Absolute requirement of spermidine for growth and cell cycle progression of fission yeast (*Schizosaccharomyces pombe*)

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Schizosaccharomyces pombe **cells that cannot synthesize spermidine or spermine because of a deletion–insertion in the gene coding for *S*-adenosylmethionine decarboxylase (Δ*spe2*) have an absolute requirement for spermidine for growth. Flow cytometry studies show that in the absence of spermidine an overall delay of the cell cycle progression occurs with some accumulation of cells in the G_1 phase; as little as 10^{-6} M spermidine is sufficient to maintain normal cell cycle distribution and normal growth. Morphologically some of the spermidine-deprived cells become spherical at an early stage with little evidence of cell division. On further incubation in the spermidine-deprived medium, growth occurs in most of the cells, not by cell division but rather by cell elongation, with an abnormal distribution of the actin cytoskeleton, DNA (4′, 6-diamidino-2-phenylindole staining), and calcofluor-staining moieties. More prolonged incubation in the spermidine-deficient medium leads to profound morphological changes including nuclear degeneration.**

The cellular polyamines are ubiquitous in nature and are absolutely required for eukaryotic cell growth (1, 2). Although many studies have shown a variety of effects of polyamines, most of these have been conducted in *in vitro* systems, and relatively few specific molecular functions of polyamines have been demonstrated *in vivo*. [For a review of these studies, see the exhaustive summary by S. S. Cohen (3).]

In our previous studies with *Saccharomyces cerevisiae*, we showed that Δ*spe2* mutant cells, which cannot synthesize spermidine or spermine because of the absence of *S*-adenosylmethionine decarboxylase, stopped growing after depletion of their internal polyamines (4). After prolonged incubation in polyamine-deficient medium, numerous morphological abnormalities including cellular enlargement, increase in the size and number of vesicle-like bodies in the cytoplasm, delocalized chitin and actin distribution were noted (4). Studies on the polyamine-depleted Δ*spe2* cells implicated spermidine in some aspects of protein biosynthesis. Thus, polyamine-deficient cells showed increased +1-ribosomal frame-shifting (5) and sensitivity to paromomycin (6). Spermidine has also been shown to be a precursor of the hypusine moiety of translation elongation factor eIF5a (7, 8).

Recently, Chattopadhyay *et al.* (9) described a comparable Δ*spe2* mutant of *Schizosaccharomyces pombe*, which also required spermidine for growth. *S. pombe* is evolutionarily distant from *S. cerevisiae*; given its regular rod-shaped cell and highly polarized cell growth, fission yeast is an attractive system to study cell form, because abnormal patterns of growth are readily detectable from a simple visual screen (10). *S. pombe* cells also have elements reminiscent of animal cell division, such as contractile rings and cell division by binary fission. In our current studies we have used the Δ*spe2* mutant of *S. pombe* to study the changes in cell cycle progression that occur as a result of polyamine deprivation. *S. pombe* was also somewhat more suitable for these studies because the cells developed signs of polyamine deprivation after transfer to polyamine-deficient media much more rapidly than we had observed with the Δ*spe2* mutant *of S. cerevisiae* (4).

In this article, using flow cytometry [fluorescence-activated cell sorter (FACS)] techniques, we show that, even in relatively early stages (24 h) of spermidine depletion, the cell cycle slows with some accumulation of cells in the G_1 phase. At this stage some of the spermidine-deprived cells are spherical, indicative of an inability to polarize growth; these cells also divide slowly. After longer incubation (48 h or more) in polyamine-deficient medium, cells showed more accumulation in the G_1 phase of the cell cycle with numerous morphological changes including lack of cell division, disruption of the actin network, diffuse calcofluor staining, absence of cell septa, and eventually disintegration of nuclear structure as observed by 4′,6-diamidino-2-phenylindole (DAPI) staining. As little as 10^{-6} M spermidine in the medium is enough to maintain normal cellular network, growth, and cell cycle progression.

