

# **The Acidic Protein Binding Site Is Partially Hidden in the Free *Saccharomyces cerevisiae* Ribosomal Stalk Protein P0**

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## **Abstract**

The ribosomal stalk is essential for translation; however, its overall structure is poorly understood. Characterization of the region involved in the interactions between protein P0 and the 12kDa acidic proteins P1 and P2 is fundamental to understand the assembly and function of this structure in the eukaryotic ribosome. The acidic protein content is important for the ribosome efficiency and affects the translation of specific mRNAs. By usage of a series of progressively truncated fragments of protein P0 in the two-hybrid test, a region between positions 213 and 250 was identified as the minimal protein part able to interact with the acidic proteins. Extensions at either end affect the binding capacity of the fragment either positively or negatively depending on the number of added amino acids. Deletions inside the binding region confirm its *in vivo* relevance since they drastically reduce the P0 interacting capacity with the 12 kDa acidic proteins, which are severely reduced in the ribosome when the truncated protein is expressed in the cell. Moreover, recombinant His-tagged P0 fragments containing the binding site and bound to Ni<sup>2+</sup>-NTA columns can form a complex with the P1 and P2 proteins, which is able to bind elongation factor EF2. The results indicate the existence of a region in P0 that specifically interacts with the acidic proteins. These interactions are, however, hindered by the presence of neighbor protein domains, suggesting the need for conformational changes in the complete P0 to allow the assembly of the ribosomal stalk.

## **Introduction**

The recent resolution of the crystal structure of different prokaryotic ribosomes represents a dramatic step forward in understanding the function of this fundamental cellular particle (1-3). Unfortunately, there are few structural features that, apparently due to their high mobility, are not resolved in previously reported 3-D structures. One of them is the so-called ribosomal stalk (4).

The stalk is one of the lateral protuberances of the large ribosomal subunit, which has been studied in detail in bacteria but scarcely approached in eukaryotic organisms. A direct and essential involvement of the stalk in the translocation step was soon reported (5) and has

recently been confirmed (6-8). In addition, its implication in the function of initiation (9) and termination factors (10) has been reported as well.

The bacterial stalk is a very flexible structure formed by a protein complex of two dimers of the acidic protein L7/12 and protein L10, which binds through the L10 amino terminal region to the highly conserved 23S rRNA GAR<sup>1</sup> region. This protein pentamer is extraordinarily stable, resisting high urea concentrations (11); nevertheless, it can be easily removed from the ribosome by high-salt washing (12).

The eukaryotic ribosomal stalk seems to perform the same basic functions as the bacterial structure regarding the activity of the elongation factors (13-15), although it presents important differences compared to its prokaryotic counterpart (16, 17). Thus, the unique bacterial acidic protein has evolved to two phosphoprotein families, P1 and P2, which, depending on the species, are formed by one or several members. In *Saccharomyces cerevisiae*, there are two members of each type, P1 $\alpha$ , P1 $\beta$  and P2 $\alpha$ , P2 $\beta$ . In addition, the role of bacterial L10 is played by the protein P0, which contains a carboxyl extension that structurally resembles the acidic proteins (18) allowing P0 to perform functions similar to those of P1 and P2. Because this function is duplicated, the eukaryotic acidic proteins, in contrast to the bacterial L7/L12, are not essential for cell viability (19).

The plurality of its components has raised questions about the actual composition of the eukaryotic stalk, especially in species with multiple acidic proteins. In organisms with only one member in each acidic protein family, a bacteria-like pentameric model based on one monomer, P0, and two dimers, P1<sub>2</sub> and P2<sub>2</sub>, has been postulated (20, 21), although the presence of P1/P2 heterodimers has also been reported (22, 23), and different alternative assembly models have been proposed (24). In *S. cerevisiae*, it has been shown that the prokaryotic model is not valid and the stalk is formed by P0 and one protein of each family, which form two preferred associations, P1 $\alpha$ /P2 $\beta$  and P1 $\beta$ /P2 $\alpha$  (25, 26). The stalk structure in species with more than two members in each acidic protein family or even a third type, P3 (27), is still unsolved.

More relevant from a functional point of view is the instability of the eukaryotic stalk. The eukaryotic pentamer is rather weak in contrast to the strong bacterial complex. Thus, conditions that remove L10-((L7/L12)<sub>2</sub>)<sub>2</sub> from the bacterial ribosome easily release the P1 and P2 proteins from the eukaryotic ribosome (28) but leave protein P0 tightly bound to the rRNA (29). It has also been shown that the ribosome-bound P1/P2 proteins are interchangeable with free proteins from the cytoplasm pool during protein synthesis (30-32). Moreover, not all the ribosomes in the cell seem to carry a full complement of acidic proteins (26), and particles lacking some of these components accumulate in certain metabolic conditions such as stationary phase in yeast (33). All these data clearly indicate that the eukaryotic stalk is a highly dynamic structure. The mobility of the P1/P2 proteins, together with the capacity of protein P0 to perform the stalk functions, confers a regulatory potential to the acidic stalk components (16).

