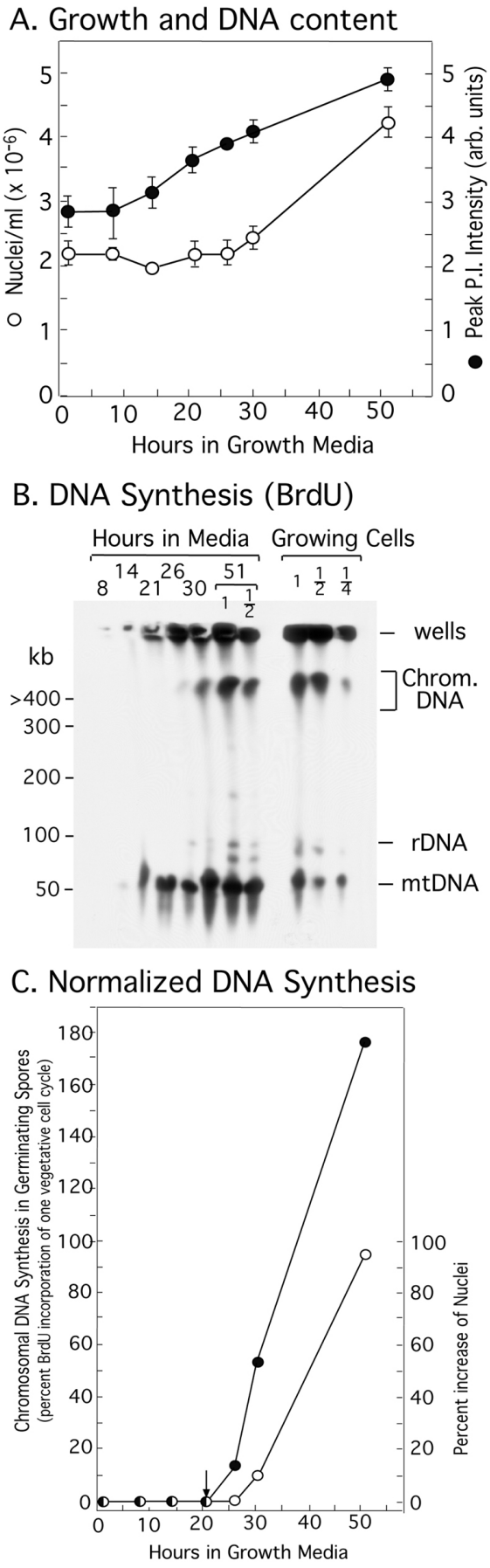

Fig. 4. DNA Synthesis during spore germination and recovery to growth phase. Spores were heat-shocked, and allowed to recover in liquid growth media. (A) The increase in nuclei and the peak level of the DNA content profile of mononucleated cells is plotted over the time of the first cell doubling. (B) DNA synthesis was measured by BrdU incorporation as in Fig. 3, using growing cells that had been labeled for one cell cycle as a control. (C) The percentage of chromosomal DNA synthesis in germinating spores relative to one cell cycle of growing cells was estimated from the BrdU incorporation in B. The values are normalized to the amount of chromosomal DNA on the blot, determined by Southern hybridization as described in Fig. 3. At the 30-hour time point, there was 52% the BrdU incorporation observed in growing cells after only a 10% increase in the number of nuclei. At 21-hours, some BrdU incorporation into chromosomal DNA was detectable upon longer exposures of the blot (arrow).

DNA synthesis during germination

If a spore germinates from G1 phase, it must proceed through two S phases prior to its second cell division. The first S phase would be required to achieve G2 prior to its first mitosis and the second would occur immediately following the first mitosis. To further test the idea that spores encapsulate as G1-arrested cells, we followed chromosome synthesis during the first cell cycle after germination in nutrient media containing BrdU. The number of nuclei doubled between 26 and 51 hours after the induction of germination and after this time the population began to grow exponentially (Fig. 4A). The mtDNA was the first DNA to be synthesized at 14-21 hours and substantial chromosomal DNA synthesis was observed just before cell division (Fig. 4B). Significant BrdU incorporation into chromosomal DNA occurred before 26 hours, prior to any cell division. BrdU incorporation was also observed after 21 hours on longer exposures of the film. Quantification of the 30 hour sample indicated that 52% of the chromosomal DNA was synthesized by the time 10% of the cells had divided, suggesting that S phase was preceding mitosis (Fig. 4C).