Materials and Methods

Yeast Strains, Culture, and Growth. The Δ*spe*2 strain of *S. pombe* used in these studies was described by Chattopadhyay *et al.* (9) and was a derivative of *S. pombe* strain JY 745 (wild-type) (h^- *ura*4-D18 *leu*1–32 *ade*6-M 210). Polyamine-free minimal medium was prepared from BIO 101 media components (EMM, Catalog no. 4110–022) with supplements of amino acids when required. This medium was filter-sterilized. For the growth experiment described in Fig. 1, *S. pombe* cells were inoculated from YES plates (0.5% yeast extract, 3% dextrose, and amino acid supplements) to EMM medium containing 10^{-6} M spermidine and grown for 48 h. The cells were then diluted to 0.04 OD_{600} in the presence or absence of the indicated concentration of spermidine and incubated in air with shaking at 30°C. When necessary, the cultures were diluted into media supplemented with and without the same concentration of spermidine to keep the OD_{600} less than 1.0.

Flow Cytometry Analysis. Cells (5×10^6) were collected, washed in Na-citrate buffer, and fixed in 70% ethanol before staining with Sybr Green I (1:1,000 dilution of stock solution, Molecular Probes) as described (11). DNA content was measured by a Coulter Epics FACS (XL-MCL, Hialeah, FL). Control experiments were performed with nitrogen-starved cells to identify the peak corresponding to 1C DNA (where C = one chromosome complement) content, with exponentially growing wild-type cells to identify the 2C DNA peak, and with cells grown under glucose-starved conditions (mostly 2C). A brief sonication was performed to remove clumping. Analyses of DNA histograms to determine the distribution of cells in each cell cycle phase were

Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; FACS, fluorescence-activated cell sorter.

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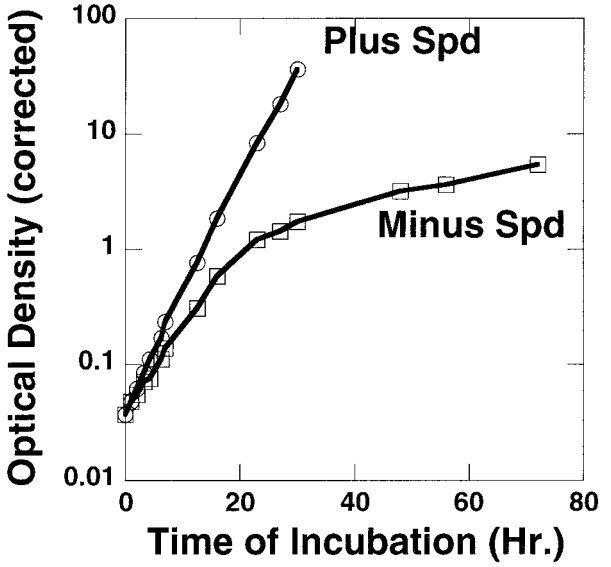

Fig. 1. Growth curve of Δ*spe*2 cells grown in the presence or absence of 10^{-6} M spermidine (Spd). The cultures were grown in 10^{-6} M spermidine and diluted into media containing no spermidine or 10^{-6} M spermidine, as described in *Materials and Methods*. The cultures were diluted into media with and without spermidine when necessary to keep the OD_{600} 0.6–1.0. The optical densities presented have been corrected for these dilutions.

performed by using the MODFITLT software program (Verity Software, Topsham, ME).

Microscopic Studies. Cell wall and septum were visualized after staining with Calcofluor white M2R (Sigma) as described (12). Cells were fixed with formaldehyde (EM grade methanol-free, Polysciences). Then one-tenth volume of 1 mg/ml Calcofluor and 0.3 mg/ml *p*-phenylenediamine ("antifade") were added. This mixture was incubated for 10 min at room temperature and washed with PBS and resuspended in 50% glycerol containing PBS before microscopic examination.