Obviously, important changes in the binding affinity of the acidic proteins for P0 must have occurred during evolution, which facilitates the exchange of the P1/P2 proteins. In addition, the higher affinity of P0 for the rRNA must be the result of alterations at the protein

RNA-binding site, since the GAR domain, where P0 binds, is highly conserved and even functionally interchangeable between eukaryotes and prokaryotes (15, 34, 35). Unfortunately, as commented previously, a high-resolution model of the stalk, which would facilitate an interpretation of these changes in the stalk properties at the molecular level, is not available.

A first step toward understanding the functional peculiarities of the eukaryotic stalk is to characterize in detail the interaction among its different components. We have focused on the acidic protein binding site in the yeast protein P0. An important role of the P0 carboxyl terminal domain in the stalk assembly has already been defined (36, 37). In this report, the smallest P0 fragment involved in the interaction with the acidic proteins, which must play a central role in the binding site, has been characterized, and its implication in the assembly and recycling of the P1/P2 proteins is discussed.

## Experimental Procedures

**Bacterial and Yeast Strains.** *Escherichia coli* DH5 $\alpha$  strain, used for plasmid manipulations, was grown in LB medium. *E. coli* BL21pLys (38), used for preparation of histidine-tagged P0 fragments, was grown in M9ZB medium.

*Saccharomyces cerevisiae* PJ69-6, was the reporter host strain for the two-hybrid assay. *S. cerevisiae* W303dGP0, a P0 conditional null mutant (39), was used to express exogenous P0 proteins and fragments, and *S. cerevisiae* W303 (MAT  $\alpha$ , *leu2-3, 112, ura3-1, trp1-1, his3-11, 15, ade2-1, can1-100*) was used as a wild-type control strain. Yeast strains were grown at 30 °C in YEP medium (1% yeast extract, 2% peptone) with either glucose (YEPD) or galactose (YEPG) (2%) as a carbon source.

**Cell Transformations.** Bacterial transformations were performed according to the method described by Hanahan (40). Yeasts were transformed using lithium acetate as described previously (41). The transformed cells were selected on SD plates lacking the required amino acids.

**Cell Fractionation.** Exponentially grown *S. cerevisiae* W303dGP0 cells in 20 mM Tris-HCl (pH 7.4), 10 mM MgCl<sub>2</sub>, 60 mM NH<sub>4</sub>Cl, and 5 mM 2-mercaptoethanol (buffer 1) were broken with glass beads in a FastPrep instrument (Bio101). Cell extracts were then centrifuged 15 min at 30 000g, and the supernatant was centrifuged again at 100 000g for 2 h yielding a ribosomal pellet and an S-100 fraction. The ribosomes were then washed by centrifugation through a discontinuous 20 and 40% sucrose gradient in 20 mM Tris-HCl (pH 7.4), 100 mM MgCl<sub>2</sub>, 0.5 M NH<sub>4</sub>Cl, and 5 mM 2-mercaptoethanol and dissolved in buffer 1.

The 12 kDa acidic proteins were obtained by washing ribosomes with 50% ethanol and 1.5 M ammonium chloride as described previously (28), obtaining the split protein fraction (SP1.5).

*S. cerevisiae* EF2 was purified by standard chromatography according to a previously described method (42).

*Estimation of  $\beta$ -Galactosidase Activity.* A biochemical estimation of induced  $\beta$ -galactosidase activity was carried out in total cell extracts of YEPD grown transformants as previously described (37).

*Plasmids. Two-Hybrid Constructs.* The *S. cerevisiae* *RPP1A*, *RPP1B*, *RPP2A*, *RPP2B*, and *RPP0* genes encoding proteins YP1 $\alpha$ , YP1 $\beta$ , YP2 $\alpha$ , YP2 $\beta$ , and P0, respectively, were cloned in the two-hybrid vectors pGBT9 and pGAD424 (Clontech, Palo Alto, CA) as previously described (37). The different RPP0 fragments used in the work were obtained by PCR using custom-made complementary oligonucleotides (Table 1) and similarly cloned in pGBT9 and pGAD424. Plasmids were used to transform *S. cerevisiae* PJ69-6.

*Affinity Chromatography Constructs.* The tagged P0f48-His, P0f53-His, and P0p55'-His fragments were obtained by cloning the corresponding RPP0 fragments, obtained by PCR using the specific oligonucleotides BamHIP098-P048HIS, BamHIP098-P053HIS, and P055'HIS- P048HIS (Table 1), in the *Bam*HI and *Eco*RI sites of pRSETB (43).

*Mutagenesis.* Site-directed mutagenesis and deletions were carried out by PCR (44) using overlapping mutagenic oligonucleotides (Table 1). The resultant mutated genes, P0LS, P0LE, P0LV, P0 $\Delta$ L, P0 $\Delta$ 7aa, P0 $\Delta$ L/7aa, as well as wild-type P0wt, were cloned into the *Eco*RI and *Bam*HI restriction sites of pFL39 (45).

