Acta Biochimica Polonica

Vol. 46 No. 4/1999

901–910

QUARTERLY

Extraribosomal function of the acidic ribosomal P1-protein YP1α from *Saccharomyces cerevisiae*[o]

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Received: 05 July, 1999; accepted: 04 November, 1999

Key words: acidic ribosomal P-protein, translation, transactivation, yeast

The yeast acidic ribosomal P-proteins YP1α, YP1β, YP2α and YP2β were studied for a possible transactivation potential beside their ribosomal function. The fusions of P-proteins with the GAL4 DNA-binding domain were assayed toward their transcriptional activity with the aid of reporter genes in yeast. Two of the P-proteins, YP1α and YP1β, exhibited transactivation potential, however, only YP1α can be regarded as a potent transactivator. This protein was able to transactivate a reporter gene associated with two distinct promoter systems, *GAL1* or *CYC1*. Additionally, truncated proteins of YP1α and YP1β were analyzed. The N-terminal part of YP1α fused to GAL4-BD showed transactivation potential but the C-terminal part did not. Our results suggest a putative extraribosomal function for these ribosomal proteins which consequently may be classified as "moonlighting" proteins.

The function of ribosomal proteins is not restricted solely to ribosome building and active participation in protein biosynthesis. Several ribosomal proteins take part in the process of replication, transcription, DNA repair, malignant transformation, regulation of development (Chan *et al.*, 1993; 1994; Jeffery, 1999; Rice & Steitz, 1989; Wool, 1996) and therefore they may be regarded as "moonlighting" proteins (Jeffery, 1999).

The large ribosomal subunit of all organisms contains a unique set of acidic proteins that are involved in the activity of ribosomes. They form a characteristic flexible lateral protuber-

[o]This work was supported by Grant No. 6 P04A 019 14 from the State Committee for Scientific Research (KBN, Poland) to N.G.

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Abbreviations: BD, GAL4 DNA-binding domain; AD, GAL4 activation domain; 3-AT, 3-amino-1,2,4-triazole; ONPG, *o*-nitrophenyl β-D-galactopyranoside; UAS, upstream activating sequence.

ance (called "stalk") on the surface of the ribosome. The bacterial (*Escherichia coli*) "stalk" is composed of two dimers of acidic proteins L7 and L12 in a complex with a single copy of ribosomal protein L10. This ribosomal structure plays an important role in the interaction of the ribosome with translation factors during protein synthesis (Liljas, 1991).

In eukaryotic cells, the acidic proteins from the 60S ribosomal subunit seem to be functionally equivalent to their bacterial counterparts, however, they have numerous features that distinguish them from bacterial L7/L12. The most significant one is the capability to be phosphorylated *in vivo* as well as *in vitro*, and for this reason they are called P-proteins. There are two types of P-proteins, P1 and P2. The amino-acid sequences of P-proteins family are extremely well conserved at the carboxyl end in all organisms – from yeast to human (Ballesta *et al.*, 1993; Ballesta & Remacha, 1996). In *Saccharomyces cerevisiae*, four acidic proteins from the 60S ribosomal subunit were identified and genes for them have been cloned (Mitsui & Tsurugi, 1988; Newton *et al.*, 1990; Remacha *et al.*, 1988). According to a proposed uniform nomenclature, the yeast P-proteins are named: YP1α, YP1β, YP2α and YP2β. On the basis of their primary structure similarity to mammalian P-proteins, the two proteins YP1α and YP1β were classified into the P1 subgroup and YP2α and YP2β into the P2 subgroup (Wool *et al.*, 1991). Recently, a new nomenclature of acidic ribosomal P-proteins has been proposed, i.e.: P1A, P1B, P2A and P2B (Mager *et al.*, 1997). The key role in the interaction of P1 and P2 proteins with the ribosomal structure is played by the non-acidic 38 kDa protein called P0, which belongs to 60S ribosomal core proteins. The P0 interacts with the acidic ribosomal proteins and forms a pentameric complex: P0-$(P1)_2$/$(P2)_2$ (Santos & Ballesta, 1994).