For rhodamine-phalloidin and DAPI staining, cells were fixed in formaldehyde for 5 min at room temperature. The cells were centrifuged, washed three times in 35 mM K-phosphate, pH 7.0/0.5 mM $MgSO_4$ (PM buffer) and permeabilized by resuspension in 1% Triton-X-100 in PM buffer. The cells were then stained by the addition of 3 units of rhodamine-phalloidin (Molecular Probes) as described (13). After 1-h incubation at room temperature, the cells were centrifuged and resuspended in 30 μl of DAPI solution (1 μg/ml DAPI and 1 mg/ml *p*-phenylenediamine in PM buffer). The cells were observed under the fluorescence microscope. The cells in the same field were also visualized by differential interference contrast (Nomarski) microscopy; all photographs were taken in a Zeiss Axiophot microscope equipped with a digital camera (Cool Snap HQ, Photometrics, Tucson, AZ).

Results

Inhibition of Cell Cycle Progression in Spermidine-Deprived Cells. Studies on the effect of spermidine deprivation *in vivo* are always complicated by the fact that the starting inoculum contains a substantial intracellular spermidine concentration because the Δ*spe*2 cells were necessarily maintained on spermidine-containing media. This spermidine level can be depleted only by prolonged growth in spermidine-free medium. Consequently, in such experiments the effects seen are necessarily gradual, and not easily comparable with the usual studies with conditional *cdc* mutants.

