Phosphorylation of the Acidic Ribosomal P Proteins in *Saccharomyces cerevisiae*: A Reappraisal[†]

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ABSTRACT: Previous reports had pointed to serines 62 and 71/79 as possible phosphorylation sites in the yeast acidic ribosomal proteins YP1α and YP2α, respectively. However, it has been found that mutation of these serine residues did not affect the phosphorylation level of the proteins. A detailed examination of the YP2α tryptic digest from the *in vivo* labeled protein demonstrates the existence of a totally trypsin-insensitive site at lysine 88 that led to a misinterpretation of previous results. The unique YP2α tryptic phosphopeptide obtained contains, in addition to serines 71 and 79, a serine at position 96 near the carboxyl end, which automatic Edman degradation confirmed as the phosphorylated residue. In addition, by using *Staphyloccocus* protease V8, it was possible to obtain phosphopeptides containing only serine 96, whose phosphorylation has likewise been confirmed by radioactive labeling as well as by chemical methods. A similar analysis of the other 12 kDa acidic proteins, YP1α, YP1*β*, and YP2*β*, has shown the presence of equivalent phosphorylation sites in the four P proteins, which correspond to position 96 in proteins YP1α, YP1*β*, and YP2α and position 100 in YP2*β*. This conclusion has been confirmed by the fact that mutation of serine 96 in proteins YP1α and YP2α abolishes their capacity to be phosphorylated *in vivo*. The mutation of the phosphorylation site of the individual acidic proteins seems not to alter their interaction with the ribosome. However, it has been found that the level of phosphorylation of the stalk proteins has an effect on the response of the cells to some specific metabolic conditions, indicating that it may modulate the translation of specific proteins.

The overall structure of the ribosome is generally accepted to have been well-conserved during evolution, in spite of specific ribosomal structural features that can be used as useful evolutional markers (Lake et al., 1982). The stalk, a lateral protuberance of the large ribosomal subunit involved in the function of the supernatant factors during the translation process, is among the most conserved ribosomal structural elements. The stalk is built by a pentameric protein complex made of a rRNA-binding protein and two dimers of a set of very acidic proteins. In bacteria, protein L10 interacts with the 23S rRNA and proteins L7 and L12 (L7 is the N-terminal-acetylated form of L12) are the acidic components. The eukaryotic counterparts of the bacterial stalk components have been called by different names depending on the organisms, but they tend to be generically called P proteins, because they are found mostly phosphorylated in the ribosomes. Protein P0 is the eukaryotic L10 equivalent, and the acidic proteins belong to two closely similar but not identical types, called P1 and P2. In lower eukaryotes (yeast, protozoa, plants) there are multiple forms of the P1 and P2 proteins [see Ballesta and Remacha (1996) for a recent review]. In *Saccharomyces cerevisiae*, two P1's, YP1α and YP1*β*, and two P2's, YP2α and YP2*β*, have been described (Newton et al., 1990).

Although the stalk components seem to be structurally and functionally analogous in all systems, there are clear differences among them that, in the case of eukaryotes, suggest the evolutional acquisition of new functions probably related to the regulation of the ribosomal activity (Remacha et al., 1995a,b). One of the most interesting characteristics of the eukaryotic stalk system is the phosphorylated state of its components. In *S. cerevisiae* the acidic ribosomal proteins are found phosphorylated in a single position (Sanchez-Madrid et al., 1981), while in mammalians, multiple phosphorylation states of the P1 and P2 proteins have been found (Lin et al., 1982). In addition, it was reported that *in vitro* dephosphorylation of the acidic proteins from mammalians (MacConnell & Kaplan, 1982) and yeast (Juan-Vidales et al., 1984) inhibited the capacity of the polypeptides to bind to the ribosome.

The presumable relevance of the P protein phosphorylation led us to the identification of the phosphorylated sites in the yeast acidic proteins. Characterization of tryptic digests derived from ^{32}P-labeled proteins indicated that peptides containing serines 62, 73, and 19 were radioactive in YP1α, YP1*β*, and YP2*β*, respectively (Naranda & Ballesta, 1991; Naranda et al., 1993). In protein YP2α, the presence of two serine residues in the labeled peptide, serines 71 and 79, did not allow a precise identification of the phosphorylation site, and in order to unambiguously identify the phosphorylated serine in this protein and to analyze its functional implication, a site-directed mutational study was carried out. The results,

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showing new aspects of the P protein phosphorylation, are included in this report.

MATERIALS AND METHODS

Strains and Growth Media. *S. cerevisiae* W303-1b (α; *leu2-3,112, trp1-1, ura3-1, his 3-11,15, ade2-1, can1-100*) and *S. cerevisiae* 142 (**a**; *his3, can1*) were used as control strains. *S. cerevisiae* D45 (α; *leu2-3, 112, trp1-1, rpYP2α(L44)::URA3, rpYP2β(L45)::HIS3)* and *S. cerevisiae* D67 (α; *ura3-1, his3-11,15, rpYP1α::LEU2, rpYP1β(L44′)::TRP1*) were derived from W303 by gene disruption (Remacha et al., 1992). Yeasts were grown in either YEPD (1% yeast extract, 2% peptone, and 2% glucose) or YNBD (0.67% amino acid-free yeast nitrogen base and 2% glucose). *Escherichia coli* DH5α, grown in LB medium (1.0% bactotryptone, 0.5% yeast extract, and 0.5% NaCl), was used for transformation and propagation of plasmids.

Cell Transformation. *E. coli* cells were transformed according to Hanahan (1985). *S. cerevisiae* transformations were performed either by the lithium acetate method (Ito et al., 1983) or by electroporation in a Bio-Rad Gene pulser following the manufacturer's instructions.

Recombinant DNA Techniques. Restriction endonucleases, T4 DNA ligase, Klenow DNA polymerase I fragment, and other enzymes were purchased from Boehringer Mannheim (Germany), New England Biolabs, or Amersham (U.K.).

DNA preparation, restriction enzyme digestions, agarose gel electrophoresis, ligation of DNA fragments, Southern blots, etc., were carried out according to standard techniques (Sambrook et al., 1989). DNA was sequenced by the dideoxy chain termination method using universal primers and complementary oligonucleotides.