The polypeptide chains of acidic ribosomal proteins can be divided into two functionally relevant parts. The N-terminal and C-terminal parts consist of about 60 and 40 amino acids, respectively. The N-terminal part possesses a hypothetical bilateral zipper (Tsurugi & Mitsui, 1991) which closely resembles the bZIP structure characteristic for various transcription factors with prominent examples such as Jun and Fos proteins (Curran & Franza, 1988; Ransone & Verma, 1990). The primary structure of the N-terminal part shows less similarity among the P-proteins family and is used as a point of reference in classifying acidic ribosomal proteins. It has been shown that this part of the polypeptide chain is responsible for dimer formation of P1/P2 proteins and for the interaction with the ribosome (Jose *et al.*, 1995). The C-terminal part has an alanine-glycine rich region, the so-called flexible hinge, and an acidic region which is composed of acidic and hydrophobic amino acids. The acidic region closely resembles activation domains in known transcription factors such as VP16 (Cress & Triezenbergm, 1991) or BRCA1 (Monteiro *et al.*, 1996) in which acidic as well as hydrophobic amino acids play an important role in transactivation (Cress & Triezenbergm, 1991; Ma & Ptashne, 1987). The function of the acidic domain in the C-terminal part of P-proteins has not been studied yet. Recent studies on the tertiary structure of the yeast YP2β protein revealed an absence of the usual rigidity of the completely folded protein. These features suggest that YP2β possesses a native conformation closely related to a molten globule (Zurdo *et al.*, 1997). This conformation has been found in active transcription activation domains in the absence of their ligands. It is important to note that most of these largely unstructured domains in native-like conditions are acidic (Dahlman-Wright *et al.*, 1995; Donaldson & Capone, 1992; Schmitz *et al.*, 1994). Moreover, some nonacidic transcription activation domains seem to be extensively unstructured, implying that this is a frequent feature among transcription factors (Cho *et al.*, 1996).

Considering the properties of yeast acidic ribosomal P-proteins, it seemed interesting,

whether P-proteins have transcription factor characteristics and activity. We assessed the transactivation potential of yeast P-proteins by creating fusion proteins with the known GAL4 DNA-binding domain. Additionally, truncated proteins were analyzed in order to find the domain responsible for transactivation.

MATERIALS AND METHODS

Materials, media and yeast strains. All chemicals used for yeast handling and β-galactosidase assay were from Sigma. Restriction and modifying enzymes were purchased from MBI Fermentas. Two strains of *Saccharomyces cerevisiae*, HF7c (*MAT***a**, *ura3-52, his3-200, lys2-801, ade2-101, trp1-901, leu2-3, 112, gal4-542, gal80-538, LYS2::GAL1-HIS3, URA3::(GAL4 17-mer)$_3$-CYC1-lacZ*) (Feilotter *et al.*, 1994) and SFY526 (*MAT***a**, *ura3-52, his3-200, ade2-101, lys2-801, trp1-901, leu2-3, 112, can^r, gal4-542, gal80-538, URA3::GAL1-lacZ*) (Bartel *et al.*, 1993a), were used (Clontech Laboratories Inc.). Yeast-*E. coli* shuttle vector, pGBT9 carrying yeast GAL4 binding domain (Bartel *et al.*, 1993b) was obtained from Clontech Laboratories Inc. as a part of MatchMaker Two-Hybrid System. The QIAquick PCR purification kit was from QIAGEN.

Yeast transformation, colony-lift filter and liquid β-galactosidase assay. HF7c or SFY526 transformants were streaked on two solid synthetic minimal media. The first medium, for the analysis of transformants in SFY526 host cells, was tryptophan-free. The second one was free of tryptophan and histidine but supplemented with 5 mM 3-amino-1,2,4-triazole (3-AT) for the selection of transformants in HF7c host cells. The growth was evaluated after 3 days. All media were used as described (Sherman, 1991). Competent yeast cells were prepared according to supplier's (Clontech Laboratories Inc.) instruction (lithium acetate method).

The colony-lift filter β-galactosidase assay was performed using SFY526 or HF7c transformants. The reaction was carried out at 30°C for up to 24 h. The liquid β-galactosidase assay was done with *o*-nitrophenyl β-D-galactopyranoside (ONPG) as a substrate according to Clontech Laboratories procedure with SFY526 as the host strain. The activity of β-galactosidase was measured after Miller (Miller, 1972).