*Affinity Purification of P0 Fragments.* Histidine-tagged P0f38, P0f48, P0f53, and P0p55' protein expression was induced in the *E. coli* strain BL21(DE3) pLysS ( $A_{600}$  = 0.4-0.8) at 37 °C for 45 min with 1 mM IPTG. The cells were collected and disrupted by repeated freeze-thaw cycles after adding 2.5  $\mu$ g/mL of DNase and RNase. The total sample was taken in buffer P (10 mM Tris-HCl, pH 8, 50 mM Na<sub>2</sub>HPO<sub>4</sub>, 100 mM KCl), containing 8 M urea, 1 mM PMSF, 5  $\mu$ g/mL of leupeptin, 7  $\mu$ g/mL of pepstatin, and 10  $\mu$ g/mL of aprotinin and centrifuged at 12000g for 10 min at 4 °C. The tagged proteins were affinity-purified from the supernatant on Ni<sup>2+</sup>-NTA resin (Clontech) according to the manufacturer's instructions. The yield of P0f38 was extremely low and did not allow us to perform further experiments.

*Binding of Acidic Proteins to P0 Fragments.* About 5  $\mu$ g of purified P0f48-His6, P0f53-His6, or P0p55'-His6 fragments were bound to 500  $\mu$ L of Ni<sup>2+</sup>-NTA resin in buffer P. The resins were incubated with either 1 mg of S-100 fraction, containing about 4  $\mu$ g (333 pmol) of acidic proteins (46), or an equivalent amount of ribosome extract (SP1.5 fraction) for 2 h at room temperature and then packed in a column. The flow-through was collected and passed again 3 times through the resin. The packed resin was washed with 2.5 vol of buffer P, then with 1 vol of buffer P at pH 5.9 and urea 8 M, and finally with 2 vol of buffer P at pH 4.5 and urea 8 M. The proteins in the different collected fractions were analyzed as described in Electrophoretic Methods.

*Binding of EF2.* Purified EF2 (75 pmol), previously incubated with 50 mM GTP for 25 min at 30 °C in 20 mM Tris-HCl, pH 7.4, 5 mM magnesium acetate, 2 mM NH<sub>4</sub>Cl, and 2 mM DTT (buffer G), was passed through a Ni<sup>2+</sup>-NTA column containing the prebound tagged P0 fragment and acidic proteins. The resin was washed 3 times with 4 vol of buffer G, followed by another washing with the same buffer containing increasing concentrations of NaCl (0.1 and 0.25 M). Resin carrying tagged P0 fragments but not acidic proteins was used as a control.

Twenty-five percent of each fraction was resolved by SDS-PAGE, and Western blots were carried out using a rabbit serum raised against EF2 and a monoclonal antibody to the C-terminal part of the acidic proteins (3BH5) (46).

*Electrophoretic Methods.* Proteins were resolved either by 15% SDS-PAGE or by isoelectrofocusing (47) and detected by silver staining, autoradiography, or Western blotting using antibodies specific to the stalk proteins (46).

## Results

### Definition of the Minimal P0 Acidic Protein Binding Site.

Previous data have excluded a direct interaction of the first 176 amino acids and the last 50 amino acids of the 312 amino acid-long P0 sequence with any of the acidic proteins, while a strong interaction was detected with the 100 amino acid long C-terminal region starting at position 212 (37). These results strongly suggested that an internal region of the protein, specifically the fragment containing residues 212-262, is directly involved in the interaction. Using this fragment as a starting point, we have carried out a series of deletions and extension at both ends trying to precisely define the binding site for each acidic protein (Figure 1).

First, a series of sequentially shorter fragments starting at position 213 and ending from position 237 to position 265 have been assayed in the two-hybrid system with each one of the acidic proteins. When tested with both P1 proteins, a maximum  $\beta$ -galactosidase activity was detected in the case of P0f53, which is close to the activity previously reported for a P0 fragment containing the last 100 amino acids (37). A progressive reduction of the induced  $\beta$ -galactosidase activity was detected as the fragments got smaller, with a dramatic drop occurring from fragment P0f38 to P0f25 (Figure 2A). It seems that removal of 13 amino acids at the P0f38 C-terminal end eliminates the minimal structural features required to interact with these proteins. Unexpectedly, the inducing capacity of the fragments is the reverse when they are tested with protein P2 $\alpha$ , which in all cases showed a lower activity than the P1 proteins. The enzymatic activity increases from P0f53 to P0f38, although it is also lost in the P0f25. No significant interaction with P2 $\beta$  was detected with any of the fragments.

Taking P0f38 as nearly the smallest part of P0 able to interact with acidic proteins, a second series using P0f38 as the starting fragment was constructed to explore the effect of modifications at the amino terminal (Figure 2B). In this case, removal of seven amino acids from P0p38 (P0f38) to yield P0p31 results in a total loss of capacity to bind to all the acidic proteins. However, the extension of nine residues in P0p47 significantly increases the interaction with all the proteins except P2 $\beta$ , which again did not show an interaction in any instance. A further extension of eight residues has a different effect in the interacting capacity of P0p55 with the different proteins. Interaction with P1 $\alpha$  and P2 $\alpha$  suffers a substantial decrease, while interaction with P1 $\beta$  is practically unaffected. Any additional extension at the amino end results in a further decrease of the interaction with the proteins approaching the values previously reported (37) for the complete P0 protein (Figure 2B).

The inverse constructs, namely, the P0 fragments in pGAD424 and the acidic proteins in pGBT9, were also obtained and tested. In general, the  $\beta$ -galactosidase activity detected in this case was lower, but the results are qualitatively similar to those previously presented (data not shown).