If spores are in G1, the expected chromosomal BrdU incorporation per cell after one population doubling should be 150% that of the incorporation observed in cycling G2 cells. The chromosomal DNA synthesis in the germinating spores that had divided once was 178% that of exponentially growing cells (Fig. 4C). This level of chromosomal DNA synthesis and its timing relative to the first cell division indicates that amoebae emerging from germinating spores undergo S phase prior to cell division. These results support the notion that spores have a G1 DNA content.

FISH analysis of chromosome copy number in amoebae and spores

To verify that spores encapsulate as G1 cells by an independent method, we directly compared the chromosome copy number of amoebae and spores by fluorescence in situ hybridization (FISH). A single copy locus can be reliably detected in *Dictyostelium* by FISH if the probe is complimentary to greater than 10 kb of the target locus (G.C., unpublished). For this test, we used a probe corresponding to the 20 kb *tagB/C/D* locus on chromosome 4. We examined vegetative cells and 6 hour developing cells that are expected to be in the G2 phase of the cell cycle. We also inferred the chromosome copy number in spores by carrying out FISH on amoebae that had just emerged from germinating spores. By allowing spores to germinate in growth media for 12 hours, we obtained robust FISH signals and we could examine their nuclei well before any chromosomal DNA replication had occurred. Most vegetative cells and 6 hour developing cells had two *tagB/C/D* loci (Fig. 5A and data not shown). We also examined several independent spore samples and found that more than 90% of the emergent amoebae displayed a single *tagB/C/D* locus (Fig. 5B). Although detection of chromosome copy number by FISH is complicated by variable signal intensity and the possibility of overlapping signals, these results appear to be in general agreement with the experiments described above. The FISH