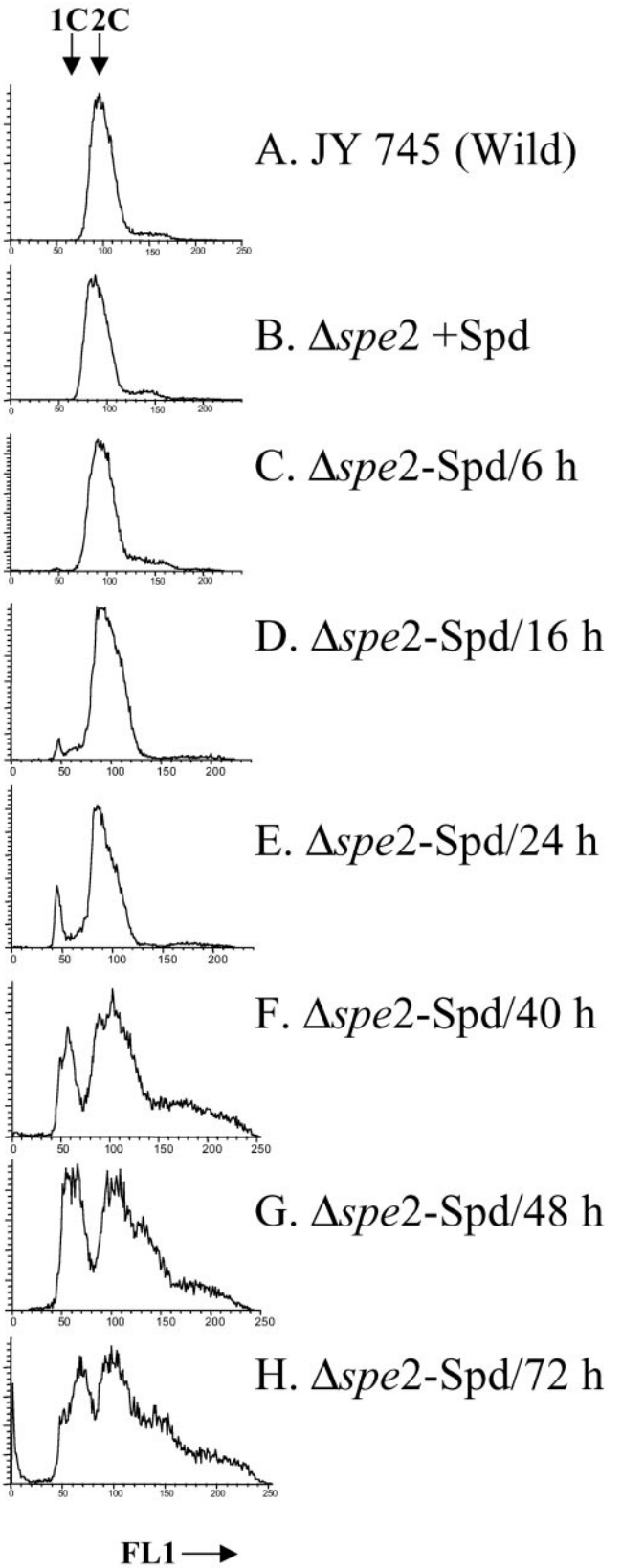

Fig. 2. Histogram showing the FACS profile of spermidine (Spd)-supplemented and spermidine-depleted cells. Samples were obtained after growing the cells for different times in the polyamine-deficient medium (Fig. 1). Cells (5×10^6) were harvested, fixed, and stained with Sybr Green I as detailed in *Materials and Methods*. The samples were then analyzed for DNA content by a Coulter Epics FACS (XL-MCL, Hialeah, FL).

