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Biochemistry

Phosphorylation of the yeast equivalent of ribosomal protein S6 is not essential for growth

(phosphoprotein/gene disruption/site-directed mutagenesis/cloned gene/protein synthesis)

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ABSTRACT **The yeast equivalent of ribosomal protein S6, known as S10, can be modified by the addition of two phosphates. The two adjacent serines that are likely to be subject to phosphorylation were deduced by comparison with the known sites of phosphorylation on rat liver S6. Using oligonucleotide mutagenesis, we altered the gene for S10 to replace these two serines with alanines. This mutant gene was introduced into a diploid yeast cell heterozygous for each of the two S10 genes. After sporulation, we obtained colonies in which the mutant gene was the only intact S10 gene. Although the ribosomes of these cells contained a full complement of S10, no phosphorylation of S10 was detected. These cells grow exponentially with a doubling time about 50% greater than that of control cells. We conclude that the phosphorylation of S10 is not essential for growth. However, the mutant gene in such cells is very unstable, frequently reverting to wild type, presumably by interaction with the disrupted host genes. We suggest that at some stage of the growth cycle there is strong selection for S10 that can be phosphorylated.**

In vertebrate cells, ribosomal protein S6 is frequently found in a multiply phosphorylated form. The degree of phosphorylation is somehow related to cell proliferation. Phosphorylation of S6 is stimulated by a number of conditions or agents: for example, partial hepatectomy (1), growth factors (2), or tumor-promoting agents (3). Transformed cells no longer require growth factors to maintain the phosphorylation of their S6 (4), due to the action of an oncogene (5), apparently working through the indirect activity of a viral tyrosine protein kinase (6).

Although a great deal is now known about the phenomenology of the phosphorylation of S6, very little is known about the function either of the protein or of its phosphorylation. *In vivo*, ribosomes with highly phosphorylated S6 appear to have a slight advantage in forming polysomes (7, 8). Extensive efforts to demonstrate an effect of phosphorylation *in vitro* have been sometimes successful (9) and sometimes unsuccessful (10).

The yeast, *Saccharomyces cerevisiae*, has its own version of ribosomal protein S6, referred to as S10. The homology was originally deduced from two-dimensional gel electrophoresis (11–13). Comparison of the sequences now available (Fig. 1) not only establishes the homology of the two proteins but suggests the location of the phosphorylation sites on S10. The rat liver protein is longer by some 10 amino acids, which include several potential phosphorylation sites. This presumably explains why yeast S10 carries no more than two phosphates, whereas metazoic S6 can carry at least five (16).

The physiological aspects of the phosphorylation of yeast S10 have not been thoroughly studied, although it is clear that in growing cells most S10 is phosphorylated, while in non-growing cells it is not. The exchange of phosphate on S10 is rapid, occurring in a matter of minutes, even in the absence of protein synthesis (11).

Because of the potential importance of the phosphorylation of S6 to regulation of cell growth and because of the lack of success in determining its molecular effects, we have undertaken to apply to this protein the powerful genetic techniques available in yeast. In an approach analogous to that used by Wallis *et al.* (17) to study the function of altered histones in yeast, we have employed gene disruption and site-directed mutagenesis to develop a strain of yeast whose only gene for S10 codes for a protein in which two serines, suspected of being the phosphorylation sites, have been replaced by alanines (Fig. 1). The mutant protein is not detectably phosphorylated *in vivo*. Nevertheless, the cells grow reasonably well, demonstrating that phosphorylation of S10 is not essential for normal growth.

MATERIALS AND METHODS

Strains. *S. cerevisiae* strain W303 (*MATa/α ade2-1, his3-11, leu2-3,112, trp1-1, ura3-1, can1-100*) is a homozygous diploid obtained from R. Rothstein (Columbia University). Haploid *MATa* and *MATα* strains were derived from spores of W303. *Escherichia coli* strains C600 mK^+rK^- and JM101 were obtained from R. Davis (Stanford University) and J. Messing (Rutgers University), respectively. Yeast generally were grown in minimal drop-out media to maintain plasmids (18). For labeling with $^{32}PO_4$, a culture was grown in broth medium depleted of inorganic phosphate (19). Growth rates (Table 1) were determined in YPD medium (1% yeast extract, 2% peptone, 2% dextrose).

