Phosphorylation of the *Saccharomyces cerevisiae* Equivalent of Ribosomal Protein S6 Has No Detectable Effect on Growth

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The phosphorylation of mammalian ribosomal protein S6 is affected by a variety of agents, including growth factors and tumor promoters, as well as by expressed oncogenes. Its potential role in the regulation of protein synthesis has been the object of much study. We have developed strains of *Saccharomyces cerevisiae* in which the phosphorylatable serines of the equivalent ribosomal protein (S10) were converted to alanines by site-directed mutagenesis. The S10 of such cells is not phosphorylated. Comparison of these cells with the parental cells, whose genomes differ by only six nucleotides, revealed no differences in the lag phase or logarithmic phase of a growth cycle, in growth on different carbon sources, in sporulation, or in sensitivity to heat shock. We conclude that in *S. cerevisiae* the phosphorylation of ribosomal protein S10 may play no role in regulating the synthesis of proteins. This conclusion leads one to ask whether certain protein phosphorylations are simply the adventitious, if easily observable, result of the imperfect specificity of one or another protein kinase.

Ribosomal protein S6 is the major phosphoprotein of eucaryotic ribosomes. It can be phosphorylated at multiple sites (two in *Saccharomyces cerevisiae* and at least five in vertebrates), and the degree of phosphorylation is somehow related to cell proliferation. A variety of stimuli and agents have been observed to affect the phosphorylation of S6, including insulin (35, 40, 44), growth factors (40), serum treatment (40), viral transformation (7), phorbol esters (3), and fertilization (2, 26). Phosphorylation of the S6 protein is carried out by a number of kinases, one of which is apparently specific for S6, and is activated by at least two regulatory mechanisms (4).

In *S. cerevisiae* the homologous ribosomal protein, referred to as S10, is phosphorylated after transfer of a stationary culture to fresh nutrient medium (16). Phosphorylation also occurs at an early stage of germination (37). As in other eucaryotes, the protein is dephosphorylated during heat shock (16).

At present, much is known about the phenomenology of S6 phosphorylation, but little about its function. Generally, as phosphorylation of protein S6 increases, the level of protein synthesis increases (14, 40), and as dephosphorylation of S6 occurs, the level of protein synthesis decreases (13, 34). However, phosphorylation of S6 has been observed to be stimulated under conditions in which protein synthesis increased, decreased, or remained unchanged (14, 17, 41). In serum-stimulated 3T3 cells, ribosomes containing highly phosphorylated S6 are preferentially incorporated into new polysomes, suggesting a role in mRNA recruitment (8, 40). Such a role is supported by the localization of S6 in the mRNA-binding site of 80S ribosomes (39). In vitro attempts to demonstrate an effect of S6 phosphorylation have been successful in some cases (6) and unsuccessful in others (20, 25).

Because of the potential importance of the phosphorylation of S6 to the regulation of cell growth and because of the lack of success in determining its molecular effects, we

approached this problem by using certain powerful techniques of molecular genetics available only for yeasts. Previously we had disrupted each of the S10 genes, *RPS10A* and *RPS10B*, and used site-directed mutagenesis of *RPS10A* to develop a strain of *S. cerevisiae* with a single S10 gene that codes for a protein in which the two serines which are phosphorylated were replaced by alanines (19). These cells grew reasonably well even though the mutant protein was not phosphorylated. However, the mutant gene in such cells was very unstable, frequently reverting to wild type, presumably because of the genetic constructions used in developing the strain.

We have constructed a new strain in which each of the two chromosomal copies of the S10 gene has been replaced with an altered gene. The resulting cells were compared with those of the parental strain, which differed only in the four codons for the phosphorylated serines. During all growth conditions and all shifts in growth, no differences were found between the parent and mutant strains. These results suggest that in *S. cerevisiae*, phosphorylation of ribosomal protein S10 does not play a critical role in the regulation of protein synthesis.