Plasmids

pFL36-L44 Series. The plasmids were constructed by subcloning either the wild-type (pFL36-L44) or different mutated rpYP2α genes (pFL36-L44cys71, pFL36-L44cys79, pFL36-L44cys71/79) from BS-L44 in the *Bam*HI-*Sma*I sites of the centromeric vector pFL36 (Bonneaud et al., 1991). BS-L44 was obtained by cloning in the *Eco*RI site of Bluescript KS, a 2.3 kbp *Eco*RI-*Eco*RI fragment from plasmid pMRE44 (Remacha et al., 1988) containing the wild-type rpYP2α gene. Site-directed mutagenesis was carried out by PCR (Dieffenbach & Dveksler, 1995) on plasmid BS-L44 using mutagenic oligonucleotides prepared by Isogen Bioscience (Maarsen, The Netherlands). Serines 71 and 79 were replaced by cysteine, and serine 96 was replaced by alanine either independently, as in pFL36-L44cys71, pFL36-L44cys79, and pFL36-L44ala96, or simultaneously, as in pFL36-L44cys71/79, pFL36-L44cys79/ala96, and pFL36-L44cys71/79/ala96. At the same time, a new restriction site, *Pst*I in the case of serines 71 and 79 and *Sac*II in the case of serine 96, was introduced close to the mutated residues without affecting the amino acid sequence of the protein. Mutations were confirmed by restriction analysis as well as by direct sequencing of the mutated genes.

pFL38-L47 Series. This series, carrying the wild-type and mutated YP1α genes, was obtained in a way similar to the previous one. The plasmid BS-L47, used as a source of the YP1α gene, was obtained by subcloning a 2.0 kbp *Eco*RI−*Bam*HI fragment from plasmid pSceL12IIA (Newton et al., 1990) into Bluescript KS. Mutagenesis was carried out by PCR on BS-L47 replacing serine 62 by cysteine and/or serine 96 by valine; at the same time, new *Pst*I and *Sal*I sites were introduced near the respective mutated sites. The genes, as a *Bam*HI-*Eco*RI fragment, were transferred to pFL38 yielding the plasmids pFL38-L47, pFL38-L47cys62, pFL38-L47val96, and pFL38-L47cys62/val96.

Cell Fractionation. Ribosomes were obtained as previously described (Naranda et al., 1993). In summary, late logarithmic-phase cells were broken by shaking with glass beads in a vortex or by grinding with sea sand in 20 mM Tris-HCl (pH 7.4), 80 mM KCl, 10 mM $MgCl_2$, 2.5 mM β-mercaptoethanol including a cocktail of protease inhibitors (0.5 mM phenylmethanesulfonyl fluoride, 10 μg/mL aprotinin, 2 μg/mL leupeptin, 2 μg/mL pepstatin A), and phosphatase inhibitors (10 mM NaF, 40 mM β-glycerophosphate, 5 mM EGTA). The ribosomes and S-100 supernatants were prepared from the extracts by high-speed centrifugation. The particles were resuspended in 20 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 50 mM KCl, and 5 mM β-mercaptoethanol and stored at −20 °C.

Acidic proteins (SP fractions) were extracted by washing the ribosomes with 0.5−1 M NH_4Cl in 50% ethanol (Sanchez-Madrid et al., 1979).

P Protein Purification. Acidic proteins were purified from SP fractions by reverse-phase HPLC[1] on a Spherisorb C3 column (300 Å, 5 μm, 75 × 4.6 mm) (Saenz-Robles et al., 1988). Elution was performed using a 30−50% acetonitrile−trifluoroacetic acid gradient at room temperature at a constant flow rate of 0.5 mL/min. Proteins were detected by UV absorption at 224 nm and analyzed either by indirect ELISA using monoclonal antibodies (Vilella et al., 1991) or by isoelectrofocusing.

Electrophoretic Methods. The acidic protein fractions ($SP_{0.5}$) were analyzed on horizontal 5% polyacrylamide/8 M urea isoelectrofocusing gels in the 2.5−5.0 pH range (Juan-Vidales et al., 1984). Whole ribosomes (1 mg) were directly analyzed in similar conditions but in vertical gels after treatment with RNase A (20 μg) for 60 min at 0 °C. The lyophilized samples were dissolved in 10−15 μL of 6 M urea and 0.65% ampholytes (2.5−5.0 pH) and used for isoelectrofocusing. Proteins were either detected by silver staining or blotted to either PVDF or nitrocellulose membranes by electrophoresis in a semidry system using Novablot LKB buffer. Proteins in membranes were immunodetected using specific P protein monoclonal antibodies (Vilella et al., 1991) following standard procedures (Towbin et al., 1979).

Protein Phosphorylation. Proteins were phosphorylated *in vivo* by adding 1 mCi of $^{32}PO_4^{3-}$ to mid-log-phase cells growing in 100 mL of a low-phosphate medium (Rubin, 1973). Cells were allow to grow for 90 min, collected by centrifugation, and processed for preparation of ribosomes as usual.

Proteolysis of Acidic Proteins. *Trypsin Treatment*. The acidic proteins (20 μg), purified by HPLC, were dissolved in 25 μL of 8 M guanidine and diluted before treatment up to 200 μL with 0.1 M Tris-HCl, pH 8.0, to a final concentration of 1 M guanidine. The enzyme-to-protein ratio ranged from 1:10 up to 10:1 depending on the conditions (see text). Treatment was performed at 37 °C for 18 h.

[1] Abbreviations: HPLC, high-pressure liquid chromatography; ELISA, enzyme-linked immunosorbent sandwich assay.

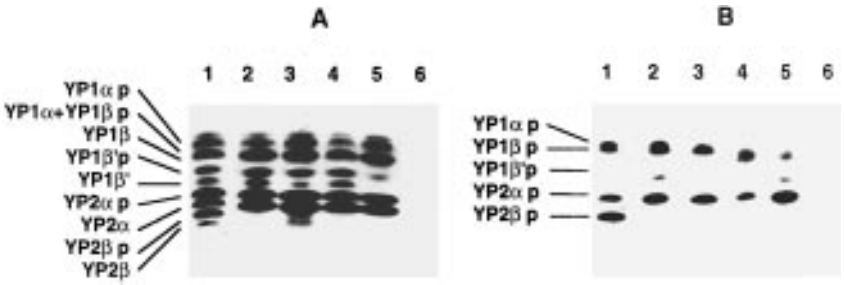

FIGURE 1: Isoelectrofocusing of acidic proteins in ribosomes from wild-type *S. cerevisiae* (lane 1) and D45 strain transformed with pFL36-L44cys71/79 (lane 2), pFL36-L44cys79 (lane 3), pFL36-L44cys71 (lane 4), and wild-type rpYP2α (lane 5): (A) silver-stained gel, (B) autoradiography of gel from *in vivo* ^{32}P-labeled ribosomes. The acidic proteins were extracted from the ribosomes by NH_4Cl/ethanol and resolved by isoelectrofocusing in a pH range from 2.5 (top) to 5.0 (bottom). Only the relevant proteins are indicated. The phosphorylated forms of the proteins are marked by a "p".