Construction of vectors expressing the yeast P-proteins fused to Gal4 DNA-binding domain. The genes for yeast P-proteins were amplified by PCR using a yeast cDNA library in the vector pBSIISK-. The library was obtained as a generous gift from Dr. Johannes Regenbogen, Laboratorium für Molekulare Biologie, Genzentrum der Universtität (München, Germany). The following oligonucleotide primers were used: YP1α forward primer - 5′ GGG GAA TTC AGG AGA GAA GAA ATG TCT ACT GAA TCC 3′, reverse primer – 5′ GGA GGG GAT CCT TTC TTC TAA ACA GTG CGG CA 3′; YP1β, forward primer – 5′ GGG GAA TTC AGG AGG AAG AAA ATG TCT GAC TCT ATT A 3′, reverse primer – 5′ GGA GGG GAT CCT TTA AAT ACT GAT TGA TTA GAG GT 3′; YP2α, forward primer – 5′ GGG GAA TTC AGG AGT ACA AAA ATG AAG TAC TTA GCT GC 3′, reverse primer – 5′ GGA GGG GAT CCT AAC CAG TAA AAC AAT CGG TTT GA 3′; YP2β, forward primer – 5′ GGG GAA TTC AGG AGA ACA GAA ATG AAA TAC-3′, reverse primer – 5′ GGA GGG GAT CCT AAA ATG AAG GAA AAC-3′. The truncated forms of proteins YP1α and YP1β were constructed with PCR using following primers: YP1α N-terminal part (amino acids 1 to 62), forward primer 5′ GCG GAA TTC ATG TCT ACT GAA TCC GCT TTG 3′, reverse primer 5′ GCG GGA TCC TTA GCT GAA GTT GAC CAA TAA GTC 3′; C-terminal part (amino acids 63 to 106), forward primer 5′ GCG GAA TTC GCT GGT GCT GCT GCC CCA 3′, reverse primer 5′ GCG GGA TCC TTA ATC AAA TAA ACC GAA ACC 3′; YP1β N-terminal part (amino ac-

ids 1 to 62), forward primer 5′ GCG GAA TTC ATG TCT GAC TCT ATT ATT TCC 3′, reverse primer 5′ GCG GGA TCC TTA GTT ATG GAA ACC AGA TAG GAT 3′; C-terminal part (amino acids 63 to 106), forward primer 5′ GCG GAA TTC GCT GGC CCT GTT GCT GGT 3′, reverse primer 5′ GCG GGA TCC TTA GTC GAA TAA ACC GAA ACC 3′. All primers used for PCR contained suitable endonuclease restriction sites at either ends, *Eco*RI at the 5′ end and *Bam*HI at the 3′ end. All DNA fragments, after PCR amplification were purified using QIAqick PCR purification kit. The resultant fragments were digested with suitable restriction endonucleases and were ligated into the yeast expression vector pGBT9 in frame to the GAL4 DNA-binding domain. The junctions and sequences of all constructs were sequenced on the Macrophor sequencing system (Pharmacia Biotech) using Sequenase™ Version 2.0 DNA Sequencing kit. Vectors were named as follows: pT9-YP1α and pT9-YP1β carry intact genes for YP1α and YP1β proteins, respectively, fused to GAL4 binding domain (GAL4-BD), pT9-N-YP1α and pT9-N-YP1β represent N-terminal parts of YP1α and YP1β proteins, respectively, fused to GAL4-BD, pT9-C-YP1α and pT9-C-YP1β correspond to C-terminal parts of YP1α and YP1β proteins, respectively, fused to GAL4-BD.

RESULTS

Transcription activation of reporter genes by the acidic ribosomal proteins YP1α and YP1β, utilizing two promoter systems *GAL1* and *CYC1*

Based on theoretical considerations, that yeast acidic ribosomal proteins might possess a transactivation potential, all genes for yeast P-proteins were fused to GAL4 DNA binding domain. Transcription activation in yeast was assayed with the aid of the β-galactosidase reporter gene. Plasmids expressing YP1α (pT9-YP1α), YP1β (pT9-YP1β), YP2α (pT9-YP2α), and YP2β (pT9-YP2β) were transformed into yeast host strains SFY526 and HF7c. The transformants were selected on two media. The first medium depleted of tryptophan was used for the selection of transformants (SFY526 or HF7c host cells) which were analysed for β-galactosidase activity. The second medium without tryptophan and histidine but supplemented with 5 mM 3-AT was used for the selection of transformants (HF7c host cells) on the basis of their growth ability.

