Eukaryotic Acidic Phosphoproteins Interact with the Ribosome through Their Amino-Terminal Domain†

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ABSTRACT: Variable-size fragments of the four yeast acidic ribosomal protein genes *rpYP1α*, *rpYP1β*, *rpYP2α* and *rpYP2β* were fused to the LacZ gene in the vector series YEp356-358. The constructs were used to transform wild-type *Saccharomyces cerevisiae* and several gene-disrupted strains lacking different acidic ribosomal protein genes. The distribution of the chimeric proteins between the cytoplasm and the ribosomes, tested as β-galactosidase activity, was estimated. Hybrid proteins containing around a minimum of 65−75 amino acids from their amino-terminal domain are able to bind to the ribosomes in the presence of the complete native proteins. Hybrid proteins containing no more than 36 amino terminal amino acids bind to the ribosomes in the absence of a competing native protein. The fused YP1−β-galactosidase proteins are also able to form a complex with the native YP2 type proteins, promoting their binding to the ribosome. The stability of the hybrid polypeptides seems to be inversely proportional to the size of their P protein fragment. These results indicate that only the amino-terminal domain of the eukaryotic P proteins is needed for the P1−P2 complex formation required for interaction with the ribosome. The highly conserved P protein carboxyl end is not implicated in the binding to the particles and is exposed to the medium.

The large ribosomal subunits of cytoplasmic ribosomes from all organisms have a set of very acidic proteins (p*I* <5.0) generically designated as A proteins (P proteins in eukaryotes). They are the only ribosomal proteins known to be present in more than one copy per ribosome [for a review, see Liljas (1991)]. *Escherichia coli* L7 and L12 are the best studied among this ubiquitous protein family. L7 is the NH_2-terminus acetylated form of L12, meaning that both are encoded by the same gene, *rplL* (Terhorst et al., 1973). Bacterial ribosomes have four copies of A proteins (Subramanian, 1975), assembled as a pentameric complex, made of two L7/L12 dimers and one molecule of protein L10, which binds to 23S rRNA and forms the lateral flexible stalk of the 50S ribosomal subunit (Brot & Weissbach, 1981; Möller & Maassen, 1986). This complex is highly stable, standing up to 8 M urea (Pettersson et al., 1976), and it can be removed easily from the ribosome by salt−ethanol washing (Highland & Howard, 1975).

Both L7/L12 dimers are needed for optimal rates of protein synthesis and for binding of elongation factors (Möller & Amons, 1985) and aminoacyl-tRNA to the ribosome (Brot & Weissbach, 1981; Möller & Maassen, 1986), as well as for factor-dependent GTP hydrolysis (Koteliansky et al., 1978; Lavergne et al., 1992). They have also been postulated as transducers of the energy required for peptidyl-tRNA translocation (Möller, 1991) and implicated in translation accuracy (Kirsebom & Isakson, 1985). The available evidence from in vivo experiments suggests that proteins L7 and L12 have similar functions because, despite that their ratio changes as log-phase cell cultures reach stationary phase (Ramagopal & Subramanian, 1974), the acetylation of ribosomal protein L12 remains constant during the cell growth cycle (Subramanian & Nehls, 1975), and cell growth is not altered in a mutant without L12 acetylase activity (Isono & Isono, 1981).

Eukaryotic P proteins differ from eubacterial L7/L12 in four main aspects. First, they are encoded by independent genes (Maassen et al., 1985; Rich & Steitz, 1987; Mitsui & Tsurugi, 1988a,b; Remacha et al., 1988; Beltrame & Bianchi, 1990; Newton et al., 1990; Wool et al., 1991). Second, they are phosphorylated on ribosomes (Zinker & Warner, 1976; Juan-Vidales et al., 1984; Naranda & Ballesta, 1991; Naranda et al., 1993), which is why they were named P proteins (Tsurugi et al., 1978), and dephosphorylated in a cytoplasmic pool (van Agthoven et al., 1978; Zinker, 1980; Mitsui et al., 1988). Third, during the process of protein synthesis, exchange between the ribosomal phosphoproteins and their cytoplasmic non-phosphorylated counterparts takes place (Zinker & Warner, 1976; Tsurugi & Ogata, 1985) most probably under control of more than one protein kinase (Naranda et al., 1993) and of dephosphorylation mechanisms. Fourth, their ribosomal and cytoplasmic amounts change according to the metabolic state of the cell (Saenz-Robles et al., 1990).

The acidic protein genes from human cells (Rich & Steitz, 1987), rat liver (Wool et al., 1991), *Artemia salina* (Maassen

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[1] Abbreviations: aa, amino acid; bp, base pair; kbp, kilobase pair; β-gal, β-galactosidase; ELISA, enzyme-linked immunosorbent assay; ORF, open reading frame; ONPG, *o*-nitrophenyl β-D-galactopyranoside; PMSF, phenyl methanesulfonyl fluoride.

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et al., 1985) *Drosophila melanogaster* (Qian et al., 1987), *Schysosaccharomyces pombe* (Beltrame & Bianchi, 1990) and *S. cerevisiae* (Mitsui & Tsurugi, 1988a,b; Remacha et al., 1988; Newton et al., 1990) have been cloned. Their primary DNA sequences demonstrate that the encoded proteins are of two similar classes, P1 and P2, that have been extremely conserved during evolution (Shimmin et al., 1989). The multicellular organisms have one gene of each class, whereas yeasts have two. In contrast to the bacterial A proteins, none of the four *S. cerevisiae* genes or their encoded proteins, called YP1α, YP1β, YP2α, and YP2β in a unifying nomenclature (Wool et al., 1990), are indispensable for ribosome activity and cell viability (Remacha et al., 1990, 1992). On the other hand, on the basis of physicochemical, immunological, and functional criteria, it is accepted that eukaryotic P proteins serve the same function as eubacterial L7/L12 (Wool & Stöffler, 1974; Howard et al., 1976; Sanchez-Madrid et al., 1981).