Trypsin (Sigma sequencing grade) was divided into three aliquots that were added at the beginning of the reaction, after 2 h, and finally after 14 h of incubation. The reaction mixture was directly used for HPLC separation of peptides.

Staphylococcus V8 Protease Treatment. Protein (20 μg) was dissolved in 50 μL of 50 μM NH_4HCO_3, pH 8.0, and digested with 0.5 μg of protease for 12 h at 25 °C. The samples were directly resolved by HPLC.

Peptide Analysis. The tryptic digests were separated by HPLC with a Waters Delta Pack C18 (300 Å, 5 μm, 150 × 3.9 mm) column using a nonlinear water/acetonitrile (0−60%) gradient in 0.1% trifluoroacetic acid at a flow rate of 0.5 mL/min. Detection of peptides was carried out at 214 nm. The peptide peaks were collected, concentrated by vacuum centrifugation, and stored at −20 °C.

For ELISA evaluation of peptides an aliquot of the peak before concentration, proportional to the A_{214} of the sample, was diluted in 50 mM sodium carbonate buffer, pH 9.6; 100 μL of the diluted sample was bound to plastic microtitration plates by incubation at room temperature for 30 min and then overnight at 4 °C. After washing, the plates were treated with a specific anti-P protein C-terminal monoclonal antibody as described previously (Vilella et al., 1991).

Peptide Sequencing. Peptides obtained from HPLC separation of protein digests were concentrated and sequenced by Edman degradation in an Applied Biosystems 447 automatic peptide sequenator at the Centro de Biologia Molecular Protein Sequencing Service.

Immunological Techniques. The acidic proteins were detected in S-100 fractions, ribosomal extracts, and HPLC fractions using monoclonal specific antibodies by indirect ELISA as previously described (Vilella et al., 1991).

Other Techniques. Protein concentration was estimated according to Bradford (Bradford, 1976).

RESULTS

Effect of Mutation of Putative Phosphorylation Sites in Proteins YP1α and YP2α on Protein Activity. Previous data suggested that serine 62 and either serine 71 or 79 correspond to the phosphorylation sites in proteins YP1α and YP2α, respectively (Naranda et al., 1993). To distinguish between the two YP2α sites and to confirm the YP1α identification, mutation of the respective sites was carried out in both proteins.

In the first case, *S. cerevisiae* D45, a strain defective in proteins YP2α and YP2β (Remacha et al., 1992), was transformed with the constructs carrying the YP2α gene mutated at either serine 71 (rpYP2α-71cys) or serine 79 (rpYP2α-79cys) or at both positions simultaneously (rpYP2α-71/79cys). As a control, the cells were transformed with the rpYP2α wild-type gene. All the transformed strains had a similar doubling time in rich and minimal medium of around 95 and 145 min, respectively. Ribosomes from the different strains were prepared and the acidic proteins identified by isoelectrofocusing (Figure 1) and estimated by ELISA (Table 1). Untransformed strain D45 does not contain acidic proteins because the P1 proteins present in the cell do not interact with the particle in the absence of P2 proteins (Remacha et al., 1992) but recovers these proteins when transformed with the wild-type rpYP2α gene (Table 1 and Figure 1A, lanes 5 and 6). Either independent or simultaneous mutations at serines 71 and 79 did not affect the capacity of YP2α to bind itself to the particle and to induce the binding of YP1β (Table 1 and Figure 1A lanes 2−4). Moreover, the phosphorylated form of protein YP2α is present in the ribosomes irrespective of the strain, indicating, therefore, that the mutated proteins are susceptible to phosphorylation in the cell. This point was confirmed further when cells were labeled with $^{32}PO_4^{3-}$, the radioactive form of YP2α being present in the ribosomes from all the samples (Figure 1B).

Table 1: Effect of YP2α Mutations on Protein Binding to the Ribosome

strain	transforming plasmid	P proteins in ribosomes estimated by ELISA[a] (A_{450}) YP1β	YP2α	YP2β
142 (wild-type)		1.30	1.41	1.57
D45		0.02	0.00	0.01
D45-YP2αw-t	pFL39leu44	1.34	1.45	0.02
D45-YP2αcys71	pFL39leu44/cys71	1.06	1.48	0.01
D45-YP2αcys79	pFL39leu44/cys79	1.30	1.50	0.00
D45-YP2αcys71/79	pFL39leu44/cys71/79	1.32	1.23	0.04

[a] Ribosomes were obtained from cells grown in YEPD. P proteins were estimated in the split protein extracts ($SP_{0.5}$) by ELISA using specific monoclonal antibodies. The A_{450} in the peroxidase reaction is proportional to the amount of protein in the extracts.

Similar results were obtained when *S. cerevisiae* D67, lacking proteins YP1α and YP1β (Remacha et al., 1992), was transformed with constructs carrying the YP1α gene mutated at serine 62 (rpYP1α-62cys) (lane 4, Figure 4A).

Characterization of Tryptic Phosphopeptides from HPLC-Purified Proteins. These results apparently contradict the initial identification of the phosphorylation sites in the yeast acidic ribosomal proteins (Naranda et al., 1993). The existence of alternative phosphorylation sites which are modified upon mutation of the original one might explain this apparent contradiction. To explore this possibility,

Table 2: Tryptic Peptides Derived from Protein YP2α Resolved by HPLC

peak	t_R (min)	exptl amino end sequence	theoretical tryptic peptide[a]	radioactivity[b] (cpm × 10^{-3})	reactn with anti-C-terminal antibody[c] (A_{450})
1	38.0	YLAAY	3-YLAAY-7	0.13	0.02
		LLLN...	8-LLLN...DATK-21		
2	41.2			0.14	0.01
		SVDE...	49-SVD...NEK-60		
3	43.0	MKYLAAY	1-MKYLAAY-7	0.17	0.01
4	52.5	VSSVL...	38-VSS...EGK-48	0.33	0.03
5	54.0	AILESVGIEIEDEK...	24-AILE...DEK-37	2.24	0.03
6	58.0	LAAVPAAGPASAGGAAAASG...	61-LAA...EEK-88	69.97	1.05
7	59.7	LAAVP...	61-LAA...EEK-88	3.86	0.58
8	62.1	YLAAYLL...	3-YLAA...ATK-21	1.71	0.08
9	71.2	AILESVGI...	24-AILE...DEK-37	1.35	0.04

[a] See Figure 2A. [b] Data from a representative experiment. The different specific radioactivity of the original labeled proteins in four experiments makes the average values irrelevant. [c] Peptides were estimated by ELISA as indicated in Materials and Methods using a monoclonal antibody specific for the highly conserved C-terminal peptide of the P proteins. The data correspond to the average of four experiments.

