A new model for disruption of the ornithine decarboxylase gene, *SPE1*, in *Saccharomyces cerevisiae* exhibits growth arrest and genetic instability at the *MAT* locus

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Ornithine decarboxylase (ODC) is a rate-determining enzyme of the polyamine-biosynthetic pathway. We sought to produce cells with impaired ODC function in order to study the biological functions of polyamines. *Saccharomyces cerevisiae* strains were obtained by one-step gene replacement of a 900 bp fragment of the yeast ODC gene (*SPE1*) with the yeast *URA3* gene. Spores derived from *SPE1/spe1* cells germinated at reduced efficiency relative to *SPE1/SPE1*. Sustained growth of *spe1* haploid mutants in polyamine-free medium led to intracellular polyamine

depletion, reduction in budding index, G1 arrest and cessation of growth, and cells that were large and misshapen. All of these effects were completely reversed by adding polyamines to the medium, even after 5 days of polyamine starvation. A diploid yeast strain bearing two copies of disrupted *spe1* lost heterozygosity at the mating-type locus more often when grown in the absence of polyamines than when grown in their presence, indicating that polyamine deficiency leads to either chromosome loss or to mitotic recombination.

INTRODUCTION

The naturally occurring polyamines putrescine, spermidine and spermine are low-molecular-mass aliphatic amines that are positively charged and ubiquitous in eukaryotic cells [1–4]. Although polyamines have prominent regulatory roles in growth and differentiation [1–6], precise molecular functions are not well established [7]. The polyamine biosynthetic pathway begins with two parallel reactions: (1) ornithine decarboxylase (ODC) produces putrescine from ornithine, and (2) *S*-adenosylmethionine is decarboxylated by *S*-adenosylmethionine decarboxylase (AdoMetDC). These enzymes are vital for cell proliferation [8], and overexpression of ODC yields a partially malignant phenotype [9]. Polyamines induce at least two major changes in DNA conformation *in vitro*: (1) B-to-Z transition and (2) sequence-specific bending followed by DNA condensation and aggregation [10]. We hypothesize that similar structural alterations take place *in vivo*, and that some biological functions of polyamines relate to their ability to alter DNA conformation.

Studies with inhibitors of polyamine biosynthetic enzymes [11] and mutant polyamine auxotrophs [12–14] have shown that cell growth, DNA and protein synthesis, and chromosome stability, all depend on polyamine levels. When α -difluoromethylornithine is used to inhibit ODC, cell growth and proliferation are suppressed [11]. When inhibitors of polyamine biosynthesis are present and polyamine levels are very low, the rates of protein and nucleic acid synthesis are diminished [14,15], fidelity of trans-

lation is impaired [16] and chromosome disintegration may occur [17–21]. Pharmacologic polyamine depletion can also alter the expression of specific genes and affect the sensitivity of cell nuclei to digestion by bovine pancreatic DNase I and micrococcal nuclease [22,23].

Pharmacological studies of polyamine depletion have certain limitations. Inhibitors of polyamine biosynthesis do not completely deplete intracellular polyamines, even though they can inhibit cell growth [11]. Polyamine analogues can deplete natural polyamines, but their abilities to substitute for polyamine function differ. Some analogs allow normal growth after natural polyamines have been depleted, while others inhibit cell growth and produce cytotoxicity [24,25]. This behaviour makes studies of polyamine function that make use of polyamine analogues difficult to interpret. Do growth-inhibitory polyamine analogues act at a polyamine functional site or at a completely different site? Do cytotoxic effects directly relate to growth inhibition? Studies based exclusively on polyamine analogues and/or inhibitors have not provided consistent conclusions concerning molecular functions of polyamines. Therefore, a polyamine-free system free of pharmacological constraints is needed.

Various polyamine auxotrophs of the yeast *Saccharomyces cerevisiae* have been isolated [12,13,26]. These strains are mutated in genes that encode enzymes of polyamine biosynthesis. In *S. cerevisiae*, putrescine is obtained only from ornithine. Strains (*spe1*) mutated in *SPE1* cannot synthesize putrescine, and, consequently, spermidine or spermine. Mutants in polyamine

Abbreviations used: ODC, ornithine decarboxylase; AdoMetDC, *S*-adenosylmethionine decarboxylase; URA, uracil; YEPD, yeast extract/phosphate/dextrose; YNB, yeast nitrogen base; SD, amino-acid-supplemented minimal media; PFM, polyamine-free media; 5FOA, 5-fluoro-orotic acid; PBS-CM, PBS containing Ca^{2+} (0.1 mg/l) and Mg^{2+} (0.1 mg/l).