MATERIALS AND METHODS

Strains. *S. cerevisiae* W303 (*MATa/α ade 2-1 his 3-11 leu2-3,112 trp1-1 ura3-1 can1-100*) is a homozygous diploid obtained from R. Rothstein (Columbia University). Haploid *MATa* (W303a) and *MATα* (W303α) strains were derived from spores of W303. *Escherichia coli* C600 was used for recombinant DNA manipulations.

Media. Yeast strains were generally grown in minimal dropout medium (30). When strains were grown on alternative carbon sources, the dextrose was replaced by 1% potassium acetate or 2% ethanol. Growth rates were usually determined in YPD (1% yeast extract, 2% peptone, 2% dextrose). For labeling with ³²PO₄, a culture was grown in broth medium depleted of P_i (33). Diploids were sporulated by replica plating onto agar plates containing 0.8% yeast extract, 0.3% peptone, and 10% glucose. After 2 days at 30°C, these were replica-plated onto sporulation agar con-

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FIG. 1. Schematic diagram of the *RPS10A* and *RPS10B* genes and their respective disruptions. The approximate positions of the restriction sites are indicated. The heavy arrows indicate the transcripts of the two S10 genes; the open portion is the intron. The hatched boxes represent the genes inserted to disrupt the S10 genes. Restriction sites: B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; H, *Hind*III; P, *Pst*I.

taining 2% potassium acetate, 0.22% yeast extract, and (in milligrams per liter) uracil, 40; lysine, 230; arginine, 20; histidine, 20; methionine, 20; tryptophan, 20; threonine, 300; leucine, 60; adenine, 20. These plates were incubated at 30°C for 4 days.

Growth curves. Cells were inoculated at 10^4 cells per ml. Logarithmic growth rates were determined by following the increase in cell density with a Klett-Summerson colorimeter (red filter) within the range of 10 to 100 Klett units.

Isolation of yeast DNA and Southern blot hybridization. Yeast DNA was isolated as described by Elion and Warner (9). The DNA was digested with restriction enzymes, electrophoresed on 0.8% agarose gels, and transferred to nitrocellulose by the method of Southern (36). At times, nitrocellulose filters were probed with the nick-translated replicative form of the filamentous phage f1 derivative CGF4 (46) containing the *RPS10A* *Eco*RI-*Hind*III fragment (Fig. 1). These filters were washed in $0.1\times$ SSC ($1\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate)-0.2% sodium dodecyl sulfate (SDS) at 60°C. Oligonucleotides were 5'-end labeled with polynucleotide kinase and filtered through a Millex-HA filter. Hybridization with the oligonucleotide (5×10^6 cpm) was done at 42°C in $5\times$ SSC- $5\times$ Denhardt solution-0.005 M sodium pyrophosphate-0.5% SDS-0.1 mg of salmon sperm DNA per ml. These filters were washed at 60°C in $5\times$ SSC-0.1% SDS.

Disruption of the *RPS10* genes. (i) *RPS10A*::*HIS3*. Deletion of the *RPS10A* gene was carried out by inserting a DNA fragment carrying the *HIS3* gene between the *Eco*RI and *Pst*I sites of the gene (Fig. 1). The *HIS3* gene was isolated as an *Eco*RI-*Pst*I fragment from pUC18-*HIS3*, which contains *HIS3* cloned in the *Bam*HI site of the polylinker. Deletion of the *Eco*RI-*Pst*I region of *RPS10A* removes all of the coding sequence except those for the two amino-terminal amino acids (21). This deletion was used to replace the chromosomal copy of the *RPS10A* gene by the one-step gene disruption procedure of Rothstein (32). A DNA fragment containing the disrupted gene was used to transform W303a and W303 α by the method of Ito et al. (15). Mitotically stable His⁺ transformants were screened by Southern analysis. Replacement of the endogenous gene was determined by the disappearance of the 4.3-kilobase (kb) *Pst*I fragment of *RPS10A* that hybridized to the probe (Fig. 2, lane 5).