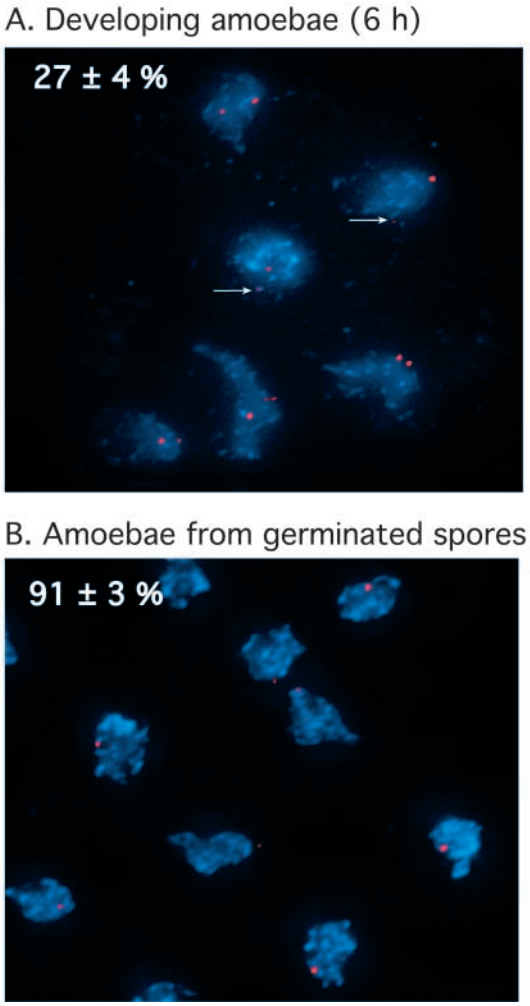

Fig. 5. Visualization of chromosome content by FISH. Fluorescence in situ hybridization was carried out with a single copy probe corresponding to the *tagB/tagC/tagD* locus on chromosome 4. The representative images are two-dimensional projections of the three-dimensional images used to score the presence of the locus. The percentage of nuclei with a single observable locus is shown. (A) Amoebae that had been developing for six hours on filters showing 4 of 6 nuclei that clearly contain two loci and two nuclei that were counted as having a single locus. Arrows point to signals within these two nuclei considered too weak to be scored as a locus. (B) Amoebae that had emerged from spores that were germinating for 12 hours showing eight nuclei that contain a single *tag/C/D* locus.

indicates that vegetative cells are mainly in G2, as expected, while spores are in G1.

Prespore cells divide prior to terminal differentiation

Spores have about one-half the DNA of 6 hour cells but similar ratios of cellular DNAs (Fig. 1C, Fig. 2C). Thus, prespore cells must undergo mitosis and arrest in G1 at some time during development. However, dissociated stalk cells also have roughly the same DNA content as spores and so they are also likely in G1 (Fig. 1C). Therefore it was important to determine the timing of mitosis in these two cells types.

We first compared the DNA content of prestalk and prespore cells in migrating slugs. We dissected slugs marked with a prestalk *ecmA/GFP* reporter into two cell populations: slug anterior tips that contain mostly prestalk cells and slug posterior ends that contain mostly prespore cells (Fig. 6A). The posterior cells gave a uniform DNA profile and lacked significant numbers of GFP-positive cells (Fig. 6B,C). The anterior cells had a DNA profile suggestive of a bimodal distribution of DNA content among the population. Although the fixation procedure reduced the GFP fluorescence about 100-fold, the remaining 10-fold difference in green fluorescence between the remaining GFP-positive cells and the GFP-negative cells allowed their separation. The flow cytometry window used to segregate GFP-expressing cells was shown to identify populations that contained >95% GFP-positive cells by visual confirmation of fluorescence in the sorted cell populations (Fig. 6C and data not shown). The GFP-positive prestalk cells had significantly higher DNA content than the GFP-negative prespore cells, suggesting that prespore cells undergo mitosis before the slug stage and that prestalk cells divide after the slug stage (Fig. 6D).

To explore the timing of prespore cell division, we sorted live cells by their GFP content and then fixed and stained them to measure their DNA content. A *cotB/GFP* reporter was used to mark prespore cells and an *ecmA/GFP* reporter was used to mark prestalk cells in separate experiments. For each marked population, a significant number of cells expressed GFP by 11 hours of development and the maximum number of expressing cells was observed by 16 hours (Fig. 7A,B). The DNA content of prespore cells decreased by 42% between 11 and 19 hours of development, the same time interval in which half of all cells undergo mitosis (compare Fig. 1A with Fig. 7C). The DNA content of prestalk cells decreased much less over the same time interval and was about 40% higher than the prespore cell DNA content at the later times, consistent with the observations from migrating slugs (Fig. 7C). These results strongly imply that prespore cells undergo mitosis and arrest their cell cycle in G1 prior to the initiation of fruiting body morphogenesis. Prestalk cells probably undergo mitosis closer to the time of their terminal differentiation and integration within the stalk. This could explain why the DNA content of mature stalk cells is similar to that of mature spores, while prestalk cells have a higher DNA content than prespore cells or spores.