Plasmids. The shuttle vector YEp24''' contains 2-μm ("2-μ") sequences and the *URA3* gene for propagation and selection in yeast, as well as most of pBR325 for propagation and selection in *E. coli*, and was derived from YEp24' (18) by removal of the *Eco*RI site and of the *Hin*dIII site that lies between 2-μ and *URA3* sequences. YEpS10A was prepared by inserting the *Bam*HI–*Hin*dIII fragment containing the gene RPS10A (Fig. 2) into YEp24'''. To facilitate subsequent handling of the gene, we destroyed the *Eco*RI site within the intron of RPS10A (indicated as E' in Fig. 2) by partially digesting the plasmid with *Eco*RI, filling in the termini with Klenow fragment of DNA polymerase, and religating. The ligation mixture was used to transform *E. coli*, and plasmid DNA was prepared from the transformants and cut with *Eco*RI to verify that the site was altered. The resulting plasmid, YEpS10A', was used for site-directed mutagenesis.

Recombinant DNA Methods. DNA was extracted from bacterial and yeast cells and subjected to restriction enzyme analysis as described (18).

Oligonucleotides. Oligonucleotides were synthesized on an Applied Biosystems Model 380A DNA Synthesizer.

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Abbreviation: kb, kilobase(s).

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

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

RPS10B  ---AAG AGA AGA GCT TCT TCT TTG AAG GCT TAA AGTTAGAAA----

RPS10A  ---AAG AGA AGA GCT TCT TCT TTG AAG GCT TAA GTATGTCTT------

RPS10A*      G AGA AGA GCT GCA GCT TTG AAG GC

S10 *  ----Lys Arg Arg Ala Ala Ala Leu Lys Ala
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FIG. 1. Rat S6 and yeast S10 sequences. The first line shows the COOH terminus of rat liver S6, with the certain and uncertain sites of phosphorylation (14). The second shows the COOH terminus of S10 of *S. carlsbergensis*, as deduced from the nucleotide sequence (15). The next two lines show partial sequences we determined for the two S10 genes of *S. cerevisiae*. The next line shows the mutant oligonucleotide, with the three mismatches underlined. (For technical reasons, the complement of this oligonucleotide was actually used for the mutagenesis; see Fig. 5.) The last line shows the COOH terminus of the hypothetical mutant S10 protein (S10*).

Site-Directed Mutagenesis. Mutagenesis was carried out following the strategy of Norris *et al.* (20). The 0.9-kilobase (kb) *Eco*RI–*Pst* I fragment of RPS10A was inserted into M13mp18. Primer extension and ligation were carried out essentially as described (21), using two oligonucleotide primers: the universal M13 primer usually used for sequencing and the mutagenic primer. Thirty pmoles of the mutagenic phosphorylated oligonucleotide was added to 2 pmol of single-stranded template DNA and 20 pmol of universal primer in 10 μl of annealing buffer (0.02 M Tris Cl, pH 7.5/0.1 M $MgCl_2$/0.05 M NaCl/1 mM dithiothreitol). The mixture was heated in a siliconized Eppendorf tube for 5 min at 95°C under a layer of paraffin oil and then cooled to 10°C. The annealing-reaction mixture was withdrawn from the tube and added to 10 μl of 20 mM Tris Cl, pH 7.5/10 mM $MgCl_2$/10 mM dithiothreitol/dATP, dCTP, dGTP, and dTTP (1 mM each)/1 mM ATP containing 3 Weiss units of T4 DNA ligase (New England Biolabs) and 2 units of *E. coli* DNA polymerase I Klenow fragment (Boehringer Mannheim). The reaction was allowed to proceed for 10 min at 10°C and then for 3.5 hr at 15°C. The reaction was stopped by heating for 10 min at 65°C. The product was cut with *Eco*RI and *Hin*dIII (which cleaves beyond the *Pst* I site in the M13 polylinker), and the resulting 0.9-kb fragment was purified by electrophoresis in a low-melting-point agarose gel. The fragment then was ligated to the large fragment of YEpS10A′ that had been cut with *Eco*RI and *Hin*dIII (Fig. 5). The resulting ligation mixture was used to transform *E. coli*, and the resulting ampicillin-resistant colonies were analyzed as described in *Results*.