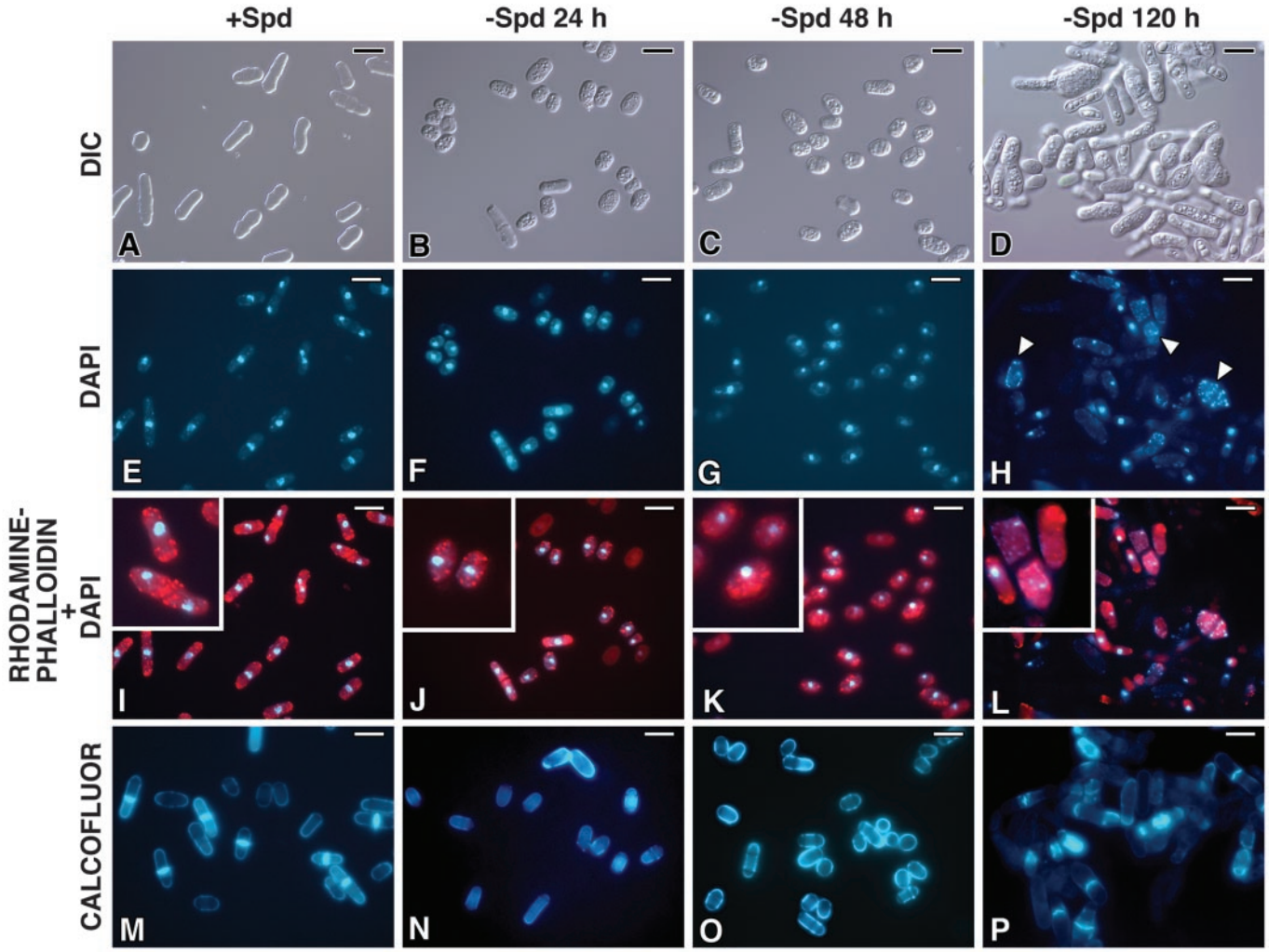

Fig. 3. The morphological features of Δ*spe2* cells during polyamine deprivation. The Δ*spe2* cells were grown as shown in Fig. 1; samples were harvested for various staining procedures and for differential interference-contrast optics (Nomarski) as described in *Materials and Methods*. (*A–D*) Nomarski; (*E–H*) nuclei staining by DAPI; (*I–L*) actin and nuclei staining by rhodamine-phalloidin and DAPI, respectively; (*M–P*) cell wall and septum staining by Calcofluor White M2R. Cells were observed under the fluorescence microscope and photographs were taken in a Zeiss Axiophot microscope equipped with a digital camera (Cool Snap HQ, Photometrics). (Bar = 10 μm.) (*Insets*) Magnified views showing the details of actin dots of the stained cells. Spd, spermidine; DIC, differential interference contrast microscopy.

In the current experiments, after a preliminary growth period in purified (EMM) medium containing 10^{-6} M spermidine for 48 h, the cells were washed and transferred to the same medium with or without 10^{-6} M spermidine (Fig. 1). At 6, 16, 24, 40, 48, and 72 h, aliquots were taken for determination of their FACS profile. The FACS profile for the cells growing in 10^{-6} M spermidine ("0 time") is shown in Fig. 2*B*; essentially all of the cells were in the G_2/M phase with 2C DNA content. This profile was the same as that seen in wild-type cells (Fig. 2*A*); in rapidly growing *S. pombe* cells, S phase begins before cytokinesis is complete, and a very short G_1 phase occurs (14). After 6 h in the deprived medium, little or no change occurred in the growth rate (Fig. 1) or in the FACS profile (Fig. 2*C*) compared with that seen with the spermidine-supplemented cells. After 16-h growth in spermidine-deprived medium, the growth rate decreased somewhat compared with the spermidine-supplemented cell, but little change in the FACS profile was observed (Fig. 2*D*). After an additional 8 h in the spermidine-deprived medium the growth rate had decreased markedly, and a definite slowing of the cell cycle occurred with a significant percentage of cells in the G_1 phase (about 18%) (Fig. 2*E*). After 40–48 h incubation in the deprived medium, the growth rate was much lower; the FACS profile is shown in Fig. 2*F*. Analysis of the 48-h DNA histogram by MODFITLT software indicated that 29% of the cells were in G_1 phase, 42% were in S phase, and 29% were in G_2/M phase. These percentages have to be considered approximate, however, because some residual clumping or possible increase in mitochondrial DNA synthesis might have affected them. Microscopic examination of these cells indicated no evidence of cell division (see below). At this time the cells were still viable, as shown by lack of staining with methylene blue (results not shown).

FACS analysis of 72-h spermidine-deprived cells (Fig. 2*H*) showed another peak at sub-G_0/G_1 phase of the cell cycle, a peak that is often observed with necrotic mammalian cells (15).