Like the bacterial acidic proteins, the eukaryotic P proteins are detected as dimers (Juan-Vidales et al., 1983), and they seem to form a pentameric complex with the eukaryotic L10-like ribosomal protein, analogous to the complex formed by L7/L12 and L10 (Uchiumi et al., 1987). This L10-like ribosomal protein is called P0 and has some sequence homology to bacterial L10 that supports their similar functional role (Shimmin et al., 1989), but it also shows important structural differences, the most important one probably being the presence of a carboxyl-terminal extension with a high sequence homology to the acidic P proteins (Shimmin et al., 1989). The eukaryotic pentameric complex is much less stable than the bacterial structure, and it easily disassembles when subjected to the same conditions that remove it from the bacterial ribosome. Under these conditions, P0 stays bound to the particles and proteins P1 and P2 are released (Sanchez-Madrid et al., 1979; Towbin et al., 1982; Santos & Ballesta, 1994).

There is evidence indicating that the bacterial (Gudkov et al., 1980; Olson et al., 1986) and archaebacterial (Köpke et al., 1992) acidic ribosomal proteins are exposing their carboxyl end to the exterior of the stalk in the large ribosomal subunit, while their interaction with the ribosome takes place through the amino terminal domain. On the other hand, amino acid sequence comparison of bacterial and eukaryotic acidic proteins led to the proposal that during evolution a sequence transposition may have taken place in such a way that the carboxyl-terminal domain in the prokaryotic protein now corresponds to the amino-terminal domain in the eukaryotic polypeptide (Lin et al., 1982). If this happened, inversion of the functional domains in the eukaryotic P proteins occurred, and these proteins should be expected, accordingly, to interact with the ribosome through their carboxyl end. Thus, it is important to establish, for the eukaryotic systems, which acidic ribosomal protein regions are involved in ribosome binding.

We report here about the role of the amino-terminal domain of these proteins in ribosome binding as studied by means of constructions that were made by deleting variable-size fragments from the 3′ end of their encoding genes, which were then fused to the *LacZ* gene.

MATERIALS AND METHODS

Yeast Strains and Growth Conditions. Clones of the parental yeast strain *Saccharomyces cerevisiae* W303-1B (MAT α, *leu2*-3, trp1-1, ura3-1, ade2-1, his3-11,15, can 1-100) and its derived mutants, D5 (*rpYP2β*::HIS3), D6 (*rpYP1β*::TRP1), and D7 (*rpYP1α*::LEU2) as well as D67 (*rpYP1α*::LEU2, *rpYP1β*::TRP1) carrying one (Remacha et al., 1990) or two (Remacha et al., 1992) disrupted acidic ribosomal protein genes, respectively, were grown in rich YPD (5% yeast extract, 10% peptone, and 2% glucose) liquid medium to stationary phase. These cultures were used as stocks. They were kept at 4 °C and renewed monthly, and they were used to inoculate minimal complete (SD) liquid medium (0.67% yeast nitrogen base without amino acids, 40 μg/mL each of the strain nutritional requirements, and 2%glucose). When required, agar was added up to 2% to prepare solid YPD and SD media. Yeast cell cultures either in liquid or in solid media were incubated at 30 °C. The host for vector and plasmid amplification was *E. coli* strain C600 (F-, *thi*-1, *thr*-1, *leu*B6, *Lac*Y1, *Tona*21, *sup*E44), which was grown at 37 °C in liquid LB (1% Bacto peptone, 0.5% yeast extract, 0.5% NaCl, and 0.2% glucose) or solid (LB + 1.5% agar) broth.

Table 1: Features of the Plasmids Used for Yeast Transformation

plasmid[a]	inserted DNA fragment[b]	gene fragment size (kbp)	aa in the fused ORF	native protein[c]	hybrid protein
YEFp47ep	*Eco*RI−*Pvu*II	0.730	69	YP1α	YP1α(69aa)-βgal
YEFp47eh	*Eco*RI−*Hpa*II	0.696	80	YP1α	YP1α(80aa)-βgal
YEFp46ph	*Pst*I−*Hinc*II	0.560	36	YP1β	YP1β(36aa)-βgal
YEFp46ep	*Eco*RI−*Pst*I	1.100	95	YP1β	YP1β(95aa)-βgal
YEFp44ep	*Eco*RI−*Pvu*II	1.300	65	YP2α	YP2α(65aa)-βgal
YEFp45bb	*Ban*II−*Ban*II	0.456	50	YP2β	YP2β(50aa)-βgal
YEFp45ph	*Pst*I−*Hpa*II	0.601	75	YP2β	YP2β(75aa)-βgal

[a] Genomic DNA fragments encoding acidic ribosomal proteins were inserted in plasmid YEp357R (Myers *et al.*, 1986). [b] Restriction sites were selected from previously reported genomic DNA primary sequences (Remacha *et al.*, 1988; Tsurugi & Mitsui, 1988b). [c] According to the proposal for a uniform nomenclature (Wool *et al.*, 1991). The equivalence to previous nomenclatures is as follows: YP1α = A1, YP1β = L44, YP2α = L44 or A2 and YP2β = L45 or YP-A1 (Remacha *et al.*, 1988; Tsurugi & Mitsui, 1988a,b). The numbering of amino acids is as follows: YP1α = 106 (Tsurugi & Mitsui, 1988b); YP1β = 106, YP2α = 106 and YP2β = 110 (Remacha *et al.*, 1988).