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biosynthesis [12,13,26] either do not grow or exhibit a marked decrease in doubling time in the absence of exogenous polyamines. Although these and other mutants of genes that code for polyamine-biosynthetic enzymes have been studied, revertants and low-activity mutant protein limit their usefulness.

We describe the construction of a mutant of *S. cerevisiae* obtained by disruption of *SPE1* using the genetic marker *URA3*. This mutant provides a novel model to study the effects of polyamine depletion in eukaryotic cells at the molecular level.

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EXPERIMENTAL

Study design

We engineered strains of yeast disrupted at the genetic locus for ODC (*spe1*). After assuring that the strains were unable to produce polyamines, we found conditions under which cells were unable to grow. We characterized the growth arrest by analysing DNA distribution and then studied the ability of diploids to mate with haploids to assess loss of function at the *MAT* locus.

Culture conditions and strains

S. cerevisiae strains (Table 1) were grown at 30 °C in liquid or solid media. Liquid cultures were grown in either 2% glucose/2% bacto-peptone/1% bacto-yeast extract (YEPD; Difco Laboratories, Detroit, MI, U.S.A.) [26] or in liquid synthetic minimal media [0.67% bacto-yeast nitrogen base (YNB) without amino acids (SD)] with appropriate supplements, or in polyamine-free media (PFM) (2% glucose; Sigma Chemical Co., St. Louis, MO, U.S.A.), 0.67% YNB (Difco) fortified with amino acid solution as described [27]. The PFM was sterilized by filtration or by boiling to prevent biogenic amine contamination from the autoclave [28]. Glassware was washed in conc. HCl to remove adherent amines, rinsed extensively in triple-distilled water to remove the HCl, and dried overnight in a 150 °C oven.

Solid cultures consisted of YEPD or SD solidified with 2% bacto-agar (Difco). The PFM was solidified with 2% agarose (Sigma) that we found to be free of polyamines, because polyamine levels in bacto-agar were significant (results not shown).

Polyamines (putrescine, spermidine and spermine) (Calbiochem, La Jolla, CA, U.S.A.) were added at appropriate times to reverse depletion.

Construction of the *spe1* null mutant

The plasmid *pSPE-d14* was provided by Dr. W. Fonzi, University of California, Irvine, CA, U.S.A. In addition to *SPE1*, *pSPE-d14* contains a 53 bp *Bam*HI--*Eco*RI sequence upstream from *SPE1* (Figure 1). The plasmid was digested with *Hind*III, which excised a 900 bp fragment that included the *SPE1* ATG initiation codon and a large portion of the gene.

An 1170 bp *Hind*III fragment containing the full length of *URA3* with a unique *Eco*RV restriction site was isolated from the pJL123 vector and ligated within the *Hind*III site of the disrupted *SPE1* construct. The resulting construct was digested with the restriction enzyme *Eco*RI, producing a 3.2 kb fragment that was transfected into an AB1 diploid strain (Table 1) by the LiCl method [27]. Transformants bearing the disrupted *spe1::URA* gene were selected on amino-acid-supplemented SD medium in the absence of uracil. In order to discard transformants with extrachromosomal copies of the constructs, we grew transformants for several generations on YEPD and then replica-plated them on plates containing 5-fluoro-orotic acid (5FOA). *URA* cells produce 5-fluorouracil from 5FOA and die. Cells bearing *URA3* extrachromosomally eventually lose the plasmid while growing on YEPD and survive 5FOA treatment, whereas cells containing *URA3* integrated into the genome cannot lose the gene, and therefore die. We selected replicas of non-surviving strains, sporulated them, and submitted them to tetrad analysis. The resulting haploids were tested for *URA* prototrophy; when all four spores were viable, a 2:2 segregation was observed.

Table 1 Listing of genotypes and sources of yeast strains used in the present study

Abbreviations: Hap., haploid; I. H., Ira Herskowitz, University of California, San Francisco; Dip., diploid; P.S., the present study.