Morphological Changes During Spermidine Deprivation. After incubation of the cells in polyamine-free medium for 24 h (Fig. 1), growth was markedly decreased. As expected, microscopic examination showed very few dividing cells (Fig. 3 *B*, *F*, and *J* vs. *A*, *E*, and *I*). These cells became spherical without polarized growth, compared with the spermidine-supplemented cells where the cells were elongated and growth occurred by apical

extension (Fig. 3 *A* and *I*). The spermidine-supplemented (10^{-6} M) cells showed normal septum development, with the formation of a highly fluorescent calcofluor-stained ring located at the cell equator (Fig. 3*M*). In contrast, the deprived cells (24 and 48 h) showed no evidence of septum formation after calcofluor staining (Fig. 3 *N* and *O*).

After more prolonged incubation (120 h) in the polyamine-deficient medium, the morphological changes became more pronounced. The cells became longer, irregular in shape, and contained large vesicle-like secretory bodies that were highly refractile (Fig. 3*D*). At this stage the calcofluor-stained material was abnormally distributed compared with the spermidine-supplemented cells (Fig. 3 *P* vs. *M*). We also followed the changes in actin localization during the deprivation period by staining with rhodamine-phalloidin and DAPI; these changes were of interest because the phalloidin selectively binds to F-actin polymers (not to monomers) and rhodamine-conjugated phalloidin allows visualization of polymerized actin within the cells and DAPI binds to DNA (16). After 24–48 h of spermidine deprivation, the actin dots were distributed in an apparently random fashion in some cells as opposed to spermidine-supplemented cells where the actin was localized either at the ends of the growing cells or at the equator, as observed in rapidly dividing cells (Fig. 3 *J* and *K* vs. *I* and *Insets*). The appearance of cells possessing delocalized actin-staining patterns paralleled the appearance of spherical cells in the population. After further spermidine deprivation (120 h), the fluorescence after rhodamine-phalloidin staining was seen in patches mostly near one side of the cell (Fig. 3*L*).

Fig. 3 *E–H* shows the changes in DAPI staining during deprivation. At 24 h, the DAPI staining revealed mostly nondividing cells (Fig. 3 *F* vs. *E*). These cells were uninucleate; some cells showed abnormal nuclear position located off center, as opposed to the spermidine-supplemented cells, where nuclear materials are equatorially placed or mitotic. After 48 h (Fig. 3*G*), more cells showed absence of cell division. When these cells were deprived further (120 h), the nuclear DNA was absent with the appearance of degenerate nuclear structure in some cells (Fig. 3*H*). These cells also showed intense dots, which presumably represent staining of mitochondrial DNA distributed near the periphery, because DAPI binds to AT-rich mitochondrial DNA with high affinity. In contrast, with spermidine-supplemented cells, all nuclei appeared as single round spots or dividing nuclear materials. Mitochondria in most of the actively dividing cells were located near the periphery as dots of far less intensity (Fig. 3*E*).

Effect of Incubation in Different Concentrations of Spermidine on the DNA Content and Morphological Changes. In these experiments we grew the Δ*spe2* cells in different concentrations of spermidine until the growth rate was constant, and then compared the growth rate with the FACS analysis and morphological changes of DAPI-stained cells (Fig. 4). The growth, morphology, and FACS profile of the Δ*spe 2* cells were normal (i.e., like wild-type cells) when grown in the presence of 10^{-6} M spermidine. Even in 10^{-7} M spermidine, the FACS profile of the cells did not change enough, even though they were already growing more slowly than the wild-type cells or Δ*spe 2* cells grown in the presence of 10^{-6} M spermidine. Marked changes in all of the parameters were noted in Δ*spe2* cells growing in 10^{-8} or 10^{-9} M spermidine. FACS profile showed accumulation of cells in the G_1 phase with a corresponding decrease in G_2/M phase cells in presence of 10^{-8} M spermidine. The FACS profile of cells continuously cultured in 10^{-9} M spermidine showed the same characteristics as described above for 72-h spermidine-deprived cells (Fig. 2*H*), namely accumulation of cells in the G_1 phase, and DAPI staining showed absence of cell division and abnormalities in nuclear staining.