Cell Transformation and Plasmid Amplification. E. coli cells were transformed according to the method of Hanahan (Hanahan, 1985). Transformants were simultaneously selected for ampicilin resistance and blue color on X-Gal containing LB plates. They were grown at 37 °C into ampicilin-supplemented (100 μg/mL) LB liquid medium and were processed to obtain plasmids according to standard methods (Birnboim & Doly, 1979). *S. cerevisiae* cells were transformed by the method of Hinnen et al. (Hinnen et al., 1978). Yeast transformants were selected as blue colonies on uracil-free X-Gal indicator plates (Rose et al., 1990).

Construction of Plasmids. Plasmid nomenclature, the size and source of inserted genomic DNA they carry, the encoded acidic ribosomal polypeptide length, and the hybrid proteins names are given in Table 1. Plasmid BS-47EB, containing a 2.0-kbp yeast nuclear DNA fragment carrying the *rpYP1α* gene inserted into the *Eco*RI−*Bam*HI sites of Bluescript plasmid (Newton et al., 1990), was separately digested with *Eco*RI/*Pvu*II and *Eco*RI/*Hpa*II. The resulting two fragments were subcloned between the *Eco*RI−*Hinc*II and *Eco*RI−*Acc*I sites of pUC18 to give YEp47Pv and YEp47Hp plasmids, repectively, which were in turn digested with *Eco*RI/*Hin*dIII. The resulting fragments were inserted into the same enzyme

restriction sites of vectors YEp356 and YEp357 (Myers et al., 1986), respectively, obtaining YEFp47ep and YEFp47eh plasmids.

To obtain plasmid YEFp46ph, the pUC18-derived pMRH46 plasmid, containing the *rpYP1β* gene in a 1.95-kbp *Hin*dIII fragment (Remacha et al., 1988), was digested with *Hinc*II/*Pst*I, and the resulting 0.5-kbp DNA fragment was ligated between the same restriction sites of YEp358R. Plasmid YEFp46ep was obtained by inserting in the *Pst*I/*Eco*RI sites of YEp356R, a 1.11-kbp *Pst*I−*Eco*RI fragment obtained from plasmid BS-46M (Naranda et al., 1993) containing 0.5 kbp of the 5′ flanking region and the coding sequence of *rpYP1β* gene up to the amino acid in position 95, where a new *Eco*RI restriction site had been introduced by site-directed mutagenesis.

Plasmid YEFp44ep was derived from pMRE44, which contains the *rpYP2α* gene in a 2.3-kbp *Eco*RI fragment (Remacha et al., 1988), by first digesting it with *Eco*RI/*Pvu*II and then inserting the resulting 1.3-kbp genomic DNA fragment between the *Eco*RI and *Sma*I sites of YEp357.

Plasmid pRVE45 carrying the *rpYP2β* gene (Remacha et al., 1988) was digested either with *Ban*II or with *Pst*I/*Hpa*II to obtain short (456 bp) and large (601 bp) subfragments, respectively. Insertion of the former into the *Ban*II site of YEp357R produced plasmid YEFp45bb, and insertion of the later between the *Pst*I and *Sma*I sites of YEp357R, after it was incubated with the Klenow DNA polymerase I to make a blunt 3′ end, produced plasmid YEFp45ph (see Table 1).

Isolation of Polysomes. The method was essentially that of Helser (Helser et al., 1981) with some modifications. Yeast cells were grown up to the mid-log phase (0.4−0.6 OD_{260nm}) in uracil-free liquid SD medium at 30 °C. Cycloheximide was added to a final concentration of 100 μg/mL, and after 1 min at 30 °C they were poured into sterile ice to abruptly stop cell metabolism. Cells were harvested by centrifugation for 10 min at 12000*g* and 4 °C, and they were washed twice with sterile ice-cold water to eliminate traces of growth medium. Cell pellets were resuspended in 100 mM KCl, 30 mM $MgSO_4$, 10 mM Tris, and 6 mM 2-mercaptoethanol, pH 7.4 (buffer A). Glass beads (0.5 mm in diameter) were added, and cells were mechanically disrupted by alternating 30 s of vigorous vortexing and 30 s of incubation in ice−water. Cell debris was eliminated by centrifugation at 12000*g* and 4 °C. Supernatants were loaded on top of 10−45% sucrose gradients in buffer A and were centrifuged at 25 000 rpm (Beckman SW 28 rotor) and 4 °C for 5 h. Fractions of 1.0 mL were collected, and the absorbance at 260 nm was continuously recorded.

Isolation of S30 and S100 Cell Fractions and High Salt Washed Ribosomes. The method was as previously described (Sanchez-Madrid et al., 1979) with slight modifications. Yeast cells were grown in uracil-free SD liquid medium to the mid-log phase. They were washed twice with ice-cold sterile water; resuspended (1 g/mL) in 80 mM KCl, 12 mM $MgCl_2$, 100 mM Tris, 5 mM 2-mercaptoethanol, and 1 mM PMSF, pH 7.4; mechanically disrupted as described above; and centrifuged at 30000*g* at 4 °C for 20 min. The pellet was discarded, and the supernatant (S30 cell fraction) was centrifuged at 50 000 rpm (rotor 60 Ti; Beckman) at 4 °C for 3 h. This supernatant (S100 cell fraction) was saved and kept at −20 °C until further use. The pellet was resuspended in 500 mM $AcNH_4$, 100 mM $MgCl_2$, 5 mM 2-mercaptoethanol, and 20 mM Tris, pH 7.4 (buffer B) and centrifuged for 10 min at 30000*g* and 4 °C to discard undisolved material. High salt washed ribosomes were pelleted by centrifuging the resulting supernatant at 50 000 rpm (60Ti rotor; Beckman) and 4 °C for 14 h through a discontinuous gradient made of 20% and 40% sucrose in buffer B.