Initially, the system with *GAL1* promoter was utilized in SFY526 yeast host strain in which transcription of β-galactosidase reporter gene is controlled by the TATA portion of the *GAL1* promoter and by the *GAL1* upstream activating sequence (UAS). Applying the colony-lift β-galactosidase filter assay for blue/white color selection, the SFY526 cells carrying plasmid pT9-YP1α gave an intensive blue color after one-hour incubation. In the case of recombinant cells carrying the plasmid pT9-YP1β, blue color appeared after twelve-hour incubation. The transformants pT9-YP2α and pT9-YP2β gave only white color even after twenty-four-hour incubation (Fig. 1). For quantitative comparison of the transcriptional activity of YP1α and YP1β proteins, the β-galactosidase liquid assay with ONPG as substrate was conducted utilizing the SFY526 host cells. As shown in Fig. 2, YP1α gives higher transactivation. In the case of YP1β, the activation of the β-galactosidase gene reached only 10% of YP1α activity.

In order to confirm the observed results and to exclude possible artifacts in the GAL4 based assay, all constructs were tested using HF7c host strain. This strain has two reporter genes, *lacZ* and *HIS3*, which are controlled by two independent promoter systems *CYC1* and *GAL1* with *GAL1* UAS, integrated into the yeast genome. The transactivation of reporter genes depends on the reconstitution of functional GAL4 or GAL4 BD associated with a protein having transactivation potential. In the first experiment, *GAL1*-driven *HIS3* reporter gene was used. Figure 3A shows the

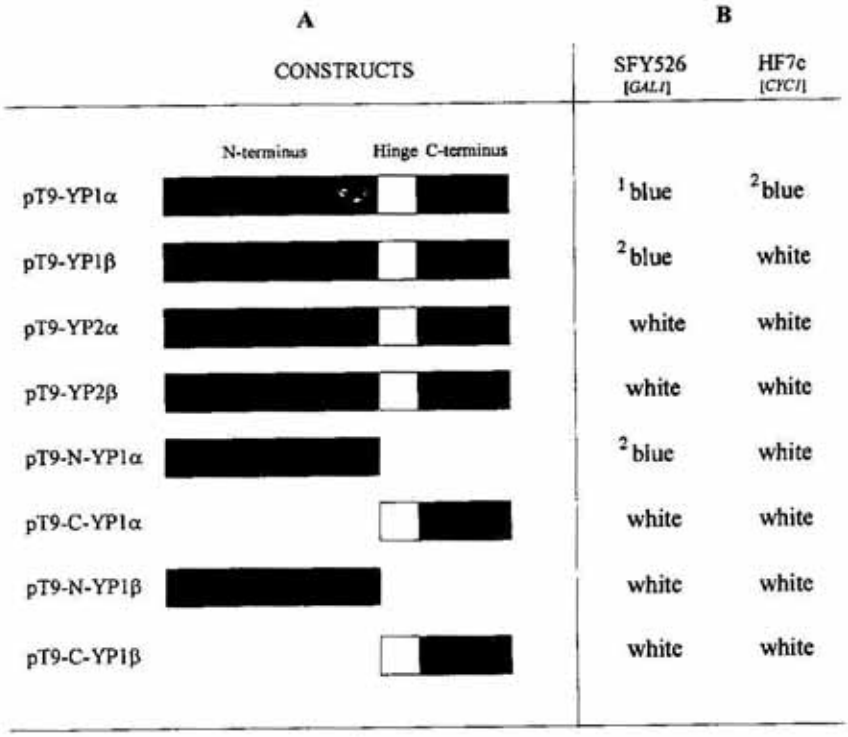

Figure 1. Transcriptional activity of acidic ribosomal P-proteins fused to GAL4 DNA-binding domain.