(ii) *RPS10B*::*LEU2*. Deletion of the *RPS10B* gene was effected by inserting a DNA fragment containing the *Pst*I fragment of the *LEU2* gene (Fig. 1), removing the entire

transcriptional unit of the gene (22). The DNA from this disruption was then used to transform W303a and W303 α to Leu⁺ prototrophy. Analysis of these transformants on Southern blots of *Pst*I-cut DNA showed the disappearance of the 1.7-kb band which represents the *RPS10B* sequences homologous to the probe (Fig. 2, lane 2).

Mutagenesis of *RPS10B*. The 1.7-kb *Pst*I fragment of *RPS10B* was cloned into M13mp8. Site-directed mutagenesis was performed by the double primer method described previously (19). The product DNA was cleaved with *Pst*I and ligated to *Pst*I-cut and dephosphorylated pUCS10B. The ligated DNA was used to transform *E. coli* C600 to ampicillin resistance, and the transformants were screened by colony hybridization (23). The nitrocellulose filters were hybridized and washed as described for the Southern blots. Plasmid DNA isolated from positive bacterial colonies was analyzed by digestion with *Pst*I. Mutated sequences now produced 1.2-kb and 0.5-kb fragments in addition to the wild-type 1.7-kb fragment. The mutant plasmid was purified by retransforming strain C600. The orientation of the *Pst*I fragment was determined by analysis of the *Eco*RI digest. Mutagenesis was confirmed by dideoxy sequencing. The mutant genes are designated *RPS10A** and *RPS10B**. The *Bam*HI-*Hind*III DNA fragment of YEpS10A* (19) and the *Bam*HI fragment of pUCS10B* were inserted into the yeast integrating plasmid YIp5 to yield YIpS10A* and YIpS10B*, respectively.

Gene replacement. One colony of either the YIpS10A* or YIpS10B* integrant was grown in 1 ml of YPD to the stationary phase. Strains that had lost sequences were selected for by growth on agar which contained 5-fluoro-orotic acid (5). The medium contained (per 100 ml): 0.7 g of yeast nitrogen base, 2% agar, 5 mg of uracil, 3 mg of tryptophan, 4.3 mg of leucine, 3 mg of adenine, 2% glucose, and 87 mg of 5-fluoro-orotic acid. A 0.01-ml portion of the

FIG. 2. Southern analysis of normal and disrupted *RPS10* genes and of integrated and popped-out mutant *RPS10** genes. In each case DNA from the appropriate culture was cut with *Pst*I and then electrophoresed in an agarose gel, transferred to nitrocellulose, and probed with the nick-translated DNA from f1 phage containing a fragment of *RPS10A* as described in Materials and Methods. The wild-type (*S10A* and *S10B*) and mutant (*S10A** and *S10B**) bands are indicated. Lanes: 1, W303a; 2, J605 α *RPS10A*/*rps10* Δ b::*LEU2*; 3, J607 α *RPS10A*(YIp*RPS10A**)/*rps10* Δ b::*LEU2*; 4, J608 α *RPS10A**/*rps10* Δ b::*LEU2*; 5, J606 α *rps10* Δ a::*HIS3*/*RPS10B*(YIp*RPS10B**)]; 6, J619 α *rps10* Δ a::*HIS3*/*RPS10B*(YIp*RPS10B**)]; 7, J609 α *rps10* Δ a::*HIS3*/*RPS10B**. Size markers were from a *Hind*III digest of phage λ DNA.

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RAT
S6 -----Arg Arg Leu Ser Ser Leu Arg Ala Ser Thr Ser Lys Ser Glu Glu Ser Gln Lys !
           P   P           P   ?   ?   ?   ?

YEAST
S10 -----Lys Arg Arg Ala Ser Ser Leu Lys Ala !
           P?  P?

RPS10B ---AAG AGA AGA GCT TCT TCT TTG AAG GCT TAA AGTTAGAAA----
RPS10A ---AAG AGA AGA GCT TCT TCT TTG AAG GCT TAA GTATGTCTT-----
RPS10*   G AGA AGA GCT GCA GCT TTG AAG GC
S10*    ----Lys Arg Arg Ala Ala Ala Leu Lys Ala !