Isolation of Antibodies. Isolation of monoclonal antibodies against acidic ribosomal proteins is described elsewhere (Vilella et al., 1991).

ELISA. In order to detect in vitro association between hybrid acidic ribosomal proteins and native acidic ribosomal proteins, ELISA assays were carried out as previously described (Vilella et al., 1991). Specific monoclonal antibodies were adsorbed to plastic microtiter plates by incubating 100 μl of a 2 μg/mL solution in 50 mM Na_2CO_3, pH 9.6 (buffer C), during 30 min at room temperature and then at 4 °C for 16 h. The plates were allowed to equilibrate to room temperature and then washed with 4 mM NaH_2PO_4, 2 mM Na_2HPO_4, 145 mM NaCl, and 0.05% Tween 20 (buffer D). The S30 cell extracts, diluted in buffer C, were deposited in the plate wells, and after a 1-h incubation at room temperature, the plates were washed once more with buffer D. The concentration of hybrid acidic ribosomal proteins retained in the plate wells was measured as β-galactosidase activity using ONPG as substrate.

Determination of β-Galactosidase Activity. The activity of β-galactosidase was measured as follows (Miller, 1972): samples in 400 μL of 100 mM potassium phosphate buffer, pH 7.0, were mixed with an equal volume of 65 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, and 40 mM 2-mercaptoethanol, pH 7.0 and incubated at 28 °C for 10 min. Then 200 μL of ONPG (4 mg/mL in potassium phosphate buffer, pH 7.0) was added, and the samples were again incubated at 28 °C. The reaction was stopped with 500 μL of 1.0 M Na_2CO_3.

Other Methods. Protein concentration was determined according to the method of Lowry (Lowry et al., 1951).

RESULTS

Ribosome and β-Galactosidase Activity Sedimentation Profiles. DNA restriction fragments (Table 1), derived from each of the four *S. cerevisiae* acidic ribosomal protein genes, comprising the promoter region and variable size lengths of the protein-coding region toward the 3′ end, were fused to the *Lac*Z gene by subcloning into the appropriate enzyme restriction sites of the YEp356-358 vector series (Myers et al., 1986). The set of rpYP1-LacZ and rpYP2-LacZ chimeric gene carrying plasmids were transformed into the W303-1B yeast strain. Transformants were grown up to the mid-log phase in uracil-free minimal synthetic liquid medium and processed to obtain the postmitochondrial S30 cell fraction.

As a first approach, we asked whether or not in vivo ribosomal binding of the YP1 type and YP2 type derived hybrids took place. To that end, the S30 cell extracts were centrifuged into sucrose gradients. Fractions from each gradient were collected, and their content of hybrid proteins was estimated as β-galactosidase activity. The results of this analysis show that the β-galactosidase activity is distributed in different parts of the sucrose gradient, although the distribution pattern was not the same for all transformants (Figure 1). An important part of the hybrid acidic ribosomal proteins was found in the top of the gradient, i.e., YP2α-

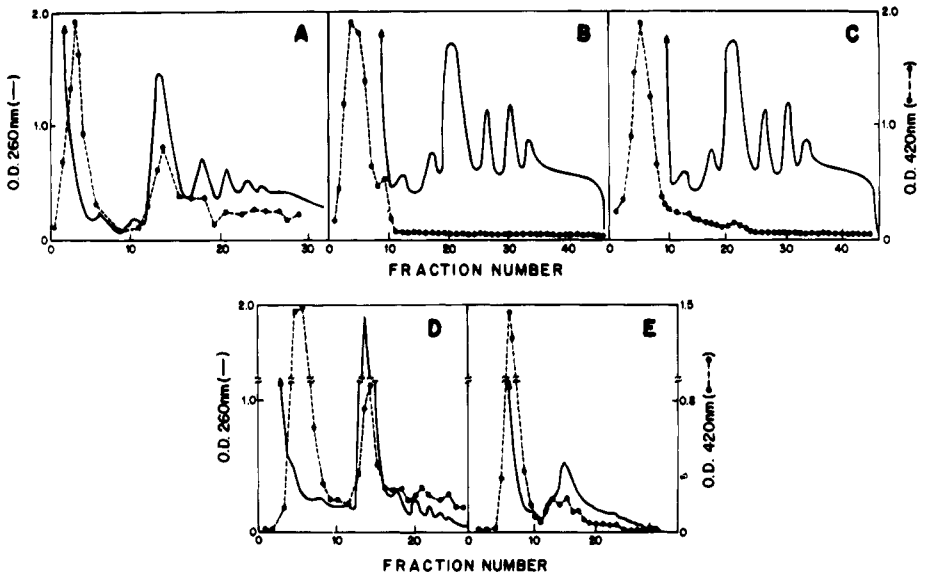

FIGURE 1: Sucrose gradient profiles of ribosomes and hybrid acidic ribosomal proteins from parental strain transformants. The set of plasmids described in Table 1 were transformed into the W303-1B yeast parental strain. Clones were grown up to mid-log phase and processed to obtain their S30 fraction. 75 OD_{260nm} from each fraction was loaded onto sucrose gradients and ultracentrifuged. Aliquots of 1 mL were collected, recording continuosly the absorbance at 260 nm (—), and the amount of hybrid acidic ribosomal proteins in each aliquot was quantified as β-galactosidase activity (- - -) as described in Materials and Methods. Representative examples of each YP type hybrid acidic protein are given. (A) YP1α(69aa)-βgal. (B) YP1β-(36aa)-βgal. (C) YP2β(75aa)-βgal postribosomal and W303-1B S30 cellular fractions mixed together before ultracentrifugation (control). (D) YP2β(75aa)-βgal. (E) YP2β(75aa)-βgal incubated with RNase A (50 μg/mL) at 36 °C for 60 min before ultracentrifugation (control).