Strain	Ploidy	Genotype	Source
YPH 258	Hap.	<i>MATa ura3-52 lys2-801 ade2-101 his3-Δ200 leu2-Δ</i>	I. H.
YPH 263	Hap.	<i>MATα ura3-52 lys2-801 ade2-101 trp-Δ1 leu2-Δ</i>	I. H.
YJL 112	Hap.	<i>MATα lys1</i>	α-Mating tester, I. H.
YJL 113	Hap.	<i>MATa lys1 cry1</i>	α-Mating tester, I. H.
YJL 114	Hap.	<i>MATα sst2-1 met1 hi6 can1 cyh2</i>	α-Halo tester, I. H.
YJL 115	Hap.	<i>MATa sst1 arg9 ilu3 ura1</i>	α-Halo tester, I. H.
AB1	Dip.	<i>MATa ura3-52 lys2-801 ade2-101 his3-Δ200+ leu2-Δ</i> <i>MATa ura3-52 lys2-801 ade2-101 + trp-Δ1 leu2-Δ</i>	P. S.
AB2	Dip.	<i>Δspe1::URA</i> <i>SPE1</i>	Disrupted AB1 strain, P. S.
AB3	Hap.	<i>MATa Δspe1::URA</i>	Sporulation of AB2, P. S.
AB4	Hap.	<i>MATα Δspe1::URA</i>	Sporulation of AB2, P. S.
AB5	Hap.	<i>MATα SPE1</i>	Sporulation of AB2, P. S.
AB6	Hap.	<i>MATa SPE1</i>	Sporulation of AB2, P. S.
AB7	Hap.	<i>MATα SPE1</i>	Sporulation of AB1, P. S.
AB8	Hap.	<i>MATa SPE1</i>	Sporulation of AB1, P. S.
AB9	Hap.	<i>MATa SPE1</i>	Sporulation of AB1, P. S.
AB10	Hap.	<i>MATα SPE1</i>	Sporulation of AB1, P. S.
AB11	Dip.	<i>MATaΔspe1::URA</i> <i>MATα Δspe1::URA</i>	Mating of AB3 and AB4, P. S.

Figure 1 Scheme showing the construction of the *spe1::URA3* knockout

pSpe-d14 was digested by *HindIII*, resulting in the deletion of a 900 bp fragment. An 1170 bp fragment containing *URA3* was ligated into the disrupted *spe1* gene. Digestion with *EcoRI* produced a 3250 bp *spe1::URA*. This construct was used to transform AB1 diploid strains and obtain the AB2 *spe1/SPE1* diploids.

Southern-blot analysis [29]

DNA was extracted from AB3–AB10 haploids, digested with the restriction enzymes *EcoRV* and *EcoRI*, electrophoresed in 0.8 % agarose gels at 35 V for 18 h, and blotted into a positive Zetabind membrane (CUNO, Meriden, CT, U.S.A.). The 900 bp fragment excised from *SPE1* and the 1170 bp fragment from *URA3* were gel-purified, labelled with ^{32}P by random priming [30] and used as hybridization probes to the blot.

Polyamine determination

Cells (1×10^7) were collected, counted in a haemocytometer, washed in neutral phosphate buffer, and sonicated for three periods of 45 s each in 10 ml of 8 % sulphosalicylic acid, which caused complete cell disruption (as judged by optical microscopy). The samples were dansylated and analysed for polyamine content using reverse-phase HPLC [31].

Cell-cycle analysis

Cells (5×10^6) were harvested at different points of the growth curve, rinsed twice in cold PBS containing Ca^{2+} and Mg^{2+} (PBS-CM) at a final concentration of 0.1 mg/ml respectively, sonicated, and fixed by dropwise addition of cold 70 % ethanol. On the day of the experiment they were washed twice in cold PBS-CM, digested at 37 °C for 1 h with 0.1 $\mu\text{g}/\text{ml}$ RNase (Sigma), stained

with 50 $\mu\text{g}/\text{ml}$ propidium iodide (Sigma), diluted in PBS-CM, incubated on ice for 30 min, and sonicated. Fluorescence intensity and forward-angle light scatter were measured in single cells using a Becton Dickinson (San José, CA, U.S.A.) FACS scan and analysed with LYSYS II software (Becton Dickinson).