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FIG. 3. Rat S6 and yeast S10 sequences. The first line shows the carboxyl terminus of rat liver S6, with the established (P) and possible (?) sites of phosphorylation (45). The second line shows the carboxyl terminus of S10 from *S. cerevisiae* as deduced from the nucleotide sequence (21). The next two lines show partial sequences for the two *RPS10* genes (19, 22). The fifth line is the mutagenic oligonucleotide, with the three mismatches underlined. For technical reasons, the complement of this oligonucleotide was actually used for the mutagenesis. The last line shows the predicted carboxyl terminus of the hypothetical mutant S10 protein (S10*).

stationary-phase culture was spread on each 60-mm plate. Approximately 50 to 100 Ura⁻ colonies were obtained per plate.

Analysis of ribosomal proteins. Ribosomes were prepared from yeast cells, and their proteins were extracted and analyzed by two-dimensional polyacrylamide gel electrophoresis (PAGE) with 8 M urea at pH 5 in the first dimension and SDS in the second dimension (43).

RESULTS

Mutagenesis of the phosphorylation site of ribosomal protein S10. The aim of this work was to determine the function of the phosphorylation of ribosomal protein S10. Our approach was to compare the growth properties of a normal yeast cell with those of a mutant which differed only by the substitution of an alanine for each of the phosphorylated serines in S10. Our strategy for generating this mutant was first to obtain cell lines in which either the *RPS10A* or the *RPS10B* gene had been deleted and then to replace the remaining wild-type gene with a mutant gene. By mating the different mutants followed by sporulation, one could obtain the desired double mutant.

To generate the precise mutation discussed above, we used a synthetic oligonucleotide to direct the mutagenesis. The sequences of both *RPS10* genes in the vicinity of the phosphorylation site are shown in Fig. 3. They were identical in the coding region for the carboxyl terminus of the protein but diverged immediately after the termination codon. The mutant *RPS10A** gene was constructed previously by using an oligonucleotide with three mismatches which not only substituted alanines for the two serines but also generated a new *Pst*I restriction site (19). Because of the identity of the two sequences, the same oligonucleotide was used to generate a mutant *RPS10B* gene (see Materials and Methods).

Gene replacement. Yeast strains containing a single copy of the mutant gene were obtained by gene replacement. The two mutant genes, *RPS10A** and *RPS10B**, were subcloned into YIp5, an integrative plasmid which contains the *URA3* gene. In these plasmids, the *Bgl*II site within each of the two *RPS10* genes was unique. The plasmids were linearized with *Bgl*II and transformed into strains whose heterologous *RPS10* gene had been deleted so that integration would occur only within the remaining homologous *RPS10* gene (Fig. 4).

Integration of the *RPS10A** gene was demonstrated by the predicted shift in the *Pst*I fragment from 4.3 kb to bands

migrating at 4.4 and 4.1 kb (Fig. 2, lane 3). Recombination between the two linked *RPS10A* genes will cause a "popout" of one gene and the associated plasmid sequences. To select for this recombination, we grew the integrant nonselectively in liquid medium to the stationary phase and then spread the cells onto agar plates containing 5-fluoro-orotic acid. The 5-fluoro-orotic acid will kill all *URA3*⁺ yeasts and therefore can be used to select for those that have spontaneously lost the plasmid (5). In a *Pst*I digest of genomic DNA of these Ura⁻ derivatives, the presence of the mutant sequence caused a shift in the migration of a genomic *Pst*I fragment from 4.3 to 4.1 kb (Fig. 2, lane 4). The mutation in the gene was confirmed by probing an *Hind*III digest with the oligonucleotide used for mutagenesis. The only DNA detected that hybridized to the probe migrated at 5.0 kb, the size of the chromosomal *RPS10A* *Hind*III fragment (data not shown).