It must be noted that, as previously shown (37), the induced enzymatic  $\beta$ -galactosidase activity is not due to differences in the expression level of the different P0 fragments.

### Effect of Deletions and Mutations at the Binding Site.

The results from the two-hybrid tests indicate that P0f38 is the smallest fragment showing any significant interaction capacity with the acidic proteins. The amino acid sequence reveals the presence in this P0 region of some features that might be relevant for the binding (Figure 1). Thus, the existence of a possible leucine zipper formed by residues L232, L239, and L246 can be easily identified. The effects of point mutations in this region on the interaction of the acidic proteins with the ribosome were tested. Substitution of the three residues by valine (P0LV), by glutamic acid (P0LE), or by serine (P0LS) was performed. Plasmids encoding the modified proteins as well as the wild-type P0wt as a control were used to transform W303dGP0, a conditional null-P0 mutant carrying the genomic *RPP0* gene copy under the control of the Gal1 promoter. When placed in a glucose medium, cells depend on the plasmid *RPP0* gene copy to survive (39). The ribosomes from the transformed strains grown in glucose were obtained and analyzed by isoelectrofocusing to check their stalk acidic protein composition (Figure 3). The results seem to exclude the involvement of a leucine zipper in the interaction since although mutation of the leucines to valines result in a slight decrease of bound acidic proteins, mutation to either glutamic acid or serine in P0LE or P0LS did not have an effect on the stalk composition. In agreement with these results, the corresponding transformed strains showed very small growth rate alterations. On the other hand, deletion of the leucine region from Leu232 to Leu247 (P0 $\Delta$ L) caused increase of the cell doubling time close to 20% and a clear reduction in the acidic protein content of the ribosomes, with proteins P1 $\beta$  and P2 $\alpha$  being preferentially affected.

The participation in the interaction of a conserved region from position 256-262, which contains a high proportion of acidic residues, was also explored. Deletion of this charged peptide in P0 $\Delta$ 7aa also caused a partial reduction on the acidic protein content (Figure 3). In contrast, the simultaneous removal of the leucine region and the charged peptide in P0 $\Delta$ L/7aa results in total absence of acidic proteins from the ribosomes. Moreover, the growth rate of the cell expressing the truncated protein was notably affected having a doubling time of 150 min, about 70% higher than the control and similar to the doubling time of acidic protein defective strains previously reported (48).

The essential role of both regions in the interaction of P0 with the acidic proteins was confirmed in the two-hybrid system. Fragments derived from P0f38 and P0p55 by removing the leucine and acidic sequences were tested for interaction with the four acidic proteins. As it is summarized in Table 2, the  $\beta$ -galactosidase inducing activity of both constructs is drastically reduced if not totally abolished when compared with the activity of the equivalent nondeleted constructs.

### *In Vitro* Binding of Acidic P1/P2 Proteins to P0 Fragments.

To confirm the biological significance of the P0 fragments, their capacity to form a complex with the P1/P2 proteins was tested. Thus, recombinant amino end His6-tagged P0f48-His6, P0f53-His6, and P0p55'-His6 were prepared and bound to a Ni<sup>2+</sup>-NTA column. Attempts to obtain P0f38-His6 failed due to a very low yield of recombinant protein. The fragments bind tightly to the resin and can be eluted from the column by washing with 8 M urea at pH 4.5 but not with 8 M urea at pH 5.9 (Figure 4). Similar amounts of each fragment, around 300 pmol, were bound to the columns as estimated from the Western blots of the eluted fractions.

A protein S-100 aliquot containing about 330 pmol of total acidic proteins was passed through columns carrying around 300 pmol of bound fragments as described in the methods section. There are discrepancies in the literature regarding the composition of the acidic protein pool in the S-100 extracts; however, our more recent estimations indicate that the four types seem to be in roughly similar amounts (49). The specificity of the acidic protein binding was tested on a control column containing resin without a bound P0 fragment and compared with a column carrying fragment P0p55'-His6, analyzed by isoelectrofocusing equivalent aliquots of each fraction (Figure 5A). While the P0p55' column retained about 80% of the P1/P2 proteins in the extract, approximately 270 pmol, the empty resin flow-through was practically identical to the original extract. The columns were then washed to eliminate nonspecifically bound proteins (fractions W1-3) and afterward eluted at pH 5.9 (fraction E1). Finally, the remaining proteins were released from the columns at pH 4.5 together with the tagged fragments (fraction E2). The four acidic proteins were found in both low-pH fractions from the P0p55'-His6 column in roughly the same proportion as in the S-100 extract. No acidic proteins were found in any control column fraction (Figure 5A). Similar experiments were carried out with columns carrying fragments P0f48-His6 and P0f53-His6, and the corresponding E1 and E2 fractions are shown (Figure 5B). Interestingly, the E2 fractions from these columns were enriched in proteins P1 $\beta$  and P2 $\alpha$ , suggesting a higher affinity of these two proteins for these shorter fragments.