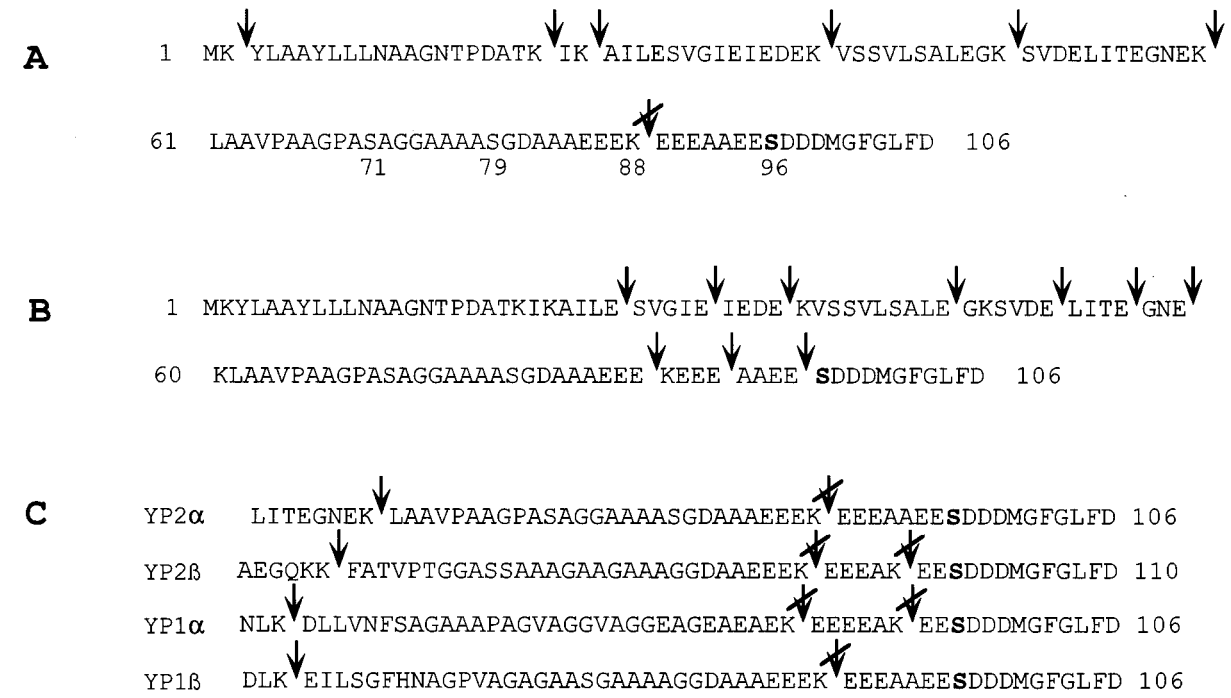

FIGURE 2: Putative protease-sensitive sites in the amino acid sequence of yeast acidic proteins: (A) trypsin-sensitive sites in protein YP2α, (B) *Staphylococcus* V8 protease-sensitive sites in protein YP2α, (C) trypsin-sensitive sites in the C-terminal domain of the four yeast acidic ribosomal proteins; down arrow, sensitive site; slashed arrow, insensitive site. In bold type is the phosphorylated serine.

peptides derived from a trypsin treatment of *in vivo* ^{32}P-labeled YP2α in conditions that should result in complete degradation were thoroughly analyzed. Nine main peaks were reproducibly obtained from all the YP2α tryptic digests resolved by HPLC and were characterized by partial amino terminal sequencing (Table 2). All the expected tryptic peptides were found except the one resulting from hydrolysis at lysine 88 (Figure 2A), which contains the last 18 amino acids from the C-terminus of the protein. The greater part of the radioactivity (91.1% average of four experiments), in the wild-type as well as in the three mutant proteins YP2α-cys71, YP2α-cys79, and YP2α-cys71/79, was associated with peak 6, which contains a peptide starting at leucine 61, confirming our previous report (Naranda et al., 1993). The peptide in peak 7 corresponds to the nonphosphorylated form of the one in peak 6 (see below).

The main radioactive peptide starting at leucine 61 must result from hydrolysis at lysine 60 and should end at lysine 88, containing, therefore, serines 71 and 79 (Figure 2A). However, phosphorylation of this peptide in the double-mutated protein YP2α-cys71/79 is obviously impossible. The most simple interpretation of the presence of radioactivity in the peptide from the mutated protein is that trypsin is unable to cut at lysine 88 in the YP2α amino acid sequence, and consequently, the labeled peptide probably extends up to the end of the protein. This possibility is in agreement with the absence in the HPLC chromatogram (Table 2) of the C-terminal peptide starting at glutamic acid 89 and is also supported by the positive reaction of peaks 6 and 7 with a P protein C-terminus specific monoclonal antibody (Table 2), confirming the presence of the YP2α carboxyl end in both peptides.

To directly confirm the failure of trypsin to cut at lysine 88, Edman degradation of the YP2α-cys71/79 phosphopeptide was carried out for 32 cycles yielding the sequence 61-LAAVPAAGPAXAGGAAAAXGDAAAEEEKEEEA-92 (X corresponds to cysteine residues undetectable by Edman degradation). This sequence confirmed the mutations at serines 71 and 79 and, more importantly, the continuity of the sequence over the putative trypsin-sensitive lysine 88.