A, schematic representation of the yeast acidic ribosomal proteins and truncated proteins cloned into the yeast vector pGBT9; B, summary of the transcription activity of each fusion protein transformed into the yeast host strain SFY526 or HF7c, promoters used in this experiment are indicated in square brackets. Transcriptional activity was determined by colony-lift filter β-galactosidase assay as described in Materials and Methods.

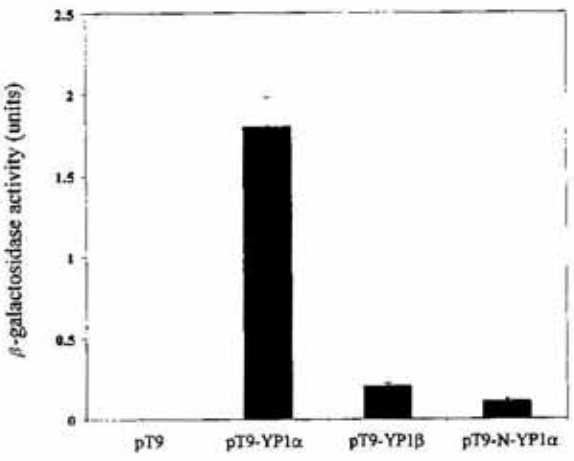

Figure 2. Graphic representation of differential transcriptional activity of acidic ribosomal P-proteins.

Liquid β-galactosidase assay was done in the transformants of yeast strain SFY526. pT9, pT9-YP1α, pT9-YP1β and pT9-N-YP1α represent: the control plasmid pGBT9 expressing GAL4 DNA-binding domain, the fusion of protein YP1α and YP1β with GAL-BD, and the N-terminal part of YP1α fused to GAL4-BD, respectively. Data represents average values from two sets of independent experiments performed in triplicate. β-Galactosidase units were quantified according to the Miller method (Miller, 1972).

growth of transformants pT9-YP1α, pT9-YP1β on SD medium free of tryptophan and histidine, and supplemented with 5 mM 3-AT in order to prevent leaky expression of *HIS3* gene. The growth of recombinant cells pT9-YP1α on the medium without histidine indicated strong transactivation of the *HIS3* reporter gene by the YP1α P-protein. In the case of pT9-YP1β, the growth of cells was poorer, but some transactivation of the *HIS3* gene could be clearly observed (Fig. 3A). No growth of the pT9-YP2α and pT9-YP2β transformants was observed (not shown). It proves the absence of transactivation of the *HIS3* reporter gene by the YP2α and YP2β P-proteins. All these results are in agreement with the experiment which was performed in SFY526 cells having the *lacZ* reporter gene.

Subsequently, the strain HF7c was used with the *CYC1* promoter system associated with the *lacZ* reporter gene in which the expression of the reporter gene is controlled by the TATA portion of the *CYC1* promoter. At this point, it is important to stress that the *CYC1* promoter is very weak and only a potent transactivator can be detected in this conditions. Applying