(65aa)-βgal and YP2β(75aa)-βgal, consistent with the well-documented notion of an acidic ribosomal protein cytoplasmic pool (van Agthoven et al., 1978; Zinker, 1980; Mitsui et al., 1988; Saenz-Robles et al., 1990). More important, as judged from enzyme activity, is that a portion of the YP1 and YP2 derived hybrid proteins (Figure 1, panels A and D) sedimented along with 80S ribosomes and polysomes. In contrast, enzyme activity on these particles was detected in trace amounts only, when a parental nontransformed strain S30 fraction and a ribosome-free cell extract (S100), containing considerable amounts of protein YP2β(75aa)-βgal, were mixed together before sucrose gradient centrifugation (control; Figure 1, panel C). Moreover, after incubation with RNase the hybrid proteins then sedimented with 80S ribosomes and the 60S ribosomal subunit (Figure1, panel E; control). These results strongly suggest that the interaction between the hybrid acidic ribosomal proteins and the ribosome is specific. The fact that, under the same experimental conditions, protein YP1β(36aa)-βgal (Figure 1, panel B) was not detected on these particles agrees with this conclusion and further suggests that the acidic ribosomal protein moiety length plays an important role in ribosome binding.

Kinetic Parameters of the Cytosol-Free Hybrid β-Galactosidase. For quantitative purposes, it was important to determine what effect the length of the acidic ribosomal polypeptide portion had on the K_m ($M \times 10^{-3}$) and V_{max} (1/h) values of the hybrid protein β-galactosidase activity. To that end, the set of plasmids described in Table 1 were transformed into the parental strain and the transformant S100 cell fractions were used as enzyme sources to perform typical enzyme kinetic analyses. K_m ($M \times 10^{-3}$) and V_{max} (1/h) were calculated from a double-reciprocal plot of the initial velocity versus the substrate concentration. The results in Table 2 show that while the K_m ($M \times 10^{-3}$) values of all hybrid proteins tested were about the same, the V_{max} (1/h) value of YP2β(75aa)-βgal was about 3.5 and 9 times higher than those of YP2α(65aa)-βgal and the two YP1α(69aa and 80aa)-βgal hybrids, respectively.

Table 2: Kinetic Parameters for Acidic Ribosomal Protein Hybrids in the S100 Cell Fraction from Parental Strain Transformants

hybrid protein	K_m ($M \times 10^{-3}$)	V_{max} (1/h)
YP1α(69aa)-βgal	0.34	0.39
YP1α(80aa)-βgal	0.35	0.41
YP1β(36aa)-βgal	0.34	0.44
YP2α(65aa)-βgal	0.32	1.07
YP2β(50aa)-βgal	0.37	0.93
YP2β(75aa)-βgal	0.35	3.65

Estimation of Ribosome-Bound Hybrid Acidic Ribosomal Proteins. On the basis of the results in Table 2, the quantitation of ribosome-bound YP1 type and YP2 type derived hybrid acidic ribosomal proteins was carried out. Advantage was taken also of the W303-1B-derived mutants D7, D6, D67, and D5 harboring disrupted, and therefore inactive, *rpYP1α*, *rpYP1β*, *rpYP1α*/*rpYP1β*, and *rpYP2β* genes, respectively (Remacha et al., 1990; Remacha et al., 1992), to determine the effect of native acidic ribosomal proteins on the amount of hybrid acidic ribosomal proteins. Cell cultures of the parental and gene disrupted mutant strains transformed with the set of plasmids described in Table 1 were grown to mid-log phase. Ribosomal pellets from cell extracts were washed through high-salt discontinuous sucrose gradients, and their molar concentration of hybrid acidic ribosomal proteins was measured.

Table 3 shows, in agreement with the results in Figure 1, that parental high salt washed ribosomes (strain W) had significant amounts of all but YP1β(36aa)-βgal and YP2β-(50aa)-βgal hybrid proteins, which seem unable to bind to the particle. Table 3 also shows that the concentration of hybrid proteins in ribosomes was affected by the presence or absence of native YP1 acidic ribosomal phosphoproteins. Reduced amounts of the YP1 hybrids are found when one

Table 3: Amount of Hybrid Acidic Ribosomal Proteins in High Salt Washed Ribosomes[a]

hybrid protein	mol of hybrid proteinb/mol of 80S ribosome,[c] yeast strain				
	W	D5	D6	D7	D67
YP1α(69aa)-βgal	0.52(±0.005)		0.52(±0.003)	0.21(±0.055)	3.19(±0.085)
YP1α(80aa)-βgal	0.61(±0.100)		0.42(±0.005)	0.35(±0.074)	4.26(±0.045)
YP1β(36aa)-βgal	<0.01(±0.002)		0.40(±0.010)	0.33(±0.010)	0.21(±0.033)
YP1β(95aa)-βgal	0.62		0.38		1.79
YP2α(65aa)-βgal	0.70(±0.039)		0.34(±0.020)	0.89(±0.010)	0.23(±0.062)
YP2β(50aa)-βgal	0.02(±0.012)	1.16(±0.023)	0.40(±0.095)	0.07(±0.017)	0.24(±0.033)
YP2β(75aa)-βgal	0.42(±0.020)	1.17(±0.015)	0.71(±0.045)	0.18(±0.015)	0.15(±0.014)