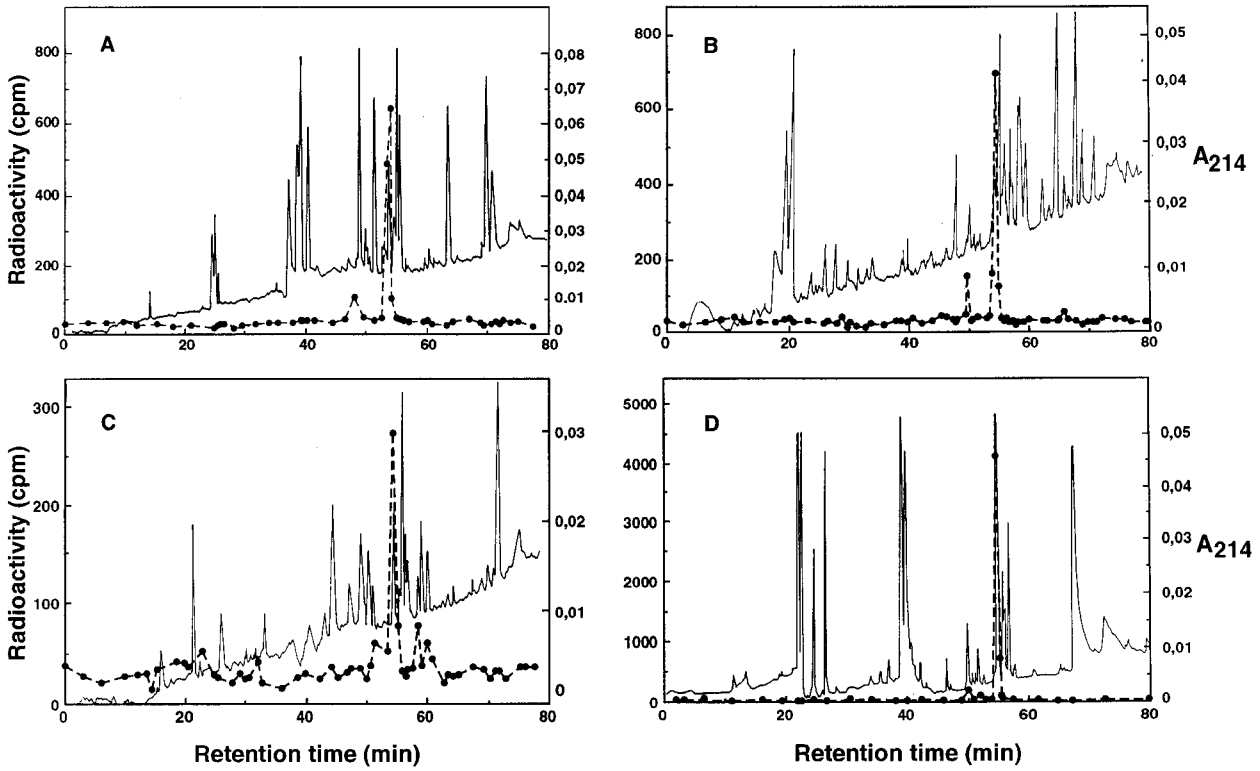

FIGURE 3: Analysis of peptides from acidic ribosomal proteins hydrolyzed by the *Staphylococcus aureus* glutamyl endopeptidase V8. YP2α (A), YP1α (B), YP1β (C), and YP2β (D) were treated with V8 protease and resolved by HPLC. Fractions were collected and tested for radioactivity (●). All radioactive peaks were collected.

Several attempts were made to force the hydrolysis of YP2α at lysine 88 under different conditions, including very high protein-to-enzyme ratios (up to 1:10), the presence of denaturing agents, and long incubation times (up to 18 h). However, the same main radioactive phosphopeptide was obtained in all cases indicating that the peptide bond at lysine 88 in YP2α is totally insensitive to trypsin.

Identification of Acidic Protein Phosphopeptides by Staphylococcus V8 Glutamyl Endopeptidase Treatment. The peptide analysis clearly indicated that trypsin is unable to hydrolyze YP2α at lysine 88, probably due to the strong acidic environment created by the surrounding glutamic residues. In addition, the results indicate that, at least in the case of the double mutant YP2α-cys71/79, phosphorylation must take place at serine 96, the only serine present in the peptide starting at alanine 61. In order to test whether this is also true for the other YP2α forms, they were treated with *Staphylococcus* V8 glutamyl endopeptidase.

The V8 protease cuts preferentially after glutamic residues (Birktoft & Breddam, 1994) and should be able to hydrolyze YP2α, separating serines 71 and 79 from serine 96 (Figure 2B). Therefore, ^{32}P-labeled wild-type protein YP2α was treated with the *Staphylococcus* protease, and the resulting peptides were resolved by HPLC (Figure 3A). As in the tryptic digest, most radioactivity appears associated with one peptide peak that had a retention time of 53.7 min and gave a positive reaction with the specific anti-P protein C-terminal monoclonal antibody. Automatic Edman degradation yielded the amino terminal sequence AAEESDDDMG, corresponding to the C-terminal peptide released upon hydrolysis at glutamic 91 (Figure 2B).

Since putative trypsin sites in an equally unfavorable environment are present in the C-terminal sequence of the remaining yeast acidic proteins (Figure 2C), the same effect may have also led to a misinterpretation of the previous tryptic analysis of these three proteins (Naranda & Ballesta, 1991; Naranda et al., 1993). To confirm this possibility, the phosphorylation site of YP1α, YP1β, and YP2β by *Staphylococcus* V8 protease treatment was analyzed.

The *in vivo* $^{32}PO_4^{3-}$-labeled proteins were digested with the protease and the peptides resolved by HPLC. A main labeled peptide with a similar retention time, around 53 min, was obtained in all cases (Figure 3B−D). The labeled peptides gave a positive reaction with the specific anti-C-end antibody, and partial amino terminal sequencing showed the same sequence, AKEESDDDMG, for those derived from YP1α and YP2β and the sequence AAEESDDDMG for the YP1β peptide. These sequences correspond to the respective C-terminus of the three proteins (Figure 2C), thus clearly indicating that the *in vivo* phosphorylated site also corresponds to the last serine in the C-termini of the three proteins.

Chemical Determination of Phosphorylated Serine Residues. A confirmation of the phosphorylated state of serine in the labeled peptides from the different proteins was obtained by estimating the formation of a dithiothreitol adduct of dehydroalanine (DTT-dhAla) in the Edman degradation cycle corresponding to the serine residue. During the degradation process, phosphoserine undergoes β elimination that splits off the phosphate group and yields dehydroalanine, which reacts with the dithiothreitol in the reaction mixture (Meyer et al., 1991); as a consequence of this reaction, the yield of the expected serine derivative is proportionally reduced. Therefore, a high DTT-dhAla-to-serine ratio in the Edman degradation reaction is a clear indication of phosphoserine presence in the sequence. This ratio has been calculated from the areas of the corresponding serine and DTT-dhAla/serine peaks in the chromatograms of the reaction products provided by the automatic peptide