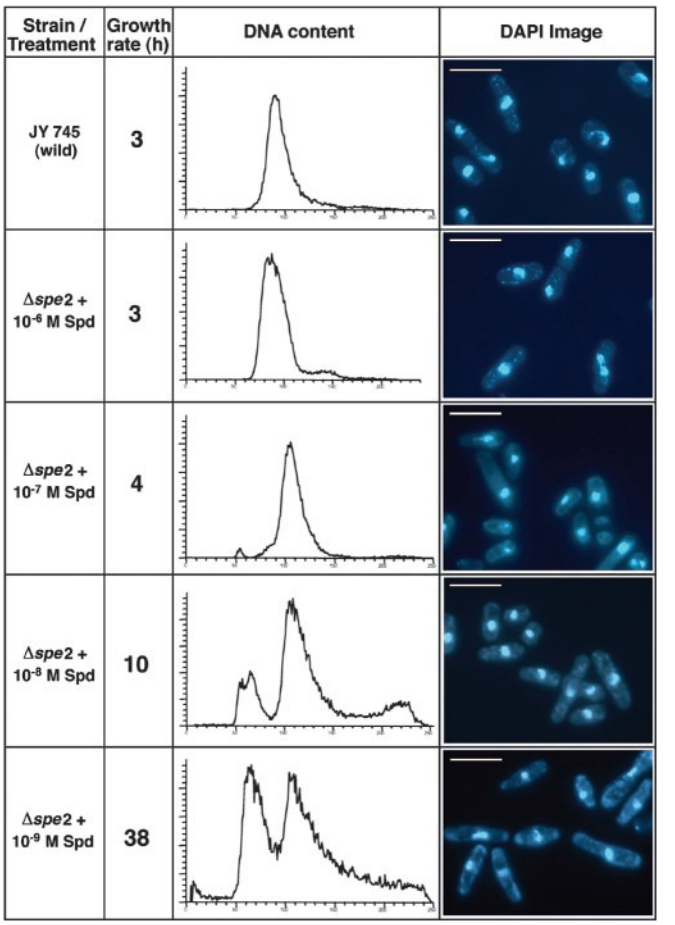

Fig. 4. Effect of different concentrations of added spermidine (Spd) on growth rate, FACS profile and DAPI staining. The Δ*spe2* cells were grown for 48 h in 10^{-6} M spermidine and diluted in different concentrations of spermidine in EMM medium. The growth rate, FACS analysis, and nuclear staining were performed when they were growing for 2–3 days in defined concentrations of spermidine. FACS analysis and DAPI staining were performed by using 70% ethanol-fixed cells. (Bar = 10 μm.)

Discussion

A Δ*spe2* mutant of *S. pombe* cannot synthesize spermidine because it lacks *S*-adenosylmethionine decarboxylase; decarboxylated adenosylmethionine is required for the conversion of putrescine to spermidine. Thus, after complete deprivation of polyamines, these Δ*spe2* mutant cells do not grow in the absence of added spermidine, even though they accumulate large amounts of intracellular putrescine. Completely normal growth is obtained upon addition of spermidine to the culture medium.

In our current studies, we were particularly interested in finding how early in the deprivation period changes in the morphology occurred and whether a specific block existed in the cell cycle phase. The cell cycle distribution at different stages of spermidine deprivation was studied by using flow cytometry after staining the cells with a fluorescent dye (Sybr Green I) that binds stoichiometrically to DNA. In the presence of as little as 10^{-6} M spermidine the cells showed normal growth and a cell cycle distribution identical with that found in wild-type cells; i.e., most of the cells were in the G_2/M phase with 2C DNA content (Figs. 1 and 2 *A* and *B*). When these cells were washed and incubated in spermidine-free medium for 6–16 h, little change occurred in the cell cycle distribution. However, after 24–48 h growth in the absence of spermidine, a definite change in the FACS profile occurred with the accumulation of cells in the G_1

phase (Fig. 2 *E–G*). As mentioned above, even though FACS analysis showed a significant percentage of cells in the G_2/M phase after 48 h, microscopic examination showed that most of the cells had already stopped cell division and showed other abnormalities in cytoskeleton.