Discussion

The data presented here support a new model for cell cycle regulation during *Dictyostelium* development. We propose that all cells pause in G2 at the onset of development and then undergo and one additional cell division with tissue-specific timing. Prespore cells divide between 12-18 hours of development, while prestalk cells appear to divide after this

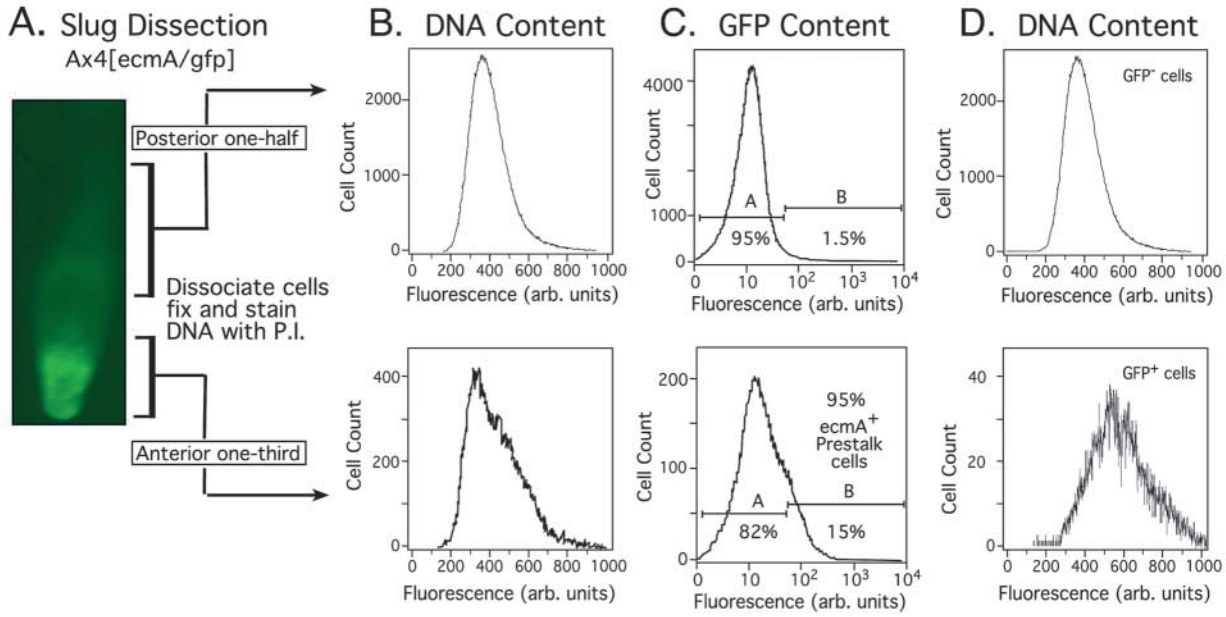

Fig. 6. DNA content in prespore and prestalk cells of migrating slugs. (A) Wild-type cells (AX4) marked with a prestalk reporter gene (*ecmA/gfp*) were developed on agar to the slug stage and dissected into two fractions enriched in anterior or posterior cells. (B) Cells were dissociated, fixed and stained with propidium iodide to obtain population profiles of their DNA content. (C) The cells were divided into GFP-negative (window A) and GFP-positive (window B) populations that were confirmed by direct visual inspection of sorted cells. The percentage of the total cell population in each window is shown. (D) The DNA content profiles of the GFP-negative cells in window A of the slug posteriors (upper panel) and the GFP-positive cells in window B of the anterior cells (lower panel).