Table 3: Chemical Identification of Phosphorylated Serine Residues by Automatic Edman Degradation

			amino acids[a]		
sequenced peptide	protein	residue	serine (peak area)	DTT-dhAla (peak area)	DTT-dhAla/serine
AILESVGIEIEDEK	YP2α	^{28}Ser	729276	91902	0.126
LAAVPAAGPASAGGAAAASG	YP2α	^{71}Ser	150833	2682	0.017
		^{79}Ser	56754	1668	0.029
AAEE**S**DDDMG	YP2α	^{96}Ser	12899	31276	2.42
AKEE**S**DDDMG	YP1α	^{96}Ser	14271	19584	1.37
AAEE**S**DDDMG	YP1*β*	^{96}Ser	10781	15556	1.44
AKEE**S**DDDMGFGLFD	YP2*β*	^{100}Ser	56307	112558	1.99
SERINE STANDARD[b]			109157	16858	0.15

[a] The area of the peaks in the chromatogram of the Edman degradation products is in arbitrary units as calculated by the automatic sequenator (A_{214} × time). [b] The serine and DTT-dehydroalanine average area, obtained from three standards containing 10 pmol of unmodified serine treated in the same conditions as the peptides, is shown.

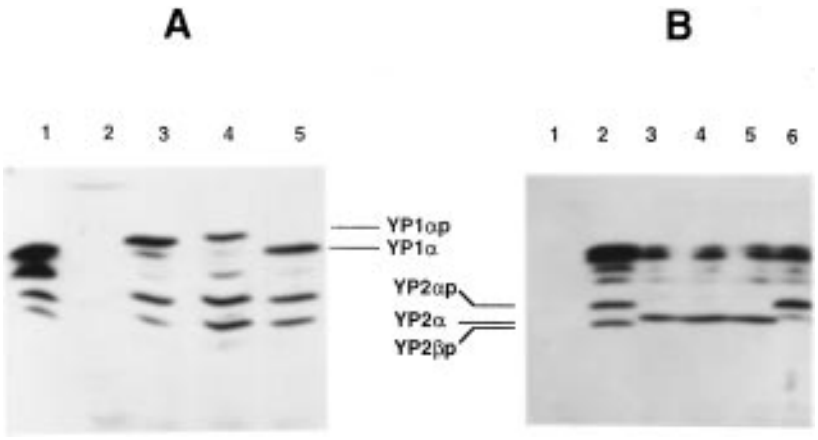

FIGURE 4: Effect of mutation of serine 96 in proteins YP1α and YP2α. Isoelectrofocusing of ribosomes from (A) *S. cerevisiae* D67 either untransformed (lane 2) or transformed with wild-type rpYP1α (lane 3), rpYP1α-cys62 (lane 4), and rpYP1α-val96 (lane 5); (B) *S. cerevisiae* D45 either untransformed (lane 1) or transformed with rpYP2α-ala96 (lane 3), rpYP2α-cys79/ala96 (lane 4), rpYP2α-cys71/79/ala96 (lane 5), and wild-type rpYP2α (lane 6). Ribosomes from wild-type *S. cerevisiae* (lanes 1A and 2B) were used as a control. Ribosomes (1 mg) digested with RNase A were directly loaded into the gels.

sequenator (Table 3). An average ratio of 0.05 was obtained in three cycles corresponding to nonphosphorylated serines, including YP2α serines 28, 71, and 79, while the average value of four modified serines, namely, serine 96 in YP1α, YP1*β*, and YP2α and serine 100 in YP2*β*, was 1.80. The ratio obtained from standards of unmodified serine treated in the same conditions, used as a control, was 0.15.

Mutation of Phosphorylation Sites in Proteins YP1α and YP2α. To confirm the biochemical data, serine 96 in YP1α and YP2α was changed to valine in the first protein and to alanine in the second one. In the case of YP2α, the substitution of serine 96 was also carried out in the gene previously mutated in serines 71 and 79. The plasmid carrying the mutated YP1α and YP2α genes (see Materials and Methods) was used to respectively transform *S. cerevisiae* D67 and D45. The two P1 protein genes are disrupted in D67, and the two P2 protein genes are disrupted in D45 (Remacha et al., 1992).

Ribosomes from the transformed strains were obtained, and the acidic proteins were analyzed by isoelectrofocusing (Figure 4). As indicated previously, the ribosomes from the untransformed D45 and D67 strains do not have acidic proteins (lanes 2A and 1B) but retrieve them when transformed with one of the missing genes. However, the protein from the mutated samples appears exclusively in the dephosphorylated form in all cases (lanes 5A and 3−5B), while in the controls, either the wild-type proteins (lanes 3A and 6B) or the protein mutated in a different serine (lane 4A) is mostly present in the phosphorylated state (lanes 3A and 6B).

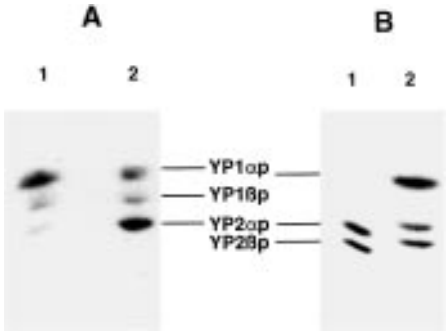

FIGURE 5: *In vivo* phosphorylation of mutated YP1α and YP2α proteins. Cells were grown in the presence of radioactive phosphate, ribosomes were obtained, and the acidic proteins were resolved by isoelectrofocusing as in Figure 4. Radioactive bands were detected by autoradiography: (A) *S. cerevisiae* D45 transformed with either rpYP2α-ala96 (lane 1) or wild-type rpYP2α (lane 2), (B) *S. cerevisiae* D67 transformed with either rpYP1α-val96 (lane 1) or wild-type rpYP1α (lane 2).

These results were confirmed by growing the cells in $^{32}PO_4^{3-}$, and it was found that the respective mutated protein in the corresponding strains did not show associated radioactivity in the autoradiograms of the isoelectrofocusing gels (lanes 1A and 1B in Figure 5).

Effect of Serine 96 Mutation on Protein Function. The previous results indicated that the mutated nonphosphorylated proteins YP1α and YP2α, respectively expressed in *S. cerevisiae* D67 and D45, are found associated with the ribosome, indicating that phosphorylation seems not to be

Table 4: Effect of Phosphorylation Site Mutation on Cell Growth Rate[a]

strain	transforming gene	doubling time (min) in YEPD	doubling time (min) in YNBD
w-t	none	90	149
D67	none	185	290
D67	rpYP1αw-t	116	147
D67	rpYP1α-val96	120	158
D45	none	217	272
D45	rpYP2αw-t	100	155
D45	rpYP2α-ala96	105	153

[a] The disruptant *S. cerevisiae* strains D67 (lacking proteins YP1α and YP2α) and D45 (lacking proteins YP2α and YP2β) were transformed with plasmid carrying the indicated genes. Strains were grown at 30 °C.