Microscopic analysis

Approx. 1×10^6 cells were spread on a glass slide and photographed through a phase-contrast Zeiss microscope equipped with a 100 $\times$ oil-immersion lens and a 10 $\times$ objective.

Loss of heterozygosity

Loss of heterozygosity was assessed by measuring quantitative mating [27] of a diploid strain obtained by crossing two haploid strains (AB3 $\times$ AB4) bearing the *spe1::URA* mutation. The strain AB11, which contained two copies of *spe1::URA*, was grown in either PFM or PFM with 1 mM putrescine, 50 μM spermidine and 50 μM spermine. AB11 cells were diluted, re-suspended, and incubated in PFM until a negligible number of budding cells were observed.

AB11 cells (5×10^6) were mixed with an equal number of a-tester cells (strain YJL 112) or α -tester cells (strain YJL 113). The cells were isolated on sterile nitrocellulose filters (0.45 μm pore

size). The filters were incubated on a YEPD plate at 30 °C for 4 h, transferred to a sterile test tube containing 5 ml of SD, vortex-mixed for 30 s and sonicated for 5 s. 0.1 ml aliquots were plated on SD and the numbers of colonies were counted.

RESULTS

Germination efficiency was reduced in *spe1* strains

The diploid strains AB1 and AB2 were sporulated. AB1 was the wild-type diploid (*SPE1/SPE1*) and AB2 contained a single copy of *spe1::URA* (*SPE1/spe1::URA*) (Table 1). In all, 31 tetrads of AB1 and 56 tetrads of AB2 were analysed. We found four viable spores in 58% (18/31) of AB1 tetrads, but in only 14% (8/56) of AB2 tetrads (Figure 2); most tetrads derived from AB2 had only two or three viable spores (Figure 2).

Figure 2 Tetrad analysis of AB1 and AB2 diploids

There were more viable spores from the tetrads of control AB1 (total 31 analysed) than from transformed AB2 (*spe1/SPE1*) (total 56 analysed).

Figure 3 Southern-blot analysis of haploid yeast strains

DNA isolated from AB3–AB6 haploid strains was digested with *EcoRI* and *EcoRV*. A 900 bp *HindIII*–*HindIII* fragment deleted from the *SPE1* gene, and an 1170 bp fragment of the *URA3* gene were used as probes. The 900 bp probe hybridized with a 3.2 kb fragment that is approximately the size of the ODC gene in AB5 and AB6 *SPE1* cells (lanes 1 and 3). This hybridization band was absent in AB3 and AB4 *spe1::URA3* haploids (lanes 2 and 4).

Figure 4 Polyamine levels of *spe1* (AB3 and AB4) and *SPE1* (AB5 and AB6) cells grown in polyamine-free medium

Polyamines were depleted in AB3 and AB4 *spe1* cells, but not in AB5 and AB6 *SPE1* cells. (The lowest detection value is less than 0.01 nmol/10⁵ cells.) (a) 1 day of incubation in PFM; (b) 2 days; (c) 3 days. Results are the means of two independent observations.

Integration of the *SPE1* substitution mutation into the *S. cerevisiae* genome

We confirmed introduction of *spe1::URA* into AB2 by Southern-blot hybridization of DNA extracted from haploid colonies. AB3, AB4, AB5 and AB6 were haploid segregants from AB2 diploids; AB7, AB8, AB9, and AB10 were haploid segregants from AB1 diploids.

DNA extracted from AB3–AB10 haploids was digested with *EcoRI* and *EcoRV* and hybridized simultaneously to a 900 bp fragment excised from *SPE1* and to an 1170 bp probe of *URA3*. Previous experiments showed that the 900 bp probe hybridized to a 3.2 kb fragment of DNA extracted from AB5–AB10 colonies. The 3.2 kb fragment in AB5 and AB6 digests (Figure 3) corresponds to the size of *SPE1* [32]. Similar experiments showed that the two bands at 6 and 7.25 kb correspond to hybridization

Figure 5 Budding index in polyamine-free medium

A representative experiment showing the budding index of haploid yeast strains AB3–AB6 subcultured by 1:100 dilution for five consecutive days in PFM. Each subculture dilution resulted in a stepwise reduction of the budding index in the *spe1* haploid strains (AB3 and AB4), while budding was not affected in the *SPE1* (AB5 and AB6) haploid strains. Similar results were observed in six different experiments.

