Transcriptional control of yeast ribosomal protein synthesis during carbon-source upshift


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ABSTRACT
Shifting a yeast culture from an ethanol-based medium to a glucose-based medium causes a coordinate increase of the cellular levels of ribosomal protein mRNAs by about a factor 4 within 30 min. Making use of hybrid genes encompassing different portions of the 5'-flanking region of the L25-gene, we could show that the increase in mRNAs is a transcriptional event, mediated through DNA sequences upstream of the ribosomal protein (rp) genes. Further analysis revealed that sequence elements are involved that many rp-genes have in common and that previously were identified as transcription activation sites (RPG-boxes or UASrp). Using appropriate deletion mutants of the fusion genes we could demonstrate that a single RPG-box is sufficient for the transcriptional upshift. In addition, both copy genes encoding rp28 which differ considerably in their extent of transcriptional activity, show the upshift effect in a proportional manner. Definite proof for the role of the UASrp in nutritional regulation was obtained by examining the effect of a synthetic RPG-box on transcription.

INTRODUCTION
One of the most intriguing aspects of ribosome biogenesis in yeast concerns its efficient adjustment to different growth conditions of the cell. Indeed, altering physiological needs for overall protein synthetic capacity primarily influence the rate of ribosome formation (reviewed in Ref. 1). Yeast ribosomes are built up out of about 80 different components (1). Therefore, tightly coordinated regulatory mechanisms must underly the maintenance of an equimolar production of this large number of ribosomal constituents.

Regulation of ribosomal protein synthesis in yeast occurs at two distinct levels (2). A variety of posttranscriptional control mechanisms operate to keep expression of individual rp-genes in balance with that of the others (3-8). On the other hand, most likely the primary and major control is the coordinate expression of the various rp-genes at the transcriptional level. Recently, compelling evidence was obtained that conserved sequence elements (RPG-boxes) occurring 5' to many rp-genes (9,10) act as transcription activation sites (11,12). In addition, by gel electrophoretic retardation assays and footprint analyses, a yeast protein factor could be identified that specifically interacts with the homologous boxes (13,14). Most likely, this protein functions as a trans-acting factor involved in the coordinate control of rp-gene transcription.

The aim of the study described in this paper was to investigate whether upstream DNA sequences, in particular the activation sites, UASrp, are engaged in the regulation of rp-gene
expression under a specific physiological condition, viz. a nutritional upshift from an ethanol-based medium to a glucose-based medium.

MATERIALS AND METHODS

Recombinant plasmids

Plasmids pBMCY138, 113 and 76-3 consist of HindIII-generated yeast DNA fragments carrying the genes for ribosomal protein L25, S10 and S33, respectively, cloned in pBR322 (15). Plasmids YCP6Δ12H and YCP1-1, kindly provided by Dr. R. Zitomer, are E. coli-yeast shuttle vectors carrying the E. coli galK gene under control of the cyc7-promoter (16,17). In YCP6Δ12H an EcoRI-site originally located immediately downstream of the galK gene has been converted into a HindIII-site (H. Klein, this laboratory). In YCP1-1 this EcoRI site has been destroyed (16). A-E (see Fig. 2) are L25-galK fusion genes identical to UR-L25 and the deletion mutants no. 5, 7, 9 and 10 respectively, described previously (12).

Oligomer insertions

Oligonucleotides carrying the RPG-box consensus sequence ACCCATACATTT (10) supplied with PstI-sticky ends were synthesized using an Applied Biosystems 381A DNA Synthesizer and subsequently inserted into the positions indicated in Fig. 2 as discussed in the text.

Preparation and analysis of DNA

Plasmids were purified as described previously (10). Restriction enzyme digestions were performed as recommended by the suppliers (Pharmacia; BRL).

Medium-upshift

Transformants of the yeast strain aBR10 (16) or HR2 (18) were grown in a medium containing 0.67% yeast nitrogen base, 0.05% yeast extract, 0.04% glucose, 2% ethanol supplemented with either 20 mg/l adenine and 50 mg/l histidine (aBR10) or 20 mg/l uracil, 50 mg/l histidine and 400 mg/l leucine (HR2) until an OD_660nm of 0.8 was reached. Then 0.1 volume of 20% glucose was added and rapidly mixed. Samples (10 ml) were taken at 0, 10, 20, 30, 60 and 120 min after this carbon-source upshift. Cells were immediately frozen in liquid nitrogen and stored at -20°C until use.