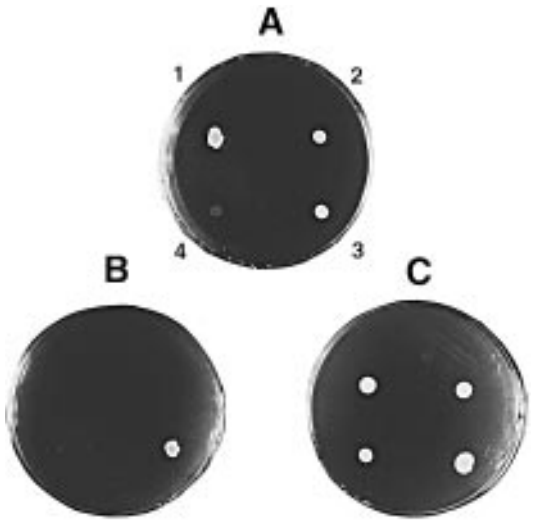

FIGURE 6: Effect of YP1α serine 96 mutation on the temperature sensitivity of *S. cerevisiae* D67. Strain D67, either untransformed (spot 4) or transformed with wild-type rpYP1α (spot 1), rpYP1α-cys62 (spot 2), and rpYP1α-ala96 (spot 3), was grown on YNBD plates at 30 °C (A), 36 °C (B), and 36 °C in the presence of 0.8 M sorbitol (C). Cells growing on YEP-galactose agar plates were resuspended in sterile water and placed in the appropriate plates which were incubated for 5 days in sealed plastic bags to avoid dryness.

an absolute requirement for binding to the ribosome. Moreover, the mutated polypeptides are also able to promote the interaction of the acidic proteins of the other type with the ribosome to a similar extent as in the wild-type controls (Figure 4). Likewise, the recovery of the respective disrupted strain growth rate induced in the transformants by the protein is similar for the mutated and the wild-type polypeptides growing at 30 °C in rich and minimal medium (Table 4).

However, it was found that only the YP1α protein carrying the serine 96 mutation, and neither the wild-type nor the serine 62-mutated protein, is able to allow growth of *S. cerevisiae* D67 at 36 °C in a galactose minimal medium. The temperature sensitivity of the D67 strain seems to be due to an osmoregulation defect since it is recovered by the presence of sorbitol in the plate (Figure 6).

DISCUSSION

An analysis of the phosphorylation site in the acidic ribosomal protein YP2α had previously indicated (Naranda et al., 1993) that the main tryptic phosphopeptide derived from the *in vivo* labeled polypeptide started at leucine 61. Assuming a complete digestion of the protein, this polypeptide would contain only serines 71 and 79 as possible phosphorylation sites (Figure 2A). Following a similar approach, serine 62 was proposed as the putative phosphorylation site in protein YP1α. However, the mutational analysis performed clearly indicated that the mutation of the previous serines did not alter the capacity of the proteins to be phosphorylated.

A total characterization of the tryptic peptides derived from purified ^{32}P-labeled YP2α has confirmed that in all cases the labeled phosphopeptide starts at leucine 61, but at the same time it has shown that the protein cannot be hydrolyzed by trypsin at lysine 88, and consequently, the peptide extends further downstream and includes the highly conserved carboxyl terminal decapeptide. Since, apart from serines 71 and 79, the only serine residue in the labeled peptide is at position 96, this has to be the actual phosphorylation site in the mutated protein.

A direct confirmation of this conclusion was obtained by treatment of the protein with the glutamyl endopeptidase from *Staphylococcus* protease V8, which by cleaving preferentially after glutamic residues (Birktoft & Breddam, 1994) is able to degrade YP2α separating serine 96 from the rest of the sequence (Figure 2B). In this way it has been shown that the main ^{32}P-labeled peptide derived from mutant as well as wild-type YP2α contains serine 96 as the unique serine residue. Moreover, characterization of the V8 phosphopeptides from the remaining acidic ribosomal proteins clearly indicates that phosphorylation takes place in all of them at the last serine in the sequence, close to the C-end of the protein, namely, serine 96 in YP1α and YP1β and serine 100 in YP2β. A chemical confirmation of this conclusion is provided by the accumulation of DTT-dehydroalanine, a product of the phosphoserine Edman degradation reaction, in the peptides proposed to be phosphorylated (Table 3).

The present results convincingly show, therefore, that the yeast P proteins are phosphorylated *in vivo* at the last serine close to the C-end, which is analogous to the position proposed to be modified in mammalian (Hasler et al., 1991) and *Artemia salina* P proteins (van Agthoven et al., 1978). Moreover, the fact that *Tetrahymena pyriformis*, the only eukaryotic organism in which phosphorylation of the acidic proteins has not been found (Sandermann et al., 1979), has P proteins that lack the last serine in the sequence (Hansen et al., 1991) supports the involvement of this residue in the protein modification. It has to be noted, however, that in the mammalian P proteins additional phosphorylation sites must be present since multiphosphorylated forms of these proteins have been reported (Lin et al., 1982), although no experimental data on their location is available.

Substitution of serine 96 in Yp1α and YP2α confirmed that this is the phosphorylated residue in the yeast P proteins but at the same time showed that phosphorylation seems not to play a role in the interaction of these proteins with the ribosome. Ribosomes from strains carrying these mutations contain apparently normal amounts of the nonphosphorylated mutated protein (Figure 4). Moreover, these results suggest that the proteins are probably phosphorylated when forming part of the stalk in the ribosome and not while they are free in the cytoplasm. The previous results indicating an effect on the P protein binding to the ribosome upon mutation of the erroneously identified phosphorylation sites (Naranda & Ballesta, 1991; Naranda et al., 1993) are probably due to

changes in the protein conformation that can affect protein−protein interactions and, consequently, its participation in the formation of the stalk.