[a] Results are the mean of three independent experiments except for YP1β(95aa)-βgal. Numbers in parentheses are standard deviations. The activity of β-galactosidase was measured as described under Materials and Methods. [b] Calculated against the specific activity (SA = units/μmol) of commercial β-galactosidase (Boehringer) under the assumption that the kinetic parameters were similar to those in Table 2. One unit = A_{420} × 1000/SE mL. The calculated SA for the comercial enzyme was 0.67. Molecular mass of β-galactosidase = 116250 Da. [c] Equivalences: 1 A_{260nm} unit = 40 μg of RNA; molecular mass of 80S ribosome = 4 × 10^6 Da, 60% of its mass being rRNA

of the native components of this protein type is missing in the cells (strains D6 and D7). On the contrary, the hybrid protein concentration is much higher in ribosomes lacking both native YP1 proteins (strain D67). Interestingly, YP1β-(36aa)-βgal does not bind to the parental particles, but it does bind, in relatively high amounts, to the ribosomes from the single gene disrupted strains D6 and D7, and its binding is considerably reduced in the ribosomes from the double disruptant strain D67.

The effect of native YP1 proteins on the YP2 hybrid protein binding to ribosomes is also interesting. The absence of YP1α (strain D7) reduces the amount of YP2β hybrids and increases that of YP2α hybrids in ribosomes; on the other hand, in the ribosomes from strain D6, which lack protein YP1β, a reduction of YP2α(65aa)-βgal and an increase of YP2β(75aa)-βgal is found. Considerable amounts of YP2β-(50aa)-βgal, which like YP1α(36aa)-βgal does not bind to wild-type ribosomes, are detected in the ribosomes from strains D5 and D6, which lack native proteins YP2β and YP1β, respectively, and in a much smaller proportion in the particles from strains D7 and D67.

Induction of Native Acidic Protein Binding to Ribosomes by the Hybrid Proteins. The acidic proteins bind to the ribosome as a P1−P2 complex, and in the absence of one of the YP protein types the remaining component is unable to interact with the particles (Remacha et al., 1992). Accordingly, the YP1 type protein deficient ribosomes from strain D67 are completely deprived of acidic proteins and the YP2 type proteins present in the cells are found free in the cytoplasm (Remacha et al., 1992). The detection of notable amounts of YP1 hybrid proteins in ribosomes from transformed D67 strains (Table 3) suggests that they are able to form the required P1−P2 complex. In order to confirm this prediction, the amount of native YP2 proteins in the particles from transformed strain D67 was estimated by ELISA using specific antibodies. The results in Table 4, in addition to confirming that the D67 ribosomes are really deprived of acidic proteins, indicate that the binding of the YP2 proteins is stimulated by the hybrid polypeptide, although the stimulatory effect is not identical in all cases. Thus, the YP1α hybrids seem to be more effective in stimulating the interaction of YP2β, while the YP1β hybrids stimulate preferentially native YP2α binding. YP1α(36aa)-βgal, however, only induces the interaction of a small amount of YP2α but has no effect on the binding of YP2β.

Effect of Native Acidic Ribosomal Proteins on the Cytosolic Concentration of Hybrid Acidic Ribosomal Proteins.

Table 4: Estimation of Native Acidic YP2 Proteins in Ribosomes from Strain D67 Transformed with Different Plasmids[a]

strain	acidic proteins expressed in cells			mol of YP2 protein in ribosomes (ELISA)[b] (A450[c])	
	YP1	YP2	hybrid	anti-YP2α	anti-YP2β
W303	+	+	−	2.5	2.7
D67	−	+	−	0.05	0.08
D67-YEFp47ep	−	+	YP1α(69)-βgal	2.0	2.7
D67-YEFp47eh	−	+	YP1α(80)-βgal	1.4	2.5
D67-YEFp46ph	−	+	YP1β(36)-βgal	0.3	0.07
D67-YEFp46ep	−	+	YP1β(95)-βgal	1.9	1.1

[a] Plasmids and the proteins they express are described in Table 1. [b] Proteins were estimated by indirect ELISA using specific monoclonal antibodies (Vilella et al., 1991). [c] The A_{450} is proportional to the amount of protein in the preparation.

The quantitation of cytosolic free concentrations of YP1 and YP2 derived hybrid acidic ribosomal proteins was also carried out. The results of these experiments are shown in Table 5.

As a whole, the amount of YP2 type hybrids was between 2 and 3 times greater than that for the YP1 type hybrids in the parental W303 strain. These results indicate, most probably, differences between the promoter strength of both P1 type and P2 type acidic protein genes. In the wild-type W303-1B strain, however, in comparing the amount of fused proteins derived from the same acidic proteins and, therefore, carrying the same promoter, it seems that the shorter the ribosomal protein fragment, the smaller the amount of the protein. In these cases, the results strongly suggest the existence of a differential effect on the polypeptide stability due to the size of the ribosomal protein portion. This effect is noticeable only when the acidic protein fragment is shorter than about 65 amino acids.