Filter Hybridization Conditions. Replica filters were made from bacterial colonies, washed, and prehybridized as described (22). The filters then were washed with 6× NET (0.9 M NaCl/6 mM EDTA/90 mM Tris Cl, pH 7.5) containing 0.1% $NaDodSO_4$ and 0.5% Triton X-100 and were added to the hybridization solution. The hybridization solution consisted of 8 ml of 6× NET/0.1% $NaDodSO_4$/0.5% Triton X-100 containing 8 mg of calf thymus DNA and 4.8 mg of calf thymus DNA that had been treated with 1 M HCl to generate small DNA fragments and then neutralized with NaOH. To the mixture, the 5′-labeled mutagenic oligonucleotide (1–5 × 10^6 cpm) was added. Hybridization was overnight at 10–20°C below the calculated T_d (23). Filters were washed in 0.9 M NaCl/0.09 M sodium citrate, pH 7, first three times at room temperature and then for 30 min at a temperature either 5–10°C below T_d (to detect both wild-type and mutant colonies) or 5°C above T_d (to detect only mutant colonies).

Analysis of Ribosomal Proteins. Ribosomes were prepared from yeast cells, and their proteins were extracted and analyzed by two-dimensional acrylamide gel electrophoresis using 8 M urea at pH 5 in the first dimension and $NaDodSO_4$ in the second dimension (24).

RESULTS

Two S10 Genes. One gene for S10, RPS10A, had been cloned previously (25, 26) and its sequence determined (15). As is true for many ribosomal proteins, however, yeast contains two copies of this gene (25–27). The second copy, RPS10B, was isolated from a genomic-clone bank derived from *S. cerevisiae* (28), using the *Eco*RI–*Hin*dIII fragment of RPS10A as a probe. The restriction map of RPS10B is compared to that of RPS10A in Fig. 2. The 3′ region of each gene was sequenced. That of RPS10A is identical to the sequence reported for the *S. carlsbergensis* gene (15) (Fig. 1). The homology of yeast S10 with mammalian S6 (14) is apparent. Within the coding region, RPS10B and RPS10A differ only in a few neutral third base changes, but they diverge completely immediately after the termination codon (Fig. 1). The two genes are identical in the vicinity of the codons for the two serines that are presumed to be the phosphate acceptors.

Either S10 Gene Is Sufficient for Growth. Both RPS10A and RPS10B give rise to mRNA when present on a multicopy plasmid (29). To verify the activity of both genes within the genome, as well as to develop a strain in which an altered S10 gene would be the only S10 gene expressed, we disrupted each of the endogenous genes by using the method of Rothstein (30) (Fig. 2). RPS10A and RPS10B, each in a plasmid, were disrupted by inserting a *HIS3* gene and a *LEU2* gene, respectively, at the *Bgl* II site located within codon 51 (15). We used linear fragments of these plasmids to transform isogeneic *MATα* and *MATa* strains, respectively, and selected for His^+ and Leu^+ phenotypes (Fig. 3). Southern analysis of the DNA of the resulting yeast strains showed that the gene disruptions were successful (Fig. 4). Integration of *HIS3* into

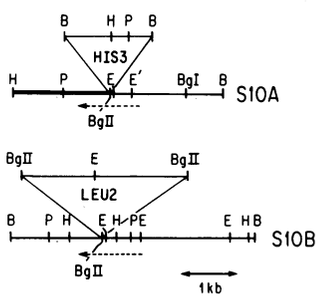

FIG. 2. Restriction maps of the RPS10A and RPS10B genes. The approximate positions of the two transcripts are indicated by the broken arrows. The *Eco*RI–*Hin*dIII fragment used to prove the Southern blots is indicated by a bold line. The *Eco*RI site in RPS10A that was destroyed to facilitate the reconstruction of the mutant gene is indicated by E′. The fragments carrying the *HIS3* and *LEU2* genes are shown at their respective sites of insertion into the RPS10 genes. Restriction sites: B, *Bam*HI; BgI, *Bgl* I; BgII, *Bgl* II; E, *Eco*RI; H, *Hin*dIII; P, *Pst* I.