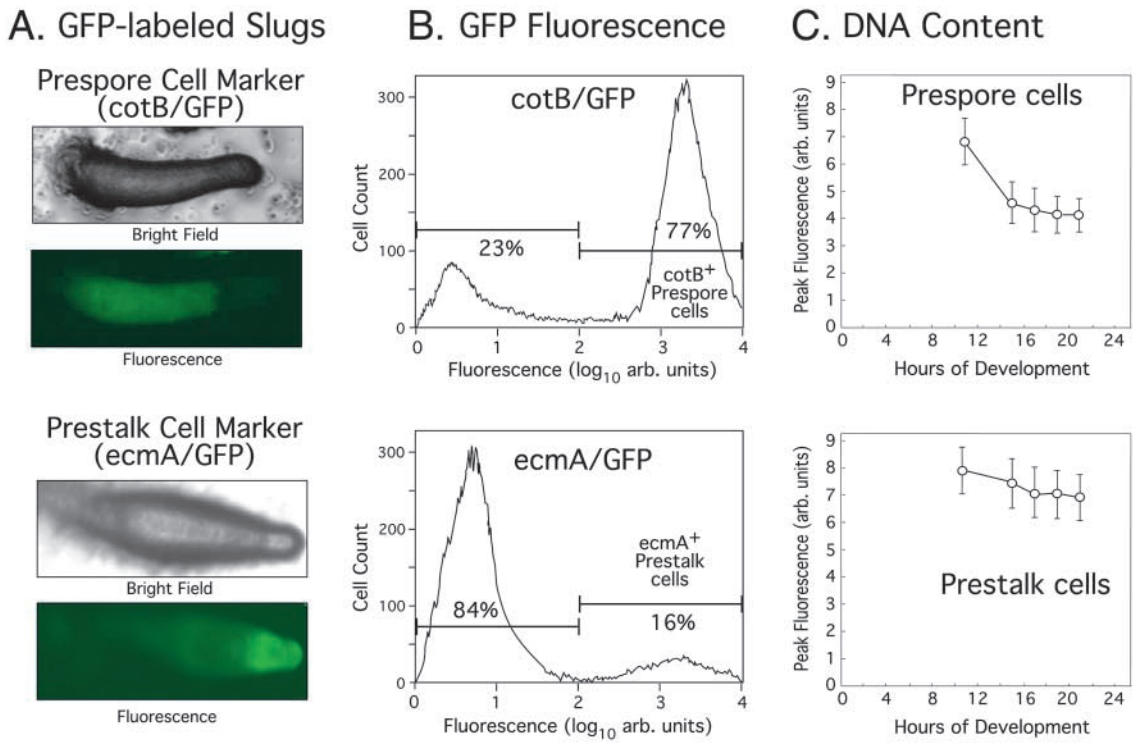

Fig. 7. Prespore and prestalk cell DNA content during development. Wild-type cells (AX4) marked with a prespore reporter gene (*cotB*/*GFP*), or a prestalk reporter gene (*ecmA/GFP*), were allowed to develop on filters (A). The *ecmA/GFP* slug is the same image as shown in Fig. 6. (B) Flow cytometry profiles of cells at 18 hours of development show the distribution of cells expressing GFP. (C) GFP-positive cells were collected by fluorescence activated cell sorting as illustrated in (B), stained with propidium iodide, and their DNA content was determined by flow cytometry. Peak values of the DNA content profiles for mononucleated cells are plotted.

time. After their final mitosis cells of both type arrest their cell cycle in G1, prior to terminal differentiation.

We have observed two stages of cell division during *Dictyostelium* development. In the first stage, between 0 and 6 hours, some cells in the population appear to be completing their final growth-stage cell cycle, as we could attribute most of the early cell division to the cytokinesis of multinucleated cells. Multinucleated cells must result from an imperfect cytokinesis mechanism, a well-known property of cells grown in nutrient broth. In the first 12 hours of development, no significant change in the number of nuclei occurs and cells appear to be arrested in G2 based on their DNA content. Actual mitosis appears to occur between 12 and 18 hours of development because about half of the cells carryout karyokinesis and cytokinesis during this period. These issues have been examined in detail for other axenic strains and our data are consistent with these reports (Sharpe et al., 1984; Zada-Hames and Ashworth, 1978a; Zada-Hames and Ashworth, 1978b). The cell division that we observe for AX4 strains is similar to that reported for AX2 and AX3 (Atryzek, 1976; Zada-Hames and Ashworth, 1978a).