Proper integration of the *RPS10B** mutation could not conclusively be shown to occur at the *RPS10B* locus by a

FIG. 4. Gene replacement with *RPS10A**. The integrating plasmid YIpRPS10A* is shown being targeted to the *RPS10A* locus by *Bgl*II linearization of the plasmid DNA. The recombination event which replaces the wild-type with the mutant sequence must occur between the two *Pst*I sites of *RPS10A**. See text for details. Restriction sites: B, *Bam*HI; Bg, *Bgl*II; H, *Hind*III; P, *Pst*I.

TABLE 1. Growth rates in YPD medium at 30°C

Strain	Relevant genotype	Doubling time (min) ^a
W303a	<i>RPS10A/RPS10B</i>	80 (4)
W303α	<i>RPS10A/RPS10B</i>	80 (3)
J601a	<i>RPS10A/rps10a::LEU2</i>	94 (2)
J602α	<i>rps10a::HIS3/RPS10B</i>	115 (2)
J605a	<i>RPS10A/rps10Δb::LEU2</i>	122 (10)
J605α	<i>RPS10A/rps10Δb::LEU2</i>	114 (7)
J606a	<i>rps10Δa::HIS3/RPS10B</i>	101 (8)
J606α	<i>rps10Δa::HIS3/RPS10B</i>	114 (8)
J608α	<i>RPS10A/rps10Δb::LEU2</i>	113 (2)
J609a	<i>rps10Δa::HIS3/RPS10B*</i>	112 (8)

^a The doubling time values are averages of the number of determinations shown in parentheses.

diagnostic restriction digest. Restriction digestion of the integrant DNA with *Pst*I did indicate the presence of the wild-type and the mutated gene as 1.7-kb and 1.2-kb fragments, respectively, on Southern blots (Fig. 2, lane 6). One of the *Ura*⁺ integrants was screened for spontaneous loss of the plasmid by growth in the presence of 5-fluoro-orotic acid as discussed above. The *Ura*⁻ isolates were again characterized by Southern analysis. Replacement with the mutated gene was confirmed by the loss of the 1.7-kb *Pst*I fragment and the presence of the 1.2-kb fragment (Fig. 2, lane 7). In an *Hind*III digest of genomic DNA from the mutant, only the 0.9-kb fragment, which is the size found in *RPS10B*, hybridized to the mutant oligonucleotide (data not shown).

The position of the crossover point in the homologous recombinant which causes the loss of plasmid sequence determines whether the wild-type or mutant sequence remains in the chromosome. Transplacement leaving the mutant *RPS10A** was expected to be a rare event since the crossover must occur in the 200-base-pair sequence downstream from the mutation and not in the 3.4 kb upstream of

FIG. 5. Southern analysis of diploids and their His⁻ Leu⁻ spores. Diploid cells were constructed from the indicated matings. These were sporulated, and His⁻ Leu⁻ segregants from each were analyzed. The DNA was isolated, cut with *Hind*III, electrophoresed in an agarose gel, and transferred to nitrocellulose. The nitrocellulose was probed with the mutator oligonucleotide, 5'-end labeled with T4 polynucleotide kinase (see Materials and Methods). The bands for each mutated gene are indicated (S10A* and S10B*). Lanes: 1, J605α × J606a diploid; 2, His⁻ Leu⁻ spore derived from J605α × J606a diploid; 3, J605α × J609a diploid; 4 and 5, His⁻ Leu⁻ spores (J615) derived from J605α × J609a diploid; 6, J606a × J608α diploid; 7 and 8, His⁻ Leu⁻ spores (J616) derived from the J606a × J608α diploid; 9, J608α × J609a diploid; 10 and 11, His⁻ Leu⁻ spores (J617) derived from the J608α × J609a diploid.