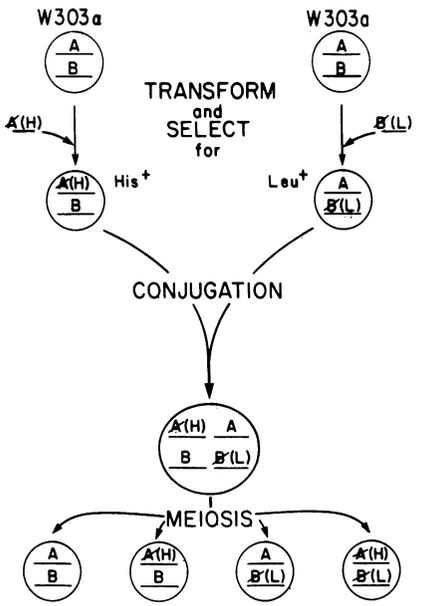

FIG. 3. Strategy for the disruption of both S10 genes. A, S10A gene; B, S10B gene; H, *HIS3*; L, *LEU2*. See text for details.

RPS10A converts a 5.0-kb band to a 2.5-kb band; integration of *LEU2* into RPS10B converts a 0.9-kb band into a 3.9-kb band. Each strain grows at a rate only slightly less than that of wild type (Table 1).

The two strains carrying the disrupted genes were mated, and the resulting diploid strain was induced to undergo meiosis. Tetrad dissection yielded no viable spores that were both Leu^+ and His^+, suggesting that S10 is an essential protein. Our subsequent strategy then was to transform such a diploid strain with a plasmid carrying a mutant S10 gene. On sporulation, one should recover His^+ Leu^+ spores only if the mutant gene is functional. Furthermore, the mutant S10 gene will be the only functional one in such cells.

Site-Directed Mutagenesis of RPS10A. To alter the presumed phosphorylation site of S10, we prepared an oligonucleotide of 24 residues, designed to hybridize to the appropriate segment of the RPS10A gene, but with three mismatches (Fig. 1). A mutant gene containing all three alterations not

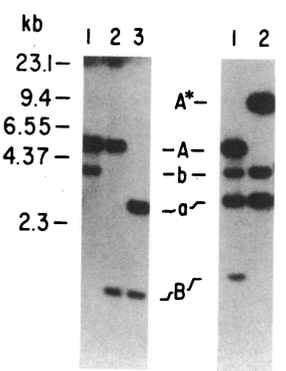

FIG. 4. Southern analysis of normal and disrupted RPS10 genes. In each case, DNA from the appropriate culture was cut with *Hin*dIII and then electrophoresed in an agarose gel, transferred to nitrocellulose, and probed with the *Eco*RI–*Hin*dIII fragment shown in Fig. 2. The wild-type (A and B), disrupted (a and b), and mutant (A*) bands are indicated. (*Left*) Lanes: 1, rps10B(*LEU2*); 2, wild type; 3, rps10A(*HIS3*). (*Right*) Lanes: 1, heterozygous diploid (see Fig. 3); 2, spore carrying two disrupted S10 genes on the genome and the mutant gene RPS10A* on a multicopy plasmid. Markers at left represent positions of fragments generated by *Hin*dIII digestion of phage λ DNA.

Table 1. Growth rate of yeast strains carrying mutant RPS10 genes

Strain	Relevant genotype	Doubling time,† min
W303a	RPS10A/RPS10B	89, 76
W303α	RPS10A/RPS10B	98, 87
J601	RPS10A/rps10B(*LEU2*)	94, 93
J602	rps10A(*HIS3*)/RPS10B	115, 115
J625‡	rps10A(*HIS3*)/rps10B(*LEU2*)/YEpS10A*	141, 130, 139
J626‡	rps10A(*HIS3*)/rps10B(*LEU2*)/YEpS10A*	140, 169, 162

†Determined in broth medium at 30°C. Each value represents a separate determination.
‡Two His^+ Leu^+ Ura^+ spores with RPS10A* as the only intact S10 gene.

only would code for alanines in place of the two serines but would also have a new *Pst* I restriction site to facilitate the identification of the mutant gene.