These studies are consistent with several studies in mammalian cells in which changes in the cell cycle progression were noted after treatment of the cells with inhibitors of either ornithine decarboxylase or of *S*-adenosylmethionine decarboxylase (17, 18) or by overexpression of antizyme or antisense inhibition of ornithine decarboxylase (19).

In this article, we also report profound changes in the microscopic appearance of the *S. pombe* cells during spermidine deprivation. At an early stage of deprivation, cells became spherical without polarized growth, and some cells contained off-centered nuclei (Fig. 3 *B*, *F*, and *J*), which suggests that, in addition to their failure to grow in a polarized manner, these cells cannot generate the signal that locates the nucleus centrally. This phenomenon was also observed in *orb*5–19 mutants of fission yeast at a restrictive temperature (10); *orb*5 encodes a fission yeast homologue of casein kinase IIα. In mammalian cells polyamines have been reported to bind directly to an essential subunit of casein kinase II responsible for phosphorylation of topoisomerase II (20).

As shown in Fig. 3, after 48 h of deprivation many cells had become spherical, showed markedly decreased cell division, and lacked directional orientation of actin. Even more marked morphological abnormalities were found after more prolonged spermidine deprivation (120 h) including disruption of the nuclei (Fig. 3*H*). These cells also showed increased dots, presumably of mitochondrial DNA after DAPI staining. Sazer and Sherwood (21) also reported increased mitochondrial DNA replication in the absence of nuclear DNA synthesis in the conditional cell division cycle mutant, *cdc10*.

The diffuse distribution of calcofluor staining (Fig. 3*P*) is similar to that found in a *S. pombe* double mutant of *myo*2-E1-*myp*2 (gene products responsible for cytokinetic actomyosin ring formation), defective in cell wall/septum biosynthesis (22) or in *cdc3*, *4*, *8* and *12* mutants (23). The changes in nuclei and in chromatin after long deprivation are consistent with many reports in mammalian cells showing the importance of polyamines for DNA replication, condensation of chromatin structure, and packaging (24–26). Although it is tempting to compare our results with those reported with various *cdc* mutants (27, 28), such a comparison is not simple in view of the long time involved in the development of the observed phenotype in our experiments. In general, however, our results seem different from the results observed with the usual *cdc* mutants, because the latter usually show arrest at a specific point in the cell cycle and block progression through the cell cycle without immediately affecting macromolecular syntheses. In contrast, our observations seem more similar to the mutations in biosynthetic genes, which can block progression through the cell cycle as an indirect consequence of blocking growth and macromolecular syntheses (14). Similarly, the spermidine-deprived cells not only show slowing of the progression of the cell cycle and accumulation of cells in the G_1 phase, but also show a slower overall growth rate and other abnormalities in cell division and cytoskeletal network.

The many studies on polyamines in the literature indicate that they probably have multiple targets *in vivo*. In the present experiments the accumulation of cells in the G_1 phase, the lack of cell division, the abnormal distribution of calcofluor staining, and the changes in cellular actin network indicate that spermidine is important for fission yeast cells for normal cell cycle progression, cell division, and polarized growth. Although the long time that is involved in these experiments because of the need to deprive the cells of their intracellular spermidine makes it very difficult to evaluate which of the effects on DNA replication, protein synthesis, cell cycle progression, or cell division are primary and which are secondary, the Δ*spe2* deletion mutant of fission yeast will be a useful tool as a model system to address this question further.

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