FIG. 6. Two-dimensional PAGE of yeast ribosomal proteins from haploid cells incubated with ³²PO₄. On the left are the Coomassie blue-stained gels, and on the right are autoradiographs of the same gels. Arrows point to the unphosphorylated form of S10; arrowheads point to rp14, which is the other phosphoprotein observed in the autoradiographs. (A) W303 *RPS10A/RPS10B*; (B) J615 *RPS10A/RPS10B**; (C) J616 *RPS10A*/RPS10B*; (D) *RSP10A*/RPS10B**.

the mutation (Fig. 4). However, of 96 *Ura*⁻ colonies examined, 34 had the *RPS10A** gene. For the *RPS10B* integration, in which there were 1.2 kb downstream and 3.0 kb upstream of the mutated sequence, 3 of 12 *Ura*⁻ colonies had *RPS10B**. This result suggests a possible hot spot of recombination within the 3' sequence of *RPS10A*.

These strains, J608 (*RPS10A**) and J609 (*RPS10B**), now each contained a single mutant *RPS10* gene. When these strains were exposed to ³²PO₄ and the ribosomal proteins were examined on two-dimensional polyacrylamide gels, no phosphorylation of the S10 protein was observed (data not shown), in keeping with previous observations of a strain carrying only *RPS10A** (19). This result confirmed the conclusion that the two serines which had been converted to alanines are the only sites of phosphorylation in S10.

The effect of the mutated gene on logarithmic growth was tested by growing both J608 and J609 in rich medium. Their growth rate under these conditions was similar to that of strains with only one normal gene, J605 and J606 (Table 1). These strains, which lack one of the *RPS10* genes, grew somewhat slowly, which may reflect their inability to compensate for the missing gene (1). If phosphorylation of S10 is important to maximize growth, then it might not be possible to observe any effect in a genetic background in which the growth rate had already been slowed.

Isolation of haploid strains with two mutant *RPS10* genes. To examine the effect of *RPS10A** and *RPS10B** in a cell otherwise identical to the parent strain, W303, we mated J608 and J609 and sporulated the resultant diploids. Additional matings were carried out between J605 and J606, J605 and J609, and J606 and J608. The frequency of sporulation was 67.4, 64.1, 66.3, and 57.9% for crosses J605 α $\times$ J606 α , J605 α $\times$ J609 α , J606 α $\times$ J608 α , and J608 α $\times$ J609 α , respectively, as determined by microscopic examination of at least 200 cells. There was no significant difference in the percentage of asci observed whether two wild-type *RPS10* genes, a wild-type and a mutant gene, or two mutant genes were present.

The His⁻ Leu⁻ spores were screened by Southern analysis of their *Hind*III restriction digests followed by hybridization with the mutagenic oligonucleotide, which will not hybridize to the wild-type gene (Fig. 5). The spores presented in this autoradiograph had bands corresponding to the predicted restriction fragment, the 5.0-kb fragment for *RPS10A** and the 0.9-kb fragment for *RPS10B**. When this blot was hybridized with the genomic probe, which hybridizes to both mutant and wild-type genes, the pattern was the same only for the spores from the J608 $\times$ J609 cross. In these cells both normal *RPS10* genes have been replaced with mutant genes.

Protein S10 of each of the spores was examined after the cells were grown in medium containing ³²PO₄. The ribosomes were isolated and their proteins were analyzed by two-dimensional PAGE (Fig. 6). The cells with two wild-type *RPS10* genes had an S10 protein which was mostly phosphorylated. Those with a mutant and wild-type gene had less of the phosphorylated form of the S10 protein. In fact, in the stained protein pattern, one could detect an elongated S10 spot indicative of phosphorylated and unphosphorylated forms of the protein. Cells with two mutant genes had no phosphorylation of S10, confirming the conclusion that the mutated serines (Fig. 3) are the only sites of phosphorylation in the yeast protein.

Mutated *RPS10* has no effect on growth. Cells containing mutant and wild-type *RPS10* genes were compared under a variety of growth conditions. The presence of one or both mutant genes caused no detectable change in exponential growth rate in medium containing glucose, acetate, or ethanol as a carbon source, nor was any effect observed at a lower temperature, 24°C (Table 2). In the media tested, the phosphorylation of S10 provided no advantage during exponential growth.