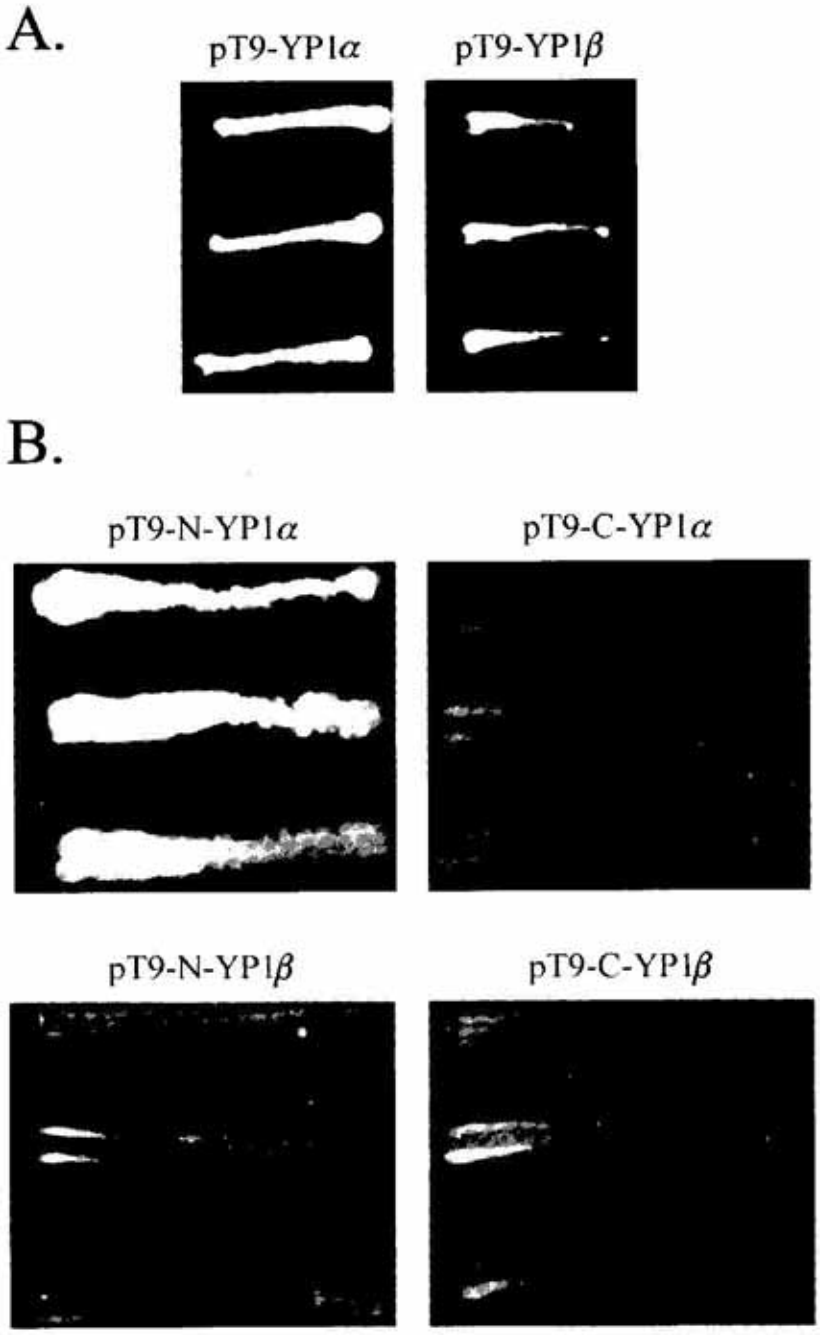

Figure 3. Transactivation of *HIS3* reporter gene by acidic ribosomal P-proteins.

Growth dependency was analyzed on SD medium without tryptophan and histidine supplemented with 5 mM 3-amino-1,2,4-triazole. Plates were incubated at 30°C for 6 days. A, growth dependency of transformants expressing full-length P-proteins YP1α and YP1β. B, analysis of truncated proteins (Fig. 1).

this method, all transformants were selected on a tryptophan-free medium. The β-galactosidase filter assay showed that only transformants carrying the plasmid pT9-YP1α gave blue color. The rest of the transformants: pT9-YP1β, pT9-YP2α and pT9-YP2β were white (Fig. 1).

Transactivation of the *lacZ* reporter gene by the deletion mutants of acidic ribosomal proteins YP1α and YP1β

Since full length YP1α and YP1β polypeptide chains possess the transactivation potential, it was interesting to learn which part

(domain) of acidic ribosomal proteins is engaged in transcription activation. Two types of truncated proteins for YP1α and YP1β proteins were prepared as described in Materials and Methods. In order to check the transactivation potential of these mutants, two reporter genes were used in two promoter systems. In the first system, SFY526 yeast strain was used with the *lacZ* reporter gene. Transformants with the pT9-N-YP1α plasmid turned blue in the colony-lift β-galactosidase filter assay after twelve-hour incubation (Fig. 1). When quantitative analysis was applied using the *lacZ* reporter gene and ONPG as a substrate (Fig. 2), pT9-N-YP1α showed only 10% of native YP1α activity. In the case of pT9-C-YP1α, pT9-N-YP1β and pT9-C-YP1β, all transformants displayed white color only in the β-galactosidase assay. Subsequently, a second system was applied with the host strain HF7c in which *GAL1* promoter was linked to the *HIS3* reporter gene. The experiment showed that pT9-N-YP1α transformant could grow on the SD medium without tryptophan and histidine. All other transformants with truncated P-proteins could not grow on this medium (Fig. 3B). The β-galactosidase filter assay gave negative results for all truncated constructs in the second system where the *lacZ* reporter gene was linked to the *CYC1* promoter (Fig. 1).