### Binding of Elongation Factor EF2 to P1/P2-P0 Fragment Complexes.

The previous results indicate that the tagged P0 fragments are able to form a complex with the acidic proteins when bound to a Ni<sup>2+</sup>-NTA column. As a confirmation of the functional relevance of this complex, its capacity to interact with the elongation factor EF2 was checked. A sample of purified factor was passed through a column containing P0f48-His6 and acidic proteins. A column with P0-bound fragment but not acidic proteins was used as a control. After washing with a low-salt buffer to remove the unbound factor, elution with increasing salt concentrations was carried out. Aliquots of each eluted fraction were resolved by SDS-PAGE, and the factor was detected by immunoblotting using a rabbit antibody to EF2. As shown in Figure 6, a fraction of EF2 is retained in the column and eluted with buffer only in the column containing both the P0 fragment and the acidic proteins.

## Discussion

The ribosomal eukaryotic stalk is a dynamic element formed by a pentamer of proteins P0, P1 $\alpha$ , P1 $\beta$ , P2 $\alpha$ , and P2 $\beta$  whose structure is still unknown. This complex participates in the translocation step as a single structure, although a translation-dependent exchange between the ribosome-bound P1/P2 proteins and the proteins present in a cytoplasmic pool has also been reported, which suggests some degree of instability (30-32). Independently of the functional meaning of this process, these data suggest that the affinity of P0 for the small acidic proteins might change in a cyclic way along the translation cycle.

In the cell, the free P0 protein is able to interact independently although not very strongly with P1 but not with P2 proteins (37). Similar results have been reported *in vitro* (24, 50). However, the *in vitro* addition of a mixture of P1 and P2 proteins to P0 results in a soluble stable pentamer (51). It seems, therefore, that the cooperative effect of the four acidic proteins induces changes in the native P0 binding sites that substantially affect their mutual interactions. Previous reports have excluded the participation of the P0 N-terminal moiety in the interaction with the acidic proteins strongly suggesting that the binding site is located in an internal region of the C-terminal domain (36, 37). The results in this report define a minimal 38 amino acid fragment from F213 to A250, P0f38/P0p38, which is able to interact with P1 $\alpha$ , P1 $\beta$ , and P2 $\alpha$  to a slightly different extent. Removal of a few amino acids at either end of this fragment practically abolished its binding capacity, indicating that this fragment probably forms the core of the acidic protein binding site. On the other hand, the interacting capacity of P0f38 is altered in different ways, depending on the protein tested, by the sequential addition of amino acids at either end.

First of all, it must be noted that induction of  $\beta$ -galactosidase activity by protein P2 $\beta$  has not been detected with any tested construct. The incapacity of P2 $\beta$  to induce enzymatic activity in the two-hybrid system is in agreement with a previous report showing its inability to bind to acidic protein-depleted ribosomes in the absence of P1 proteins *in vitro* (50) as well as *in vivo* (48).

In contrast, P2 $\alpha$  protein is able to interact with several fragments of P0. A P2 $\alpha$  binding due to the P1 proteins acting as a "bridge" is highly improbable since several P0 fragments are able to strongly interact with P1 proteins but not with P2 $\alpha$ . In addition, a similar effect should have also been expected for P2 $\beta$ , which has been shown to interact with protein P1 $\alpha$ .

These results suggest the existence of important differences in the interaction of the two P2 proteins with P0. Nevertheless, the possibility cannot be excluded that for some unknown reasons, which do not affect the other acidic proteins, the P2 $\beta$  constructs are unable to interact with protein P0 in the two-hybrid system.

Concerning the size of the acidic protein binding site in P0, extension of the P0f38 fragment at the C-terminal end increases its interaction with both P1 proteins, approaching the value reported for the P0 fragment containing the last 100 amino acids (37). These results indicate that the P0 sequence from position 265 to the C-terminal end has little effect on the P1 protein interaction. In contrast, the same extension reduces the initial induced enzymatic

activity when tested with P2 $\alpha$ , suggesting the existence of some hindrance effect in the interaction of this protein.

Addition of nine amino acids at the P0f38 amino terminal increases the interaction of the resulting P0p47 fragment with both P1 proteins and with P2 $\alpha$ . However, the presence of eight additional residues in P0p55 has an important negative effect on the interaction with P1 $\alpha$  and P2 $\alpha$  but not with P1 $\beta$ . Additional enlargements at this end progressively reduce the interaction with the acidic proteins to levels similar to those found in the complete P0 (37).

All these results indicate the presence in P0 of sites that bind proteins P1 $\alpha$ , P1 $\beta$ , and P2 $\alpha$  with a considerable affinity, which do not totally coincide but extensively overlap. A fragment from positions 206-252, which contains two  $\alpha$ -helices,  $\alpha$ -III and  $\alpha$ -IV (Figure 1), probably constitutes a common core for the three sites. The binding site for P1 $\beta$  seems to also include the small  $\alpha$ -II helix, which improves the binding capacity of fragment P0p55'. The inclusion of the preceding  $\beta$ -sheet has an important negative effect on the P1 $\beta$  binding site and increases the masking of the P1 $\alpha$  and P2 $\alpha$  sites. At the carboxyl end, the P2 $\alpha$  binding site seems to end with helix  $\alpha$ -IV, since extension into the following helix,  $\alpha$ -V, reduces the interaction. In contrast, the interaction with both P1 proteins is increased by including fragments from  $\alpha$ -V, indicating that this helix may also be part of their binding sites.