Table 5 also shows that the lack of either one or both YP1 native acidic ribosomal proteins had detectable effects on the amount of cytoplasmic YP1 hybrid acidic ribosomal proteins. Thus, compared to the parental strain (strain W), the lack of YP1α (strain D7) had opposite effects on the quantity of YP1α(69aa and 80aa)-βgal and YP1β(36aa)-βgal hybrid proteins: while the former two decreased to about half their concentration, the latter increased notably. The same reciprocal effect was apparent for the amounts of YP1α(80aa)-βgal and YP1β(36aa)-βgal in the *rpYP1β* gene disrupted mutant (strain D6). In contrast, the concentration of the YP1 hybrid proteins went up severalfold in the double-disrupted mutant (strain D67) where the two native YP1

Table 5: Amount of Hybrid Acidic Ribosomal Proteins in the S100 Cell Fractions[a]

hybrid protein	mol of hybrid protein/mol of 80S ribosome, yeast strain				
	W	D5	D6	D7	D67
YP1α(69aa)-βgal	1.18(±0.025)		1.40(±0.080)	0.34(±0.030)	4.11(±0.231)
YP1α(80aa)-βgal	1.17(±0.027)		0.58(±0.008)	0.50(±0.038)	6.67(±0.133)
YP1β(36aa)-βgal	0.27(±0.010)		1.28(±0.016)	0.97(±0.012)	1.44(±0.023)
YP1β(95aa)-βgal	1.27		0.44		1.88
YP2α(65aa)-βgal	2.96(±0.034)		3.55(±0.023)	3.09(±0.026)	3.32(±0.060)
YP2β(50aa)-βgal	1.77(±0.047)	2.72(±0.020)	1.63(±0.125)	0.58(±0.071)	3.02(±0.073)
YP2β(75aa)-βgal	3.58(±0.025)	2.56(±0.017)	5.81(±0.021)	3.43(±0.010)	3.28(±0.033)

[a] Results are the mean of three independent experiments except for YP1β(95aa)-βgal. Numbers in parentheses are standard deviations. Calculations were as described in Table 3.

acidic ribosomal protein members are missing. On the other hand, the expression of the YP2 hybrid proteins seems to be less sensitive to the absence of the native YP1 type acidic proteins, and not such important differences are detected in this case.

DISCUSSION

Due to the structural characteristics of the YEp356-358 plasmid series, which lack the promoter and the first seven codons of the *LacZ* gene (Myers et al., 1986), the β-galactosidase activity expressed by the set of plasmids transformed into the parental as well as the single (strains D5, D6, and D7) and double (strain D67) acidic ribosomal gene disrupted *S. cerevisiae* mutants is the result of transcription initiation at the promoter region provided by the acidic ribosomal DNA genomic fragments. Thus, the YP1α/β-βgal and YP2α/β-βgal hybrid proteins are made of acidic ribosomal polypeptides of various lengths toward the carboxyl-terminal end (Table 1) covalently bound to the β-galactosidase enzyme. Interestingly, the size of the acidic protein fragment seems not to affect the reaction mechanism of the β-galactosidase since the K_m ($M \times 10^{-3}$) of the reaction is not affected in the different fused proteins, indicating that the affinity for the susbtrate is not notably altered in the chimeric enzyme. Differences are found, however, in the V_{max} (1/h) of the reaction (Table 2), which can be interpreted as relatively important changes in the concentration of the cytosolic free hybrid acidic ribosomal proteins.

The presence of the relatively bulky β-galactosidase portion in the hybrid acidic ribosomal proteins had no significant deleterious effect on translation because the duplication times of the transformed and nontransformed parental strains were around 150 min in synthetic minimal medium (A. Cisneros and S. Zinker, unpublished results).

Functional Role of the Different Domains of the S. cerevisiae Ribosomal Acidic Phosphoproteins. Perhaps the most interesting conclusion from this work is the evidence for a direct implication of the amino-terminal domain in the interaction of the acidic proteins with the ribosome. A fragment containing only about 60% of their N-terminal sequence is sufficient to allow binding of both YP1 and YP2 types of hybrid proteins to the ribosome. In the range of 65−95 amino acids the amounts of ribosome-bound hybrids are similar and independent of the acidic protein fragment length, but instead of two copies of each acidic protein type, as expected, an average of 0.5 molecule of fused protein is bound per ribosome in the *S. cerevisiae* W303 parental transformed cells (Table 3). The results indicate either that the hybrid proteins unfavorably compete with the native components for binding to the particle or that the β-galactosidase moiety or the missing carboxyl-terminal portion, or both, diminishes their binding affinity to the ribosome and they are washed off the particle during preparation. In any case, the highly conserved carboxyl end of the eukaryotic acidic ribosomal P proteins is not essential for their binding to the ribosome, and they therefore interact with this particle through their amino-terminal end just like the bacterial proteins do (Gudkov et al., 1980; Olson et al., 1986). Our results, consequently, do not support the hypothesis of carboxyl end sequence transposition as a result of the evolutionary process as has been suggested (Lin et al., 1982), because that would implicate the P protein C-terminal domain in the interaction process.

Phosphorylation has been shown to be required by P proteins for binding to the ribosome (Naranda & Ballesta, 1991). That YP1β(36aa)-βgal and YP2β(50aa)-β gal fail to bind to the particle in the parental strain ribosomes and are only found free in the cytoplasm (Tables 3 and 5) could be due to lack of this chemical modification. Although this point has not been directly addressed in this study, it has to be noted that serine 19, the modified residue in protein YP2β (Naranda & Ballesta, 1991), is still present in the YP2β(50 aa)-βgal protein which may, therefore, be phosphorylated. In the case of YP1β, previously reported to be modified at either position 72 or 79 (Naranda et al., 1993), our recent data indicate that phosphorylation at a serine closer to the N-terminal domain of this protein is possible when its original phosphorylation sites are mutated (M. Zambrano and J. P. Ballesta, unpublished results). Therefore, phosphorylation of the YP1β(36aa)-βgal hybrid may also be possible. This conclusion is further supported by the fact that these short constructs do bind to the ribosomes when the native proteins from the same type are missing like in strains D5, D6, and D67, indicating that they carry most of the structural requirements for such an interaction. Furtheremore, they also have the part of the sequence that has been proposed to form a leucine zipper potentially involved in the acidic ribosomal protein−protein interactions (Tsurugi & Mitsui, 1991).