Nevertheless, these results apparently contradict previous data showing an inactivation of the acidic proteins upon *in vitro* dephosphorylation (Sanchez-Madrid et al., 1981; MacConnell & Kaplan, 1982; Juan-Vidales et al., 1984). These results showed that treatment with phosphatase of either mammalian or yeast P proteins results in an inhibition of their capacity to reconstitute core ribosome particles. Apart from trivial explanations, such as undetected effects of the *in vitro* phosphatase treatment due to possible contaminations, this apparent discrepancy could result from the fact that the mutation is performed in only one out of the four acidic proteins forming the stalk while the *in vitro* treatment affects all of them simultaneously. Therefore, the possibility cannot be ruled out that phosphorylation of the remaining components of the complex, including protein P0, may overcome the negative effects produced by the absence of phosphate in one of them. This possibility is being explored by trying to simultaneously express the mutated form of all the stalk components, although this approach presents technical difficulties due to the number of genetic markers required. At the same time, *in vitro* binding studies using purified individual acidic proteins are being performed to analyze more carefully the effect of phosphorylation in this process.

Regardless of the role that phosphorylation may have in the interaction of the different stalk components with the ribosome, the results indicate that this modification somehow affects the ribosome function. Quite unexpectedly, only the nonphosphorylatable YP1α-ser96 is able to revert the apparent osmotic sensitivity of the D67 strain at 36 °C. Probably the ribosomes carrying the unphosphorylated stalk component are able either to promote the translation of proteins required for osmotic resistance or to inhibit the expression of polypeptides responsible for the osmotic sensitivity. Similar phenotypic effects have also been found in yeast strains carrying mutations at the phosphorylation site of the ribosomal protein P0 (Rodriguez Gabriel, 1997).

Previous data have clearly indicated that the composition of the stalk selectively modifies the efficiency of the yeast ribosome in translating certain mRNAs affecting the pattern of expressed proteins (Remacha et al., 1995a). Our present results are compatible with an effect of the phosphorylation of the stalk components in the translation of certain messengers. The mechanism by which this effect may take place is not presently understood, but it is probably not related to a change in the overall translation efficiency of the unphosphorylated ribosomes, since under standard conditions the doubling time of the cells carrying the unphosphorylated protein is not different from that of the cells expressing the wild-type protein. Moreover, the presence of sorbitol allows the cell transformed with either protein to growth at a similar rate at 36 °C. The effect of phosphorylation appears to be selective, perhaps due to peculiarities of some mRNAs which are differentially detected by the ribosome depending on the stalk conformation.

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REFERENCES

Ballesta, J. P. G., & Remacha, M. (1996) *Progr. Nucleic Acid Res. Mol. Biol. 55*, 157−193.

Birktoft, J. J., & Breddam, K. (1994) *Methods Enzymol. 244*, 114−126.

Bonneaud, N., Ozier-Kalogeropoulos, O., Li, G., Labouesse, M., Minvielle-Sebastia, L., & Lacroute, F. (1991) *Yeast 7*, 609−615.

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248−254.

Dieffenbach, C. W., & Dveksler, G. S. (1995) *PCR Primer. A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Hanahan, D. (1985) In *DNA Cloning: A Practical Approach.* (Glover, D. M., Ed.) pp 109−136, IRL Press, Oxford.

Hansen, T. S., Andreasen, P. H., Dreisig, H., Højrup, P., Nielsen, H., Engberg, J., & Kristiansen, K. (1991) *Gene 105*, 143−150.

Hasler, P., Brot, N., Weisbach, H., Parnassa, A. P., & Elkon, K. B. (1991) *J. Biol. Chem. 266*, 13815−13820.

Ito, H., Fukuda, Y., Murata, K., & Kimura, A. (1983) *J. Bacteriol. 153*, 163−168.

Juan-Vidales, F., Saenz-Robles, M. T., & Ballesta, J. P. G. (1984) *Biochemistry 23*, 390−396.

Lake, J. A., Henderson, E., Clark, M. W., & Matheson, A. J. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 5948−5952.

Lin, A., Wittman-Leibold, B., McNelly, T., & Wool, I. G. (1982) *J. Biol. Chem. 257*, 9189−9197.

MacConnell, W. P., & Kaplan, N. O. (1982) *J. Biol. Chem. 257*, 5359−5366.

Meyer, H. E., Hoffmann-Posorske, E., & Heilmeyer, L. M. G. (1991) *Methods Enzymol. 201*, 169−185.

Naranda, T., & Ballesta, J. P. G. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 10563−10567.

Naranda, T., Remacha, M., & Ballesta, J. P. G. (1993) *J. Biol. Chem. 268*, 2451−2457.

Newton, C. H., Shimmin, L. C., Yee, J., & Dennis, P. P. (1990) *J. Bacteriol. 172*, 579−588.

Remacha, M., Saenz-Robles, M. T., Vilella, M. D., & Ballesta, J. P. G. (1988) *J. Biol. Chem. 263*, 9094−9101.

Remacha, M., Santos, C., Bermejo, B., Naranda, T., & Ballesta, J. P. G. (1992) *J. Biol. Chem. 267*, 12061−12067.

Remacha, M., Jimenez-Diaz, A., Bermejo, B., Rodriguez-Gabriel, M. A., Guarinos, E., & Ballesta, J. P. G. (1995a) *Mol. Cell. Biol. 15*, 4754−4762.

Remacha, M., Jimenez-Diaz, A., Santos, C., Zambrano, R., Briones, E., Rodriguez Gabriel, M. A., Guarinos, E., & Ballesta, J. P. G. (1995b) *Biochem. Cell. Biol. 73*, 959−968.

Rodriguez Gabriel, M. A. (1997) Ph.D. Thesis, Autonomous University of Madrid, Madrid, Spain.

Rubin, G. M. (1973) *J. Biol. Chem. 248*, 3860−3875.

Saenz-Robles, M. T., Vilella, M. D., Pucciarelli, G., Polo, F., Remacha, M., Ortiz, B. L., Vidales, F., & Ballesta, J. P. G. (1988) *Eur. J. Biochem. 177*, 531−537.

Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989) *Molecular cloning. A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Sanchez-Madrid, F., Reyes, R., Conde, P., & Ballesta, J. P. G. (1979) *Eur. J. Biochem. 98*, 409−416.

Sanchez-Madrid, F., Juan-Vidales, F., & Ballesta, J. P. G. (1981) *Eur. J. Biochem. 114*, 609−613.

Sandermann, J., Krüger, A., & Kristiansen, K. (1979) *FEBS Lett. 107*, 343−347.

Towbin, H., Staehelin, T., & Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4350−4354.

van Agthoven, A., Kriek, J., Amons, R., & Möller, W. (1978) *Eur. J. Biochem. 91*, 553−556.

Vilella, M. D., Remacha, M., Ortiz, B. L., Mendez, E., & Ballesta, J. P. G. (1991) *Eur. J. Biochem. 196*, 407−414.

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