The data from mutations and deletions complement the two-hybrid data and confirm the relevance of this P0 region in the acidic protein interaction. Thus, deletion of the amino end region of  $\alpha$ -V in P0 $\Delta$ 7aa causes a detectable reduction in the amount of the acidic proteins found in the ribosome *in vivo*. Moreover, the mutations introduced in  $\alpha$ -IV exclude the participation of the putative leucine zipper in the interaction of P0 with the acidic proteins but confirm its important role in assembling the acidic proteins to the ribosome. Thus, elimination of the 15 amino acids that form this segment results in a drastic reduction of the amounts of protein bound to the ribosome stressing the *in vivo* significance of this region. This deletion, however, does not affect the four acidic proteins equally, with the most substantial loss occurring in P1 $\beta$  and P2 $\alpha$ . The fact that parts of P1 $\alpha$  and P2 $\beta$  are still bound in ribosomes carrying the  $\alpha$ -helix IV-deleted P0 suggests that when bound to the ribosome, other P0 domains or even other ribosomal components can provide additional anchoring sites for these proteins. In this respect, it is relevant to note that when protein L12 is not present in the ribosome the amount of P1 $\alpha$  and P2 $\beta$  is also drastically reduced (52). Protein L12 also binds to the GAR region very close to P0 (53, 54), and its implication in the assembly and stability of the stalk is, therefore, not surprising.

The capacity of the P0 fragments to bind acidic proteins has been confirmed by affinity chromatography experiments. Caution has to be taken, however, in comparing the results from these experiments with the two-hybrid data. Both assays are carried out in very different conditions, and specially, it has to be kept in mind that in contrast to the affinity chromatography, which can be carried out in the presence of all the stalk proteins that can mutually interact, in the two-hybrid test only the interaction of the protein fused to the corresponding Gal4 fragments can be tested since the free acidic proteins are not present in the cell nucleus where the  $\beta$ -galactosidase induction takes place (55, 56).

The *in vitro* experiments have shown that resin-bound P0f48, P0f53, and P0p55' are able to retain the four acidic proteins in the column, including P2 $\beta$ , which in this case must bind through interaction with the other proteins, probably P1 $\alpha$  (50). The binding is specific since it depends on the presence of a P0 fragment prebound to the resin. Moreover, the efficiency of the binding is significant since about 1 pmol of acidic proteins is bound per pmol of P0 fragment. Interestingly, proteins P1 $\alpha$ /P2 $\beta$  seem to bind less strongly than P1 $\beta$ /P2 $\alpha$  to the shorter fragments, P0f48 and P0f53. This difference in affinity is not detected in the helix R-II-containing fragment P0p55', which suggests a role for this helix in stabilizing the interaction of the first couple. These results indicate, in addition, that in the tested conditions probably only one couple of acidic proteins is bound per P0 fragment. In fact, it is possible that the stable binding of two P1/P2 pairs may require the presence of other elements as suggested by the results from deletion mutants.

The protein complex formed on the affinity column is able to bind EF2. An interaction of the acidic proteins with EF2 has been previously shown by the two-hybrid system (37). The affinity column results indicate that the interaction with the P0 fragments does not affect the "functional" capacity of the proteins. It can be assumed, therefore, that the conformation of the P1 and P2 proteins in this minimal stalk must be not very different from the real ribosome structure.

The high-affinity acidic protein binding sites seem to be not fully exposed or even totally hidden in the case of P2 proteins, in the native P0, which interact with individual P1 proteins with low affinity and do not bind P2 protein at all (37). The two-hybrid results clearly show that the presence of additional domains results in a drastic reduction, and eventually the elimination, of the interacting capacity of P0 acidic protein site. The masking of the binding sites in the whole protein allows us to propose the hypothesis that P0 may suffer important conformational modifications during the stalk assembly, which affect the interaction with the acidic proteins. The reported initial interaction of the P1 proteins (24, 50), either free or forming part of a P1/P2 association, may induce changes in the P0 structure that expose the masked sites, allowing a high-affinity association of all the stalk components. In fact, an insoluble rat P0 preparation was taken into solution by incubation with acidic proteins (24, 57).

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## Table legends □

Table 1: Oligonucleotides Used for Cloning, Mutations, and Deletions<sup>a</sup>

Table 2: Effect of the Leucine Region Deletion on the Interaction of P0 Fragments with the Acidic Proteins<sup>a</sup>

## Figure legends

**Fig. 1.** Amino acid sequence and predicted secondary structure of the P0 region involved in acidic protein interactions. The different P0 fragments used in this report are shown. Secondary structure elements ( $\alpha$ -helices and  $\beta$ -sheets) predicted according to the PSIPred program are numbered. The sequence comprising the proposed binding site is in bold. Amino acid residues forming a putative leucine zipper are underlined. Acidic amino acid rich sequence is boxed.

**Fig. 2.**  $\beta$ -Galactosidase activity induced by the coexpression in *S. cerevisiae* of pGBT9 constructs containing the indicated P0 fragments and each of the acidic proteins cloned in pGAD424 (P1 $\alpha$ , □; P1□, ○; P2 $\alpha$ , ■; P2□, ●). Activity is expressed in arbitrary units and corresponds to the average of six experiments. (A) Modifications at the binding site carboxyl end; (B) modifications at the binding site amino end.