Mutagenesis using the two-primer method (20) was carried out as shown in Fig. 5 (see *Materials and Methods*). The double-stranded fragment with mutagenized sequences on one strand was inserted into YEpS10A'. Transformants in *E. coli* were detected by probing replicas with the mutagenic fragment, end-labeled with ^{32}P (22). Washing the filter under stringent conditions permitted identification of colonies containing mutant plasmids. These colonies in general contained a mixed population of plasmids due to propagation of the individual strands of the original mispaired DNA. Therefore, DNA was prepared from a colony and used to transform *E. coli*. Appropriate transformants were again identified by probing with the mutagenic oligonucleotide under stringent conditions. Plasmid DNA was prepared from one such colony and found to contain the predicted *Pst* I restriction site. Dideoxy sequencing primed with an adjacent oligonucleotide confirmed that all three altered bases had been incorporated into the mutant gene, which we termed RPS10A*, carried by the plasmid designated YEpS10A*.

Effect of RPS10A* in Yeast. YEpS10A* was introduced into

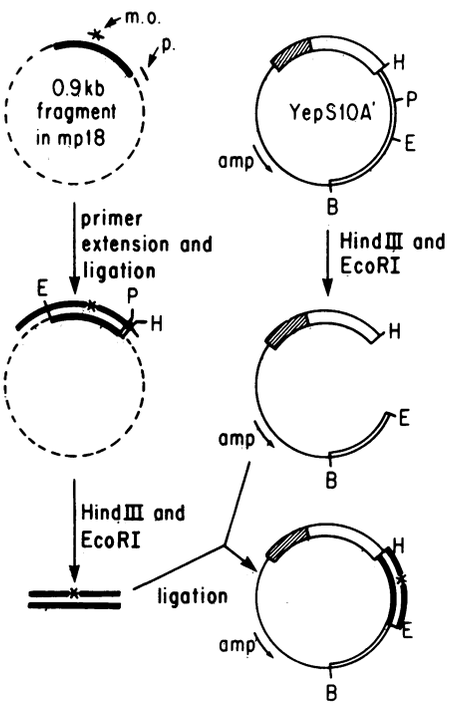

FIG. 5. Site-specific mutagenesis of RPS10A. m.o., Mutagenized oligonucleotide; p, primer; amp, ampicillin-resistance gene. Restriction sites: H, *Hin*dIII; P, *Pst* I; E, *Eco*RI; B, *Bam*HI. See text for details.

a diploid cell heterozygous for RPS10A and RPS10B (Fig. 3) by transformation and selection for Ura$^+$ colonies. The subsequent strain was shown by Southern analysis to have five genes for S10: RPS10A, rps10A(*HIS3*), RPS10B, rps10B(*LEU2*), and YEpS10A*. This strain grew well under uracil selection, showing that YEpS10A* did not have a dominant deleterious effect. Sporulation was induced in the absence of uracil. Most of the resulting spores retained the plasmid YEpS10A*. Unlike the situation where the diploid carried no plasmids, a number of Leu$^+$ His$^+$ spores were recovered. Although some of these had undergone genetic rearrangements at one or another S10 locus, two spores were shown to contain only rps10A(*HIS3*), rps10B(*LEU2*), and YEpS10A* (Fig. 4 *Right*). No wild-type gene was detectable. Since a cell is unable to survive without S10 protein, the existence of viable His$^+$ Leu$^+$ cells shows that YEpS10A* encodes a functional protein (S10A*). The plasmid was found to be mitotically stable in the absence of uracil selection, showing that S10A* is not only functional but also essential. Use of labeled oligonucleotides hybridized under stringent conditions demonstrated that large amounts of mRNA derived from the mutant gene were present in these cells.

To determine whether S10A* was subject to phosphorylation, we exposed cells carrying YEpS10A* as their only intact S10 gene to $^{32}PO_4$ [160 μCi (5.9 MBq)/ml] for 60 min. Their ribosomes were prepared, and purified ribosomal proteins were subjected to two-dimensional polyacrylamide gel analysis (Fig. 6). S10 is clearly present in such cells but has incorporated no phosphate, although several other proteins, including ribosomal protein rp14 (phosphoprotein P3 of ref. 11), have incorporated substantial amounts of ^{32}P. Thus, it seems likely that all the S10 molecules in these cells contain alanines instead of serines in the appropriate positions. Furthermore, it is clear that these two serines are the only sites at which phosphorylation occurs, or at least they must be phosphorylated before any other sites become susceptible. This finding is consistent with the work in mammalian cells in which these two serines are phosphorylated before any other residues (14).