We also observe two time periods where the DNA content of cells decreases during development. The first decrease, during the first 10 hours of development, appears to be due to loss of about half of the mtDNA. The second decrease coincides with the period of mitosis and is almost entirely accounted for by the reduction of DNA content in prespore cells carrying out mitosis. Previous reports suggesting that DNA loss after 10 hours of development is due to loss of mtDNA were based on the differences between whole-cell and nuclear flow cytometry, or by direct measurements (Durston et al., 1984; Leach and Ashworth, 1972). Our experiments suggest that mtDNA constitutes ~30% of the total DNA in vegetative cells and this decreases to ~15% by 10 hours of development, after which it remains relatively constant. These results are consistent with previous reports that mtDNA constitutes 25-42% of vegetative cell DNA (Firtel and Bonner, 1972; Soll et al., 1976; Sussman and Rayner, 1971; Weijer et al., 1984b). Because our data also indicate that only prespore cells divide before 20 hours, the ~45% decrease in DNA content in these cells cannot be accounted for simply by the loss of extrachromosomal DNA relative to chromosomal DNA because that would predict that spores are devoid of extra-chromosomal DNA. Furthermore, unchanging proportions of the major DNAs between 12 and 18 hours of development supports the idea that there is a proportionate reduction of all the cellular DNAs within prespore cells. The FISH analyses with a single-copy probe also supports the notion that spores have one copy of the chromosomes. These results are consistent with a previous report indicating that spores have 40% less DNA than 10-hour developing cells and supports our assertion that spores encapsulate as G1-arrested cells (Sharpe et al., 1984).

Earlier models suggesting that cells undergo terminal differentiation in the G2 phase are based on flow cytometry that indicated that the nuclei from growing cells have the same DNA content as the nuclei from developing cells (Durston et al., 1984). In principle, nuclear flow cytometry is the best

means of measuring chromosome content, but technical issues confound this approach in *Dictyostelium*. We carried out flow cytometry analyses on nuclei with several different protocols and we could reproduce published profiles, namely unimodal peaks that do not change during development (G.C., unpublished). However, it has never been demonstrated that G1 nuclei are as stable to purification as G2 nuclei and there is no way of telling if there is differential recovery of the two types of nuclei using the various protocols. This theoretical concern was made tenable by our finding that nuclear fragility varies at different times in development. We believe this fragility, combined with a large amount of debris in the preparations, caused erratic and aberrant DNA content profiles. Excessive debris in nuclear preparations has been reported previously to obscure the detection of G1 nuclei in flow cytometry profiles (Durston et al., 1984). Thus, the sample heterogeneity of nuclear preparations suggests that flow cytometry of nuclei is subject to artifact and is therefore unreliable.

Our estimates of chromosomal DNA synthesis further support the notion of post-mitotic G1-arrest. These BrdU incorporation studies could be misleading only if BrdU becomes inaccessible for chromosomal DNA synthesis during development. The robust mtDNA synthesis observed in prespore cells suggests that cells do have access to sufficient BrdU during development, as noted previously (Shaulsky and Loomis, 1995). However, we cannot exclude the possibility that mtDNA synthesis uses a different nucleotide pool and that BrdU is somehow excluded from the nuclear pool. We consider this possibility highly unlikely as BrdU can clearly be detected in developing nuclei by immunochemistry. Nuclear DNA synthesis has been observed in developing BrdU-labeled cells and in cells labeled with [^{3}H]-thymidine (Durston and Vork, 1978; McDonald and Durston, 1984; Zada-Hames and Ashworth, 1978a; Zimmerman and Weijer, 1993). These studies have been interpreted as evidence of an S phase late in development, but these techniques do not allow quantification of chromosome synthesis. It is likely that the observed labeling is due to DNA repair. It is also possible that mtDNA synthesis in perinuclear mitochondria has been mistaken for nuclear DNA synthesis, as pointed out previously (Durston et al., 1984; Shaulsky and Loomis, 1995).