Figure 6 Growth curves of *spe1* and *SPE1* haploids

(a) The growth rate of the *spe1* haploid strains (AB3 and AB4) was less than that of *SPE1* strains (AB5 and AB6) after 3 days of 1:100 subculture dilution in PFM. AB5 and AB6 *SPE1* colonies were diluted (1:100) after 14 h of incubation to avoid growth rate arrest due to nutrient limitation. Similar results were obtained in four independent experiments. (b) The growth rate of the *spe1* strains is essentially zero after 5 consecutive days of 1:100 subculture dilution in PFM. Dilution (1:100) was performed after 11 h of incubation to avoid nutrient limitation in AB5 and AB6 controls. Similar results were obtained in four independent experiments.

with the *URA3* probe. In AB3 and AB4 digests there was no hybridization at 3.2 kb, indicating that this segment was missing from these haploids. However, two new bands at 2.4 and 0.8 kb

Figure 7 Effect of polyamines on growth curve and budding index of *SPE1* and *spe1* haploids

AB3 (*spe1*) and AB5 (*SPE1*) cells previously grown for 5 days in PFM or in PFM with polyamines (1 mM putrescine, 50 μ M spermidine, 50 μ M spermine) added at day 0. Cell growth rate (a) and budding index (b) were determined in the absence (○, □) or presence (●, ■) of polyamines. The data are representative of nine experiments.

result from the *URA* insertion and digestion with *EcoRI* and *EcoRV* (Figures 1 and 3). We found that uracil prototrophy for AB3–AB10 was invariably linked to an absence of hybridization with the 900 bp *SPE1* fragment and to the presence of the 2.4 and 0.8 *URA3* hybridization bands. PCR analysis confirmed the Southern-blot results (not shown).

Decrease in cellular polyamine levels in *spe1* cells during media changes

Polyamine levels were measured in AB3, AB4, AB5, and AB6 cells cultured in PFM for 1 day (Figure 4a), 2 days (Figure 4b), and 3 days (Figure 4c). After 1 day in PFM, no polyamines were detected in *spe1* mutants (AB3, AB4). The same was true for polyamine levels in cells cultured for 2 and 3 days. In AB5 and AB6 cells (*SPE1*) cultured in PFM for the same time periods, we observed a general trend of increase in levels as a function of time in culture, particularly for putrescine. The patterns of polyamine

Figure 8 DNA content in *SPE1* haploids after addition of polyamines

Cells were grown in PFM for 5 days and polyamines were withheld (a, b and c) or added (d, e and f) at zero time. There were no perceptible differences in DNA distribution at 1 h (a and d), 3 h (b and e), or 6 h (c and f) after addition. The data are representative of two separate experiments.

levels in AB7–AB10 control colonies were the same as for AB5 and AB6 (results not shown).

Decrease in the budding index in *spe1* cells

Cells inoculated into YEPD were transferred to PFM and diluted 1:100 for 5 days. The budding index was defined as the ratio of the number of budding cells to the total number of cells analysed and was assessed by counting under a microscope at $400\times$. Each dilution resulted in a reduction of the budding index in AB3 and AB4 haploid cells (*spe1*). The index was $<1\%$ on day 5. AB5 and AB6 (*SPE1*) cells had a stable 68–70% budding index during equivalent consecutive media changes in PFM (Figure 5). Similar growth curves were obtained in nine independent observations (results not shown).

Decrease in the growth rate of *spe1* cells in PFM

Growth rates of *SPE1* and *spe1* cells were assessed in cells harvested on day 3 and day 5 in PFM. After 3 days of polyamine depletion, the *spe1* cells (AB3 and AB4) had a 7-fold longer doubling time than the *SPE1* cells (AB5 and AB6) (Figure 6a). After 5 days of polyamine depletion, no growth was evident in the *spe1* cell population, while *SPE1* cells doubled every 4 h (Figure 6b).

Figure 9 DNA content in *spe1* haploids after addition of polyamines

Cells were grown in PFM for 5 days and polyamines were withheld (a, b and c) or added (d, e and f) at zero time. *Spe1* cultures in which polyamines were withheld had fewer cells in G2 than *SPE1* cultures (a and Figure 8a). Addition of polyamines to *spe1* cultures enabled progressively more cells to enter into the cell cycle. Note the increasing number of cells with higher DNA content in d–f (a and d, 1 h after addition of polyamines; b and e, 3 h; c and f, 6 h). The data are representative of two separate experiments.