**Fig. 3.** Analysis by isoelectrofocusing of the acidic proteins in ribosomes containing P0 carrying different mutations and deletions as indicated in the text: P0LV, P0LE, and P0LS, leucines in putative zipper substituted by valine, glutamic acid, and serine, respectively; P0 $\Delta$ L, deletion of the putative leucine zipper; P0 $\Delta$ 7aa, deletion of the conserved acidic stretch; P0 $\Delta$ L/7aa, deletion of the putative leucine zipper and acidic stretch; P0WT, wild-type P0.

**Fig. 4.** Binding of His6-tagged P0 fragments to Ni<sup>2+</sup>-NTA resin. Recombinant P0f48-His6 and P0f53-His6 fragments were bound to the resin as described in the methods section and washed with buffer 3 times and then with 8 M urea at pH 5.9 and 4.5. Equivalent aliquots corresponding to the flow-through (FT), the third washing fraction (W3), and fractions eluted

at pH 5.9 (E1) and 4.5 (E2) were resolved by SDS-PAGE, and the fragments were detected with a commercial anti-His6 antiserum. The position of molecular weight markers is indicated to the left.

**Fig. 5.** Interaction of acidic proteins to resin-bound P0 fragments. (A) S-100 extracts were passed through resin containing either no bound P0 fragment (control) or P0p55'-His6 (P0p55'). The flow-through (FT), last washing (W3), and pH 5.9 (E1) and pH 4.5 (E2) elution fractions were collected, and equivalent aliquots of each fraction were resolved by isoelectrofocusing. An equivalent amount of the loaded S-100 fraction (S100) was also included. The position of the phosphorylated (lower band) and nonphosphorylated form (upper band) of the acidic proteins is marked. (B) Similar experiments were carried out using resins containing P0 fragments f48 and f53. Only fractions E1 and E2, resolved by isoelectrofocusing as in part A, are shown in this case. The position of the phosphorylated acidic proteins is indicated. In this preparation, the nonphosphorylated forms of the proteins are practically undetectable.

**Fig. 6.** Interaction of elongation factor EF2 with the acidic proteins/P0 fragment complex. Purified EF2 was passed through a Ni<sup>2+</sup>-NTA column containing P0f48-His6 either in the absence (-) or in the presence (+) of acidic proteins. After collecting the nonretained flow-through fraction (FT), the column was washed 3 times with low-salt buffer and then with the same buffer containing 0.1 M and 0.25 M NaCl. Aliquots of the FT fraction, the third washing fraction (W3), and each one of the NaCl-containing buffers (E1, E2) were analyzed by Western blot using EF2 specific antibodies.

## Tables

Table 1

gene/fragment	sense <sup>a</sup>	sequence <sup>b</sup>
P0f25	r	TGG ATC CGT GAC CGA CAG ATG GCA AGG
P0f38	r	TGG ATC CAA TTT CAG GGT AGT GGT AGG
P0f48	r	TGG ATC CGG CAA CAG CTA ACA AGT CCT
P0f53	r	CGG ATC CTG TCA ACC AAA TCT TCA ATT TCA GG
P0p31	f	GAA TTC ATT GCT TCT ATC TCT TTG GCT
P0p47	f	GAA TTC ATC ACC GAT GAA GAA TTG GTT TCT CAC
P0p55	f	T GAA TTC GTG TTC CCA TCT TCT ATC TT
P0p71	f	T GAA TTC CCA TTC ACT TTC GGT TTG ACT
P0p78	f	C GAA TTC TTG AAC TTG TTG AAC ATC TCT CC
BamHIP098	f	CGG GAT CCC GTT TCC GCT GTC AGC
P0f48HIS	r	T GAA TTC TTA AAT TTC AGG GTA GTG GTA GG
P0f53HIS	r	T GAA TTC TTA GTC AAC CAA ATC TTC AAT TTC AGG
P0p55'HIS	r	TG GAT CCA GGA GTG TTC CCA TCT TCT ATC TT

mutation	sense	sequence
EcoRIP0 <sup>c</sup>	f	CGAATTCCAAGTATTACTGACTGTGCGG
BamHIP0 <sup>c</sup>	r	CGGATCCTACTTCTATTGATCTCTTAAGG
P0Δ7aa	f	GTT GCC ATT GCT GCT <b>TCC GAT</b> TTG GTT GAC AGA ATT G
P0ΔL	f	TTG GCT ATT GGT TAC <b>CCA ACCΔGCT</b> GTT GCC ATT GCT GCT TCC TAC
P0ΔL	r	GTA GGA AGC AGC AAT GGC AAC <b>AGCΔGGT</b> TGG GTA ACC AAT AGC CAA
P0LV	f	C CCA ACC <b>GTG</b> CCA TCT GTC GGT CAC ACT <b>GTG</b> ATC AAC AAC TAC AAG GAC TTG <b>GTA</b> GCT
P0LV	r	AGC TAC CAA <b>GTC</b> CTT GTA GTT GAT CAC AGT GTG ACC GAC AGA TGG CAC GGT <b>TGG</b> G
P0LE	f	CT ATT GGT TAC CCA ACC <b>GAA</b> CCA TCT GTC GGT CAC ACT <b>GAA</b> ATC AAC AAC TAC AAG G
P0LS	f	G GCT ATT GGT TAC CCA ACC <b>AGT</b> CCA TCT GTC GGT CAC ACT <b>AGT</b> ATC AAC AAC TAC AAG G
P0Lmut	r	GGT TGG GTA ACC AAT AGC C