Phosphorylation of S10 is low during the stationary phase and increases when cells are induced to proliferate (16). The S10 mutation would most likely affect the cell under condi-

tions in which protein is normally being phosphorylated. Yet when a stationary-phase culture of mutant or wild-type cells was inoculated into fresh medium, the time course for each to begin growing was identical (Fig. 7A). During such a shift, ribosomes are recruited into polysomes as the rate of protein synthesis increases. At various time points following such a shift, the polysome patterns of the mutant and normal cells were indistinguishable, as determined by sucrose gradient centrifugation (data not shown), or did the growth of the mutant cell differ from that of the wild type following a nutritional shift from ethanol medium to glucose medium (Fig. 7B).

Since heat shock has been found to cause dephosphorylation of the S10 protein (16), we tested the response of the mutant to heat shock. An exponential culture growing at 30°C was shifted to 41°C for 2 h and then returned to 30°C. The effect of this stress on growth of the mutant and wild-type strains was the same (Fig. 7C). The mutant was also tested for its ability to acquire thermotolerance. Cells in early log phase were exposed to 37°C for 60 min to induce a heat shock response. They were then exposed to higher temperatures, and their ability to survive was compared with that of cells not pretreated at 37°C. No difference in survival was observed between the strains (Fig. 7D).

The methods used to observe the impact of these various conditions would not detect a subtle effect of the mutation. To do so, a competitive growth experiment was designed. Equal numbers of J617 and W303 cells were mixed and grown to the stationary phase and then diluted into fresh medium. This procedure was repeated five times; during each cycle the wild-type S10 would undergo phosphorylation and dephosphorylation. The final yeast culture was approximately 70 generations removed from the original inoculum. The fraction of mutant cells in each stationary-phase culture was determined by Southern hybridization of *Pst*I-cleaved DNA (Fig. 8). The relative intensities of the mutant and wild-type *RPS10* bands did not change, demonstrating that W303 did not outgrow J617. The selection value of the phosphorylation, under these conditions, was less than 1%.

DISCUSSION

Most analyses of S6 phosphorylation have been done with mammalian cells. At least four distinct protein kinases have been shown to phosphorylate S6 in vitro. The cyclic-AMP (cAMP)-dependent kinase can phosphorylate two or perhaps three sites (45). Three cAMP-independent kinases can phosphorylate five sites. These include protease-activated kinase II (12, 31) and protein kinase C, a Ca²⁺-activated kinase which may also be phospholipid dependent (11, 29), both of which seem to have a fairly broad substrate spectrum. Finally, several laboratories have recently purified a kinase which is apparently specific for S6 and distinct from the kinases described above by a number of criteria (3, 10, 27, 38). This kinase has substantially more activity in extracts from cells in which S6 phosphorylation has been stimulated by insulin (38), epidermal growth factor (EGF) (27), or expression of the oncogene *src* (3).

The sites of phosphorylation have been determined directly only on S6 subjected to cAMP-dependent kinase (see Fig. 3) (45). Peptide analysis suggests that the other three kinases phosphorylate the same five loci, which include the loci phosphorylated by the cAMP-dependent kinase (10–12, 24, 31). We assume that these all cluster at the carboxyl terminus of S6, as shown in Fig. 3, although there is no direct evidence on that point.

TABLE 2. Growth of Leu⁻ His⁻ spores

Cross	Relevant genotype	Doubling time (min)			
		30°C			24°C, YPD
		YPD	Acetate	Ethanol	
J605 $\times$ J606	α <i>RPS10A/RPS10B</i>	83, 86	154	244	110
	α <i>RPS10A/RPS10B</i>	84, 82	154	228	105
J605 $\times$ J609	α <i>RPS10A/RPS10B</i> *	104, 75	160	240	105
	α <i>RPS10A/RPS10B</i> *	76, 77	162	254	110
J606 $\times$ J608	α <i>RPS10A*/RPS10B</i>	81, 82	164	268	105
	α <i>RPS10A*/RPS10B</i>	81, 78	163	ND ^a	ND
J608 $\times$ J609	α <i>RPS10A*/RPS10B</i> *	76, 77	167	242	108
	α <i>RPS10A*/RPS10B</i> *	77, 79	167	258	109

^a ND, Not determined.