Cells carrying RPS10A* as their only intact S10 gene grow exponentially but at a somewhat reduced rate compared to wild-type cells (Table 1). Therefore, phosphorylation of S10 is not essential for cell growth.

However, we have found it difficult to maintain a genetically pure culture, because the S10A* gene is extraordinarily susceptible to rearrangement leading to a wild-type gene, presumably by mitotic recombination or gene conversion with sequences of one of the disrupted genes. A culture of cells carrying RPS10A* as their only intact S10 gene was spread on a plate lacking uracil. Eight single colonies were picked and subcultures were prepared. Three of the eight were found to have phosphorylated S10. Southern blot analysis showed that each of the three also had rearrangements in their S10 genes. In cultures that have gone through several cycles of growth into stationary phase, the proportion of cells with genetic rearrangements is even higher. Therefore, we suspect that there is strong selection against the mutant gene in some stage of the growth cycle, perhaps when entering or leaving stationary phase.

DISCUSSION

Yeast are unusual in that they have duplicated the genes for a number of important proteins. These include several

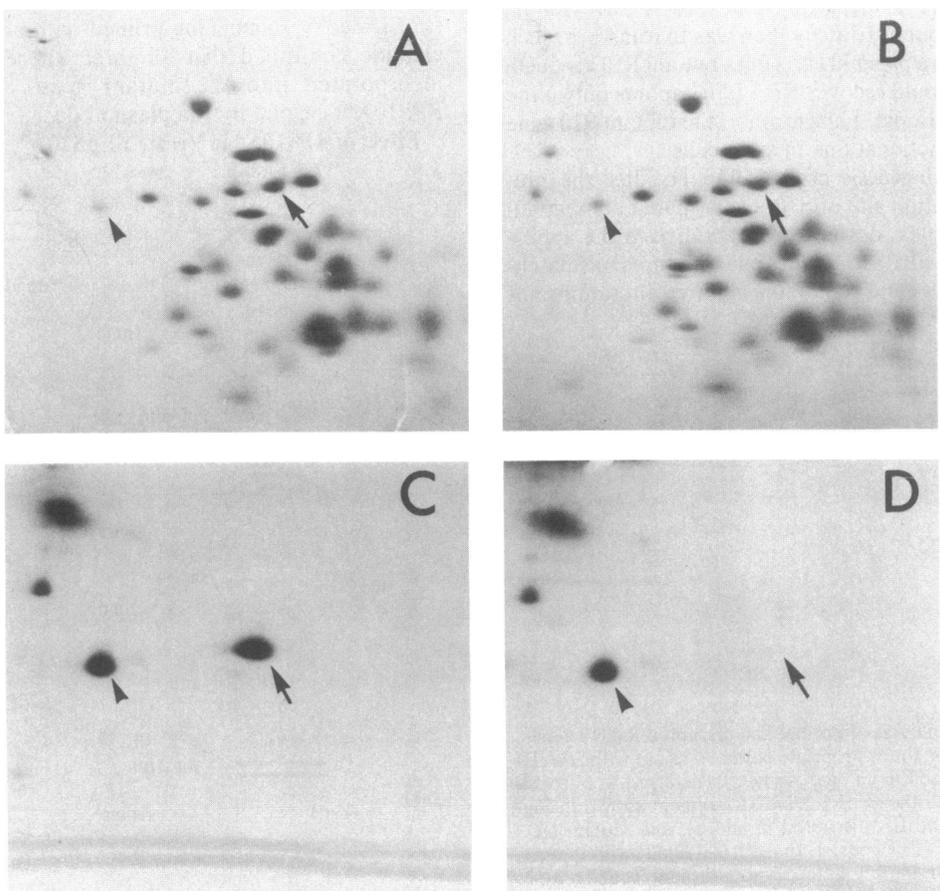

FIG. 6. Two-dimensional PAGE of yeast ribosomal proteins (see *Materials and Methods*) from control cells (*A* and *C*) and from cells derived from a spore with no intact chromosomal S10 genes (see Fig. 4 *Right*, lane 2) (*B* and *D*). Cells were incubated with $^{32}PO_4$ for 60 min. (*A* and *B*) Coomassie blue-stained gels. (*C* and *D*) Autoradiographs of dried gels. Arrows point to S10; arrowheads point to rp14.