Our measurements of DNA synthesis suggest that 5% of the cells replicate all of their chromosomal DNA, all cells repair 5% of their chromosomal DNA, or a combination of these two extremes. Chromosomal replication may occur in cells that become neither spore or stalk cells. Our ‘non-stalk cell’ sample consists of 95% spores and 5% non-spore cells. Six percent of the chromosomal DNA is synthesized in that sample, whereas only 2% of comes from spore chromosome synthesis. So it is possible that the non-spore cells complete an S phase, as they would have to account for roughly 4% of the BrdU incorporation into the chromosomes of that sample. These cells could be the cells that remain scattered outside of aggregates that do not participate in development, cells that form minority cell types, or cells that are in S phase at the start of development. In support of the latter, we detect much less BrdU incorporation into chromosomal DNA when we begin labeling cells after 4 hours of development (A.K. and G.S., unpublished). We can also estimate that prespore cells synthesize about one-third of the mtDNA synthesized during one vegetative cell cycle. The prespore expression of the small subunit ribonucleotide reductase gene, *rnrB*, is consistent with an increased nucleotide requirement to support mtDNA synthesis (Tsang et al., 1996).

It has been shown that cell cycle position at the time of starvation biases cell fate determination in *Dictyostelium*. Several studies have suggested that cells starved late in G2 phase become prespore cells, and cells starved early in G2, M or S phase become prestalk cells (McDonald and Durston, 1984; Weijer et al., 1984a; Ohmori and Maeda, 1987; Gomer and Firtel, 1987; Maeda et al., 1989; Zimmerman and Weijer, 1993; Wood et al., 1996). Cells that exit the cell cycle at different times in could be biased towards a particular cell fate by known cell-cycle regulated signals such as Ca^{2+}, or cytosolic pH (Clay et al., 1995; Gross et al., 1983; Leach et al., 1973; Maeda and Maeda, 1974; Saran et al., 1994; Thompson and Kay, 2000; MacWilliams et al., 2001). It is tempting to speculate that some condition imposed by the initial arrest impinges on the regulation of tissue specific mitosis later in development. For example, it might be that transit through G2 is merely slowed by the starvation conditions of early development and that prespore cells, which arise from cells starved later in G2, are able to achieve the competence to divide much earlier than prestalk cells. The slow increase in cdc2/cyclin B kinase activity during aggregation, followed by a decline after aggregation supports this idea (Luo et al., 1995). Distinguishing between this and other models will require additional molecular tools such as temperature-sensitive mutations in known cell cycle regulators.

The elucidation of the regulation of developmental mitosis will probably illuminate our understanding of cell type specification and maintenance. It will be important to determine whether the late mitotic events and G1-phase arrest are required for spore cell differentiation or stalk formation. Certainly, prespore-specific genes and genes that become prespore enriched are expressed in some cells well before mitosis begins so it is unlikely that G1 arrest is required for all aspects of prespore differentiation (Fosnaugh and Loomis, 1993; Good et al., 2003; Haberstroh and Firtel, 1990; Iranfar et al., 2001; VanDriessche et al., 2002; Williams et al., 1989). Microtubule destabilizing drugs that block mitosis do not block development suggesting that prespore cells need not divide prior to spore differentiation (Cappuccinelli and Ashworth, 1976; Cappuccinelli et al., 1979). However, some mutants that are blocked in development prior to the multicellular phase do not undergo mitosis (Chen, 2003; Zada-Hames and Ashworth, 1978a). In addition, dominant-negative cyclin B mutants that block the cell cycle in mitosis also block spore production (Q. Luo, PhD Thesis, The University of British Columbia, 1996) (Luo et al., 1994). However, these mutants also block the production of prespore cells, as has been pointed out for the cyclin B mutant (Weeks and Weijer, 1994). These genetic tests provide some evidence that mitosis is under control of the developmental program, but they leave unanswered the question of whether mitosis is strictly required for terminal cell differentiation.

If G1 arrest is not required for spore differentiation, how does *Dictyostelium* benefit by having prespore cells divide in the post-aggregative stage of development? For *Dictyostelium*, long-term survival is determined by the stochastic process of the spores of a fruiting body arriving at new, nutrient-rich environments (Bonner, 1982). In this regard, the selective

advantage of doubling the number of spore producing cells within each multicellular organism is likely to be greater than the potential disadvantage of spores encapsulating with only one copy of the genome.

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