FIG. 7. Effects of nutritional and temperature shifts on growth and survival. (A) A fresh stationary-phase culture of parent strain W303a (circles) or mutant strain J617a (triangles) was diluted 20-fold into fresh YPD medium. (B) Cultures of W303a and J617a were grown exponentially in ethanol medium. At the indicated time, glucose was added to 2% (wt/vol). (C) Response of cells to heat shock was determined by shifting exponentially growing cultures of W303a and J617a to 41°C for 2 h and then returning the cultures to 30°C at the times indicated. (D) The comparative ability of W303a and J617a to acquire thermotolerance was measured as follows. Cells were grown to a density of 6×10^6 to 8×10^6 cells per ml in YPD. Samples were either exposed directly to the indicated temperatures for 5 min (solid symbols) or pretreated at 37°C for 1 h prior to being exposed to the higher temperatures, again for 5 min (open symbols).

The sites of phosphorylation of yeast S10 are analogous to those subject to cAMP-dependent kinase in mammalian cells. Although cAMP-dependent protein kinase has been implicated in the phosphorylation of S10 in vivo (28) and in vitro (R. E. H. Wettenhall, personal communication), there may well be other kinases that can carry out the phosphorylations.

It has been clear for many years that phosphorylation of S6 is not essential for protein synthesis; e.g., in resting 3T3 cells only a small fraction of S6 has even one phosphate, while protein synthesis continues at a substantial rate (40). Furthermore, the phosphorylation of S6 in response to a stimulus such as EGF can be as brief as an hour or two, while the effect of EGF on total protein synthesis extends for hours or days (27). Nor is there unambiguous evidence that the phosphorylation of S6 can affect either the activity or discrimination of ribosomes. Some authors have found such effects (6), while others have not (20, 25).

Our results show that the phosphorylation of S10 in *S. cerevisiae* has no detectable effect on the initiation or continuation of growth, on sporulation, or on the response to heat shock. In short, we can find no effect whatsoever that is related to the phosphorylation of this ribosomal protein.

We cannot, of course, justify extrapolation directly from these observations to the possible effects of phosphorylation of mammalian S6, which has five sites for phosphorylation versus two for the yeast protein. One can argue that the extra three sites are responsible for all the physiological functions of phosphorylation, but this is an ad hoc proposition without experimental support.

Of all the phosphorylated proteins known, S6 is the most widely conserved across evolution, being present in all eucaryotes examined, from *Saccharomyces* to *Drosophila* to vertebrates. It is possible that a phosphorylation site present on an ancestral ribosomal protein could evolve to provide different functions in different species, being retained in some only as an evolutionary remnant. For example, starvation, which brings about dephosphorylation of S6 in most organisms, brings about phosphorylation of S6 in *Tetrahymena pyriformis* (18). It is also possible that an amino acid sequence, evolved to be useful for the function of a protein, has fortuitously acquired the recognition signal for a protein kinase.

The availability of ^{32}P makes the detection of a phosphoprotein much easier than determination of the function of the phosphate group. The findings presented in this paper, as

FIG. 8. Competitive growth of mutant and parental strains. A 25-ml culture of YPD was inoculated with approximately 10^5 cells each of W303a and J617a. When the culture reached the stationary phase, a 1- μ l portion was used to inoculate 25 ml of fresh YPD. This was repeated four times. The DNA was isolated from each stationary-phase culture, cut with *Pst*I, and analyzed by Southern hybridization. The protein bands from wild-type (S10A and S10B) and mutant (S10A* and S10B*) cells are indicated.

well as the observation that mutagenesis of the major phosphorylation site of adenovirus protein E1A has no effect on adenovirus growth (42), clearly suggest that caution be exercised in interpreting the physiological significance of the phosphorylation of proteins.

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