enzymes of the Embden–Meyerhof pathway (31), histones (32), and a number of ribosomal proteins (25–27). The results presented in Table 1 make it clear that both S10 genes can be active and that either gene alone is sufficient for growth at nearly the rate of wild-type cells. This is in some contrast to the situation for ribosomal protein rp51, in which case the disruption of one of the two genes had little if any effect on growth, but disruption of the other increased the doubling time by 50% (33).

The identity of the phosphorylated serines on S10 was presumed by homology with rat (Fig. 1) (14, 15). The observation that removal of those two serines abolishes phosphorylation of S10 substantiates our presumption. R. Wettenhall (personal communication) has found that these two serines of S10 can be phosphorylated *in vitro* by both cAMP-dependent and cAMP-independent protein kinases.

Analysis of the effect of a mutant ribosomal protein gene is complicated because each ribosomal protein not only must function within the ribosome during protein synthesis but also must be able to participate in the assembly of the ribosome. Evidently neither the terminal serines nor their phosphorylation is essential either for protein synthesis or for ribosome assembly. It is not known whether phosphorylation of S10 can occur before it is assembled into the ribosome, although newly synthesized S10 molecules are fully phosphorylated within about 5 min (34).

The clear finding of this study is that cells can grow, albeit somewhat more slowly, even though they are unable to phosphorylate their S10 molecules. This is perhaps not surprising, since nondividing mammalian cells synthesize proteins at 25–35% of their maximal rate with little if any phosphorylation of their S6 ribosomal protein (2). Nevertheless, the lack of phosphorylation of S10 in yeast does slow the growth rate and there appears to be a strong selection for cells in which genetic rearrangement has regenerated an S10 that can be phosphorylated.

These observations lead to questions at four levels. (*i*) What physiological signal causes the phosphorylation? (*ii*) What biochemical process carries out the phosphorylation? (*iii*) What biochemical process(es) does the phosphorylation affect? (*iv*) What physiological effect(s) does the altered biochemical process cause? The ability to compare wild-type cells with those unable to phosphorylate their S10 ribosomal protein will facilitate our approach to these questions.

There exists some confusion about the origin of the phosphates on S6. Wettenhall and Morgan (14) found that cAMP-dependent protein kinase can phosphorylate at least the two serines of S6 indicated in Fig. 1, using ATP as a donor. Martin-Perez *et al.* (7) found the same phosphopeptides labeled *in vitro* by cAMP-dependent protein kinase and *in vivo* by treatment of cells with growth factors. Other workers, who have not identified the residues phosphorylated, find phosphorylation of S6 *in vitro* with cAMP-dependent (35) and -independent (35–37) kinases using ATP (35, 36) or GTP (37) as a donor. The recent identification of several genes involved in cAMP metabolism and function in yeast, including the two *RAS* genes (summarized in ref. 38), suggests that it may be possible to use yeast to clarify whether cAMP-dependent or -independent kinases are operating on S6, as well as to understand the role of S6 phosphorylation in cell growth. For instance, if the cAMP-dependent phosphorylation of S6 (yeast S10) plays a major role in the cAMP repertoire, then cells with S10* should be refractory to the phenotype caused by the $RAS2^{Val\text{-}19}$ mutation, which leads to constitutively elevated cAMP levels (38).

The key to S6 phosphorylation seems to be its rapid response to changing growth conditions. This can be either a positive change such as addition of growth factors, which stimulates phosphorylation (7), or a negative change such as heat shock, which stimulates dephosphorylation (39). We presume that the selection for rearrangements leading to wild-type S10 is occurring during periods of changing growth conditions, such as entry into stationary phase or, more likely, the transition from stationary to exponential phase. We are now in a position to ask at what points in the growth cycle the ability to phosphorylate S10 provides a selective advantage.

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