Relative Expression of the Different Acidic Ribosomal Phosphoproteins. When constructs from the same gene in the parental strain are compared, it seems clear that the level of expression (cytoplasmic plus ribosome-bound protein; Tables 3 and 5) is affected strongly by the size of the acidic protein fragment in the fused polypeptide. Hybrids carrying small acidic protein parts accumulate in the cell at lower levels than those having larger fragments particularly when the fused proteins contain less than about 65 amino acids from the amino-terminal domain of the acidic polypeptide,

like YP1β(36aa)-βgal and YP2β(50aa)-βgal. This difference is probably due to a lower stability of the hybrid protein since the promoter regions are identical in the constructs. The destabilizing effect is more evident in the presence of the homologous native polypeptide (strain W) as supported by the fact that the higher amounts of β-galactosidase are found in the disruptant strains D6, D7, and D67 transformed with these short acidic protein fragment fusions.

A notable difference in the level of expression in the parental *S. cerevisiae* W303 strain is also evident when constructs carrying large stable acidic protein fragments are compared. YP2 type protein fusions are expressed to higher levels than the YP1 type. These data are in agreement with our previous report showing a higher amount of the YP2 type proteins in the S100 cellular fraction by direct estimation of acidic proteins using specific monoclonal antibodies (Vilella et al., 1991) and suggest distinct control mechanisms for these two types of ribosomal proteins that may act either at the level of promoter strength or at other regulatory levels including protein stability. These differences in the level of expression of the two acidic protein types are in some ways unexpected since both are required in the same proportion for ribosome binding and activity. Since the cytoplasmic acidic ribosomal proteins exchange with their ribosome-bound counterparts during protein synthesis (Zinker & Warner, 1976) and their cytoplasmic amount changes according to the functional state of the cell (Saenz-Robles et al., 1990), the rate of exchange may be expected to fluctuate, as suggested by the existence of nonstoichiometric amounts of each YP1 type and YP2 type acidic ribosomal protein in the cytoplasmic pool (Table 5). This is an interesting possibility that stresses the functional difference between P1 and P2.

Effects at the level of expression of the hybrid proteins are also detected in the acidic ribosomal gene disrupted strains. Differences are found for the various hybrid proteins accumulated in strains D5, D6, and D7. Their interpretation, however, is not simple since in some cases the differences are not very large and can fit into the range of experimental error, and in others the possibility of an effect at the level of both protein stability—especially in the case of the short fusions YP1β(36aa)-βgal and YP2β(50aa)-βgal, which are rather unstable as indicated above—and protein expression is open. In the case of the double-disruptant D67 strain a noticeable increase of the stable hybrid proteins is found. It seems clear, also, that in this strain a strong stimulation of the expression of the YP1 type hybrids takes place, while the expression of YP2 type hybrids is not very much affected. Since our previously reported results have indicated the existence of a mutual effect on the expression of the YP1 and YP2 proteins (Bermejo et al., 1994) a straightforward interpretation of the present data suggests the existence of some limiting factor specific for the expression of YP1. In the absence of both YP1 native genes this postulated factor would act exclusively on the fused gene, strongly stimulating its expression.

The presence of hybrid acidic ribosomal proteins in the S100 fraction of the transformed cells (Table 5) confirms the existence of an important cytoplasmic pool of these ribosomal components. However, the cytoplasmic pool size of P proteins has been a matter of controversy. For *A. salina* as much as two-thirds of the total amount of acidic ribosomal proteins was detected in the cytoplasm (van Agthoven et al., 1978). In exponentially growing cells from *S. cerevisiae* the cytoplasmic pool has been estimated by us to be about equal to the amount bound to the ribosome (Zinker, 1980; Saenz-Robles et al., 1990) and by others to be as small as 0.3% (Mitsui et al., 1988). From the present results in Figure 1 and Table 3 it can be concluded that the cytoplasmic pool size of hybrid acidic ribosomal proteins accounts for as much as 40−50% of the total in the cell, in agreement with our previous reports (Saenz-Robles et al., 1990).

Interaction between YP-β-Gal Hybrid Polypeptides and the Native Acidic Ribosomal Phosphoproteins. The missing part of the acidic protein sequence in both YP1 and YP2 types of hybrid proteins comprises a structural region, called the hinge, that confers high flexibility to the carboxyl-terminal domain (Liljas, 1991). Therefore, it is possible that the short hybrids are more rigid than the larger ones and that the steric hindrance exerted by the β-galactosidase moiety can be additionally disadvantageous in the former if the P1 hybrid−P2 or P1−P2 hybrid dimers have to compete with their native counterparts for the same anchor site on the ribosome. Thus, in the absence of both YP1 proteins an important increase of the ribosome-bound YP1 hybrids is found in strain D67. This effect is not found, however, in the case of the short hybrid YP1β(36aa)-βgal, whose binding is, in fact, reduced in the D67 ribosomes. This reduction indicates that the presence of an intact YP1 protein is important for the interaction of this chimeric polypeptide with the ribosome. Probably, YP1β(36aa)-βgal binds to the ribosome by association with other YP1 proteins and is affected in the formation of the YP1−YP2 complex required for ribosome binding. In agreement with this interpretation, while the larger YP1 hybrids are able to induce the interaction of the YP2 proteins with the other acidic proteins in D67 ribosomes, the YP1β(36aa)-βgal does so to a very limited extent (Table 5).

It is interesting to note that the YP1α derivatives seem to be more effective in stimulating the interaction of YP2β, while the YP1β hybrids induce more efficiently the interaction of YP2α (Tables 3 and 4). To our knowledge these are the first data to indicate that the associations of the YP1 and YP2 protein types that interact with the ribosome may not be totally equivalent and that ribosomes carrying YP1α−YP2β and YP1β−YP2α may be preferentially formed and have functional relevance.

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