Reversal of growth arrest by polyamines in *spe1* cells

The growth rate (Figure 7a) and budding index (Figure 7b) were measured in AB3 (*spe1*) and AB5 (*SPE1*) cell cultures grown in PFM only, or in PFM supplemented with 1 mM putrescine, 50 μ M spermidine and 50 μ M spermine. AB3 haploid cells bearing *spe1::URA* regained a nearly normal growth rate 6 h after polyamine addition (Figure 7a); no change in growth rate was observed in AB5 haploid *SPE1* cells. The budding index of AB3 cells gradually increased to 56% after 11 h of culture in polyamine-supplemented PFM (Figure 7b). AB5 cells showed the same budding index under either culture condition.

Arrest in G1 of *Spe1* cells in PFM

AB3 and AB5 cell strains were grown for 5 days in PFM supplemented with 1 mM putrescine, 50 μ M spermidine and 50 μ M spermine, and then harvested 1, 3, and 6 h after polyamines were added. Their cell-cycle distribution was determined by flow cytometry (Figures 8a–8c and 9a–9c). Distribution diagrams of the DNA content of AB5 (*SPE1*) nuclei showed two distinctive peaks. One peak had twice the DNA content of the other; the peaks were assigned to the G1 and G2/M phases of the cell cycle. There were no appreciable changes in the distribution of AB5 cells grown either in the presence or absence of

Table 2 Loss of heterozygosity at the *MAT* locus

AB11 *spe1/spe1* diploids bearing two copies of *spe1::URA3* were incubated in the presence or absence of polyamines ($\pm$ polyamines) and assayed quantitatively for mating. AB11 diploids mated with α - and α -testers less often in the presence than in the absence of polyamines. The results are the means $\pm$ S.E.M. of 19 independent observations obtained from four different experiments.

Cells and treatment	Number of colonies/ 10 ⁵ cells (means $\pm$ S.E.M.)
MAT α + polyamines	0.58 $\pm$ 0.11
MAT α + polyamines	0.74 $\pm$ 0.13
MAT α - polyamines	9.53 $\pm$ 0.98
MAT α - polyamines	12.3 $\pm$ 1.91

Figure 10 Microscopic examination (magnification 400 $\times$) of AB4 *spe1* and AB5 *SPE1* haploids after 5 days of growth in PFM

Spe1 cells (a) were larger than *SPE1* cells (b).

polyamines (Figures 8d–8f). By contrast, AB3 (*spe1*) haploids grown in the absence of polyamines showed a G1 peak, but no G2/M peak, at 1, 3, and 6 h (Figures 9a–9c). However, when the same cells were exposed for 1 h to PFM enriched with 1 mM putrescine, 50 μ M spermidine and 50 μ M spermine, a G2/M peak emerged. The same cells analysed 3 h after polyamine addition had a G2/M peak similar to that observed in control AB5 cells (Figures 9d–9f). Replicate experiments showed the same pattern in cell-cycle distribution.

More frequent loss of heterozygosity of *Spe1* cells in PFM

Since MAT α strains cannot mate with other MAT α strains, and MAT α strains cannot mate with other MAT α strains, diploid α/α strains are unable to mate. If a diploid cell loses information at one *MAT* locus, however, it gains mating ability. We used this fact to test whether polyamine depletion affects loss of heterozygosity at the *MAT* locus. Diploid *spe1/spe1* AB11 cells grown in absence of polyamines mated $\approx$ 12 times more often with α -mater tester cells and $\approx$ 15 times more often with α -mater tester cells than the same AB11 cells incubated in the presence of polyamines (Table 2).

Enlargement of *Spe1* cells when polyamines are depleted

Cells from AB3 and AB5 strains were grown in PFM until growth arrest was observed in the AB3 cells. AB3 (*spe1*) cells

were substantially larger than control AB5 (*SPE1*) cells (Figure 10).