DISCUSSION

The accumulated data on the structure of yeast ribosomal P-proteins, showing the presence of a bZIP-like structure (Tsurugi & Mitsui, 1991), an acidic region (Ballesta & Remacha, 1996), and a native molten globule-like structure (Zurdo *et al.*, 1997), all of which are characteristic for numerous transcription factors, support the concept that this group of ribosomal proteins may have a transactivation potential. In order to verify this hypothesis, a set of fusion proteins with a heterologous DNA binding domain ($GAL4_{1-147}$) was prepared and analyzed *in vivo* in yeast cells. The results obtained clearly demonstrate that two proteins, YP1α and YP1β, have a transactivation potential. However, only YP1α can be regarded as a potent transcriptional activator, due to its activity observed in the two independent systems *GAL1* and *CYC1*. The transcriptional activity of the YP1β protein is much weaker, which was shown by the lack of its activity in the system with the *CYC1* promoter linked to the *lacZ* reporter gene. The two other proteins, YP2α and YP2β, belonging to the P2 subgroup do not possess a transactivation potential. Additional information on the transactivation features of yeast P1-proteins was obtained from experiments with truncated proteins fused to the GAL4 DNA binding domain. The crucial role in transcriptional activity of YP1α is played by the N-terminal part of the polypeptide (Fig. 1). However, the C-terminal part may have a positive effect on transactivation, because the activity of the truncated YP1α protein devoided of its C-terminal part is significantly reduced.

In order to provide direct evidence that the YP1α protein undergoes a proper protein folding in this system and therefore its transcription activity is associated with the native conformation of this protein, we performed a two-hybrid assay. Assuming that YP1α can interact exclusively with YP2β (Jose *et al.*, 1995), we prepared fusion proteins of YP1α with the activation domain (AD) of the GAL4 transcription factor ($GAL4_{768-881}$) utilizing the pGAD424 vector (Bartel *et al.*, 1993b). Since YP2β and YP2α proteins do not possess a transactivation potential, they were fused to GAL4 DNA-binding domain (BD) in pGBT9 vector. The obtained co-transformants of YP1α/GAL4-AD and YP2α/GAL4-BD or YP2β/GAL4-BD were tested using the SFY526 host strain with the *lacZ* reporter gene. Positive results of the assay were obtained only for co-transformants containing YP1α/GAL4-AD and YP2β/GAL4-BD (not shown). This indicated that an interaction

took place exclusively between YP1α and YP2β.

A question arise: How can ribosomal protein YP1α act as an activator of transcription? It is thought that the activity of the transcription machinery depends on transactivators which bind DNA outside the core promoter. In many cases, transactivators exist as protein complexes in which some components recognize DNA and other components, called adapter proteins or coactivators, are responsible for the activation of the transcriptional complex (Mannervik *et al.*, 1999). Basing on the results described in this paper YP1α may act as part of a transactivator. At present, the only known protein which interacts with YP1α is the 60S ribosomal protein P0. Recent years have brought an interesting observation about an additional biological function of P0. This protein, besides its structural function as a core protein in the large ribosomal subunit (see introduction), is also involved in DNA repair and shows strong DNase activity for both single- and double-stranded DNA (Yacoub *et al.*, 1996). Recently another extraribosomal function of protein P0 has been described. This multifunctional protein is also involved in the regulation of gene expression (Frolov & Birchler, 1998). Taking into consideration the two properties of the YP1α ribosomal protein from yeast: first, that it specifically interacts with protein P0, second, that it has a transactivation potential, one may suggest that both P0 and YP1α proteins can form a multimeric structure involved in the regulation of transcription. Therefore, P0 and YP1α have two independent activity, one associated with protein translation and the second one related to regulation of a gene expression. Such behavior is characteristic of several proteins with dual activities, which are consequently called "moonlighting" proteins, whose biological function frequently can be modulated by formation of multimeric structure (Jeffery, 1999).

We are grateful to Professor O.-G. Issinger (Odense University, Denmark) for his valuable remarks and comments.

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