<sup>a</sup> The oligonucleotide sense is marked as "r" for reverse and "f" for forward. <sup>b</sup> Restriction sites are in bold characters. The mutations and deletions are in bold underlined characters. Δ indicates the position of the deleted sequence. <sup>c</sup> Oligonucleotides used at the P0 ends for all the mutations.

Table 2

acidic protein	$\beta$ -galactosidase activity (units)			
	P0f38		P0p55	
	Δ	C	Δ	C
P1 $\alpha$	0.8	262	34.1	222
P1 $\beta$	0.3	219	19.0	400
P2 $\alpha$	1.2	162	0.0	84
P2 $\beta$	0.1	3	0.0	3

<sup>a</sup> The inducing of  $\beta$ -galactosidase activity of the indicated fragments lacking the putative leucine zipper region (Δ) and their respective controls (C) containing the region was tested with each one of the acidic proteins.





Figure 5.

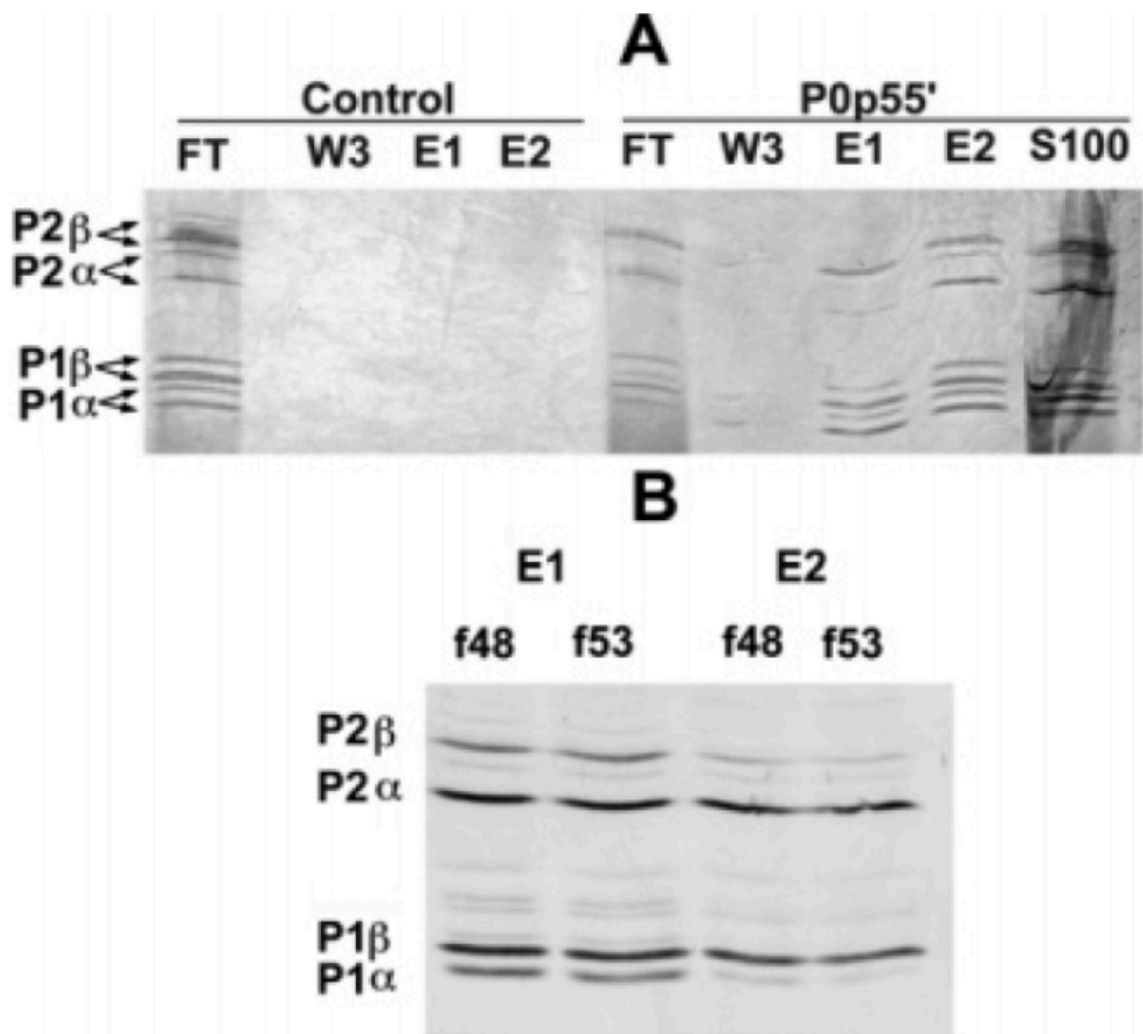


Figure 6.