DISCUSSION

These studies establish a eukaryotic-cell model and experimental conditions for depleting cellular polyamines without pharmacological manipulation. The novel cellular model is a null ODC, *spe1::URA* mutant of the yeast *S. cerevisiae*, in which the presence of the mutation can be followed by a *URA3* marker. We found that polyamine-depleted mutants arrested in the G1 phase of the cell cycle were an abnormal shape and size, and had an increased likelihood of losing heterozygosity at the mating-type locus. The viability of spores derived from *SPE1/spe1* diploids was also decreased. We thought this mutant might be an appropriate model to study the molecular actions of all three natural polyamines.

Cohn et al. [12] have isolated and characterized *spe2* mutants incapable of synthesizing spermine and spermidine. These *spe2* mutants had no measurable AdoMetDC activity, appreciable levels of putrescine, no detectable levels of spermine or spermidine, and had an absolute requirement for spermine or spermidine for growth; they had increased granularity and size, did not specifically localize chitin-like material, and were defective in sporulation in the absence of extracellular polyamines [28]. Although those strains were unable to sporulate in polyamine-deficient media, sporulation did occur after polyamines were added to media. We observed a decrease in spore viability in our *SPE1/spe1* mutant strains when they were sporulated in polyamine-rich media. These results suggest that *in situ* production of polyamines is an important determinant for sporulation.

The functional depletion of polyamines from *spe1* cells takes a long period of time. Cells must be grown in media completely free of polyamines for approx. 12 doubling times before they undergo complete growth arrest, even though polyamine levels in *spe1* cells fall below our level of detection by a 24 h incubation in PFM. The ability of cells to function with low levels of polyamines supports the hypothesis that critical cell functions rely upon tightly bound or otherwise compartmentalized polyamines.

When depleted of polyamines, *spe1* haploids reduce their budding index, grow abnormally large, and become misshapen. Large cells have been described in *spe1* mutants [1] and in a null mutant of *spe2* depleted of polyamines [28]. The fact that polyamine-depleted cells continue to enlarge suggests that the cells are not totally defective in their ability to grow. Similar to normal cells responding to certain environmental stimuli (heat shock, nutritional deprivation, mating pheromone) and to certain *cdc* mutants, they may be unable to complete an essential step in the cell cycle and consequently do not divide, although they retain the ability to enlarge. The absence of budding suggests that the polyamine-depleted cells arrest in G1, and our experiments measuring DNA content in single cells confirm this idea. However, our experiments do not address whether polyamines directly regulate the cell cycle. Miret et al. [33] have discussed evidence that *S. cerevisiae* deficient in polyamine biosynthesis have cytoskeletal defects, which could also affect cell-cycle progression. Approx. 80% of growth-arrested and polyamine-depleted cells were able to bud and duplicate 3 h after plating on YEPD. This fact, together with the evidence of rapid reinitiation of cell-cycle progression and sustained reinitiation of cell growth after polyamine replenishment, suggests that polyamine-depleted vegetative cells are viable even after 5 days of polyamine depletion. Continued viability and G1 arrest in the face of long-term polyamine deprivation and depletion corroborates findings from experiments in mammalian cells. Many studies have shown

that pharmacological depletion of putrescine and spermidine by specific irreversible inhibition of ODC activity arrests growth but is not cytotoxic, that is, the cells are able to reinitiate division upon incubation in complete medium after drug treatment [11]. On the other hand, several polyamine analogues deplete all three natural polyamines and inhibit growth, but are cytotoxic [24,34,35]. Our current studies suggest that rigorous depletion of all three natural polyamines does not, in itself, lead to cell death. The cytotoxicity seen with certain polyamine analogues is therefore more likely to be related to their direct intracellular effects and less likely to be related to simple polyamine depletion.

Diploid polyamine-starved homozygous *spe1/spe1* cells mated with α - or α -tester strains an order of magnitude more frequently than controls. This suggests that the polyamine-depleted diploids have either lost the function at one *MAT* locus or activated transcription of genes needed for the mating process. Chromosome instability followed by disintegration after polyamine starvation has been reported [17–21]. At this point, however, we have no direct evidence regarding chromosomal loss or mitotic recombination in polyamine-depleted yeast cells and cannot exclude the possibility that a specific error in the regulation of mating-type-specific genes occurs at higher frequency in polyamine-depleted diploids than in wild-type cells. Nonetheless, these results provide evidence that polyamine depletion promotes genetic instability.

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