Preparation and Northern analysis of RNA

Cells were broken with glass beads essentially as described by Bromley et al. (19). Samples of 10 μg total cellular RNA were fractionated on 1.5% agarose gels after denaturation in 1 M glyoxal and 50% dimethyl sulphoxide (20) and then blotted onto filters (Hy-bond, Amersham). GalK, L25, S10, S33 and histone H4 gene-specific probes were labelled according to the procedure of Hu and Messing (21). We used an EcoRI-generated fragment from YCp6 (i.e. galK; Ref. 16), a (HpaI+TaqI)-generated fragment of pBMCY138 (i.e. L25; Ref. 22), a (TaqI+BglII)-generated fragment of pBMCY13 (i.e. S10; Ref. 23), a HindIII+PvuII-generated fragment of pBMCY76-3 (i.e. S33; Ref. 24) and an (EcoRI+Sau3AI)-generated fragment of H4-M13mp8 (i.e. H4; Ref. 25) respectively.
Primer extension assay
In order to estimate the relative amounts of mRNA transcribed from the duplicate copy genes rp28-1 and rp28-2 (26), a primer extension experiment was performed as follows. Total RNA was incubated in the presence of a synthetic radioactive rp28 gene-specific primer which was chosen in such a way that extension in the presence of ddGTP gave rise to distinct products. After annealing 0.3 pmol primer to 6 µg total RNA in 0.1 M Tris HCl (pH 8.3), 0.14 M KCl, reverse transcriptase was added (BRL, M-MLV, 5 U/µl). The reaction was performed at 37°C. Analysis of the denatured primer extended DNA products was carried out on a 12% polyacrylamide gel.

Scanning of autoradiograms
The intensity of the hybridization signals was measured using a Kipp light scanning densitometer.

RESULTS AND DISCUSSION
Carbon-source upshift causes coordinate increase of rp mRNA levels
Shifting a yeast culture from a non-fermentable carbon-source like ethanol to a fermentable carbon-source like glucose causes a coordinate increase of the rate of synthesis of yeast ribosomal proteins by about a factor 3 within 30 min (27). We applied a similar nutritional upshift to assess whether this increased r-protein synthesis is based upon elevated cellular concentrations of rp-mRNAs. Northern analysis of yeast mRNA using several rp-gene-specific probes (see Materials and Methods) indicated that, indeed, the rp-mRNA levels increase approximately 4-fold as an immediate response to the upshift (see Fig. 1).

The results of RNA-hybridizations shown in Fig. 1 and the subsequent figures represent typical examples of these analyses. In addition, we have included a graphical representation obtained by averaging the data from several experiments.

The pattern of the histone H4 mRNA level, analyzed as a control, is clearly different from that of the rp-mRNAs (Fig. 1). Within 30 min. after the medium upshift no increase of the H4 mRNA can be observed, which appears to be consistent with the previously observed temporary arrest of cell growth following a nutritional upshift (27,28). Clearly, despite the lack of an absolute standard (i.e. an mRNA whose level is not affected by a shift in carbon-source), it is quite possible to perform a reliable analysis of the specific coordinate effects of a nutritional upshift on rp-mRNA levels.

Apparently, the major contribution to the higher expression of yeast rp-genes at a medium upshift is not supplied by an adjustment of the translation efficiency but rather by elevated mRNA levels. Two mechanisms might account for this increase in the steady state levels of rp-mRNAs: enhanced transcription of the rp-genes or higher stabilities of the rp-mRNAs.

Construction of fusion genes
To determine whether the coordinate control operates at the level of transcription and, more
Fig. 1. Effect of an ethanol-glucose medium upshift on cellular levels of yeast rp mRNAs. RNA was isolated from yeast cells grown in ethanol-based medium (0) as well as 10, 20, 30, 60 and 120 min. after glucose addition. Northern hybridization using gene-specific probes was performed as described in Materials and Methods. The autoradiograms shown represent the results of typical experiments. It is difficult, however, to precisely estimate the amount of RNA loaded on the gel. Therefore these Northern analyses were repeated several times. In the graphical representation average mRNA levels quantified by scanning and estimated relative to the ethanol-grown situation (0 min. = 100%) were plotted against the incubation time.

Specifically, whether the rp-gene-specific upstream activation sites (RPG-boxes; see Ref. 10) are involved in this regulation, we made use of several fusion gene constructs. In Fig. 2 these constructs are depicted schematically.

The upstream region of the gene encoding L25 harbors two adjacent conserved nucleotide elements, designated as RPG1 and RPG2 (Fig. 2). We have previously reported on deletion studies that provided proof for the transcription activating function of these boxes (12). Several deletion mutants were used in the present investigations. The complete L25 upstream region (Fig. 2A) as well as upstream regions carrying deletions up to the 5'-side of the boxes (Fig. 2B), or removing the gene-distal box (Fig. 2C), or removing both boxes (Fig. 2D), or
extending to a position downstream of the T-stretch (Fig. 2E), were fused to the galK marker gene in the vector YCpR6A12H (see Materials and Methods). Transcription of all hybrid genes obtained is directed by the L25-promoter and initiation sites, since the fusion point is localized within the 5'-untranslated leader region of the L25-gene. The transcripts synthesized from these hybrid genes comprise only 10 (or maximally 14; Ref. 22) nucleotides of the leader sequence of the L25 mRNA. Occasionally synthetic oligonucleotides were inserted in the (M13-derived) PstI-site (see below). In addition, we made use of a cycJ-galK fusion gene cloned in the vector YCpRl-1 (16,17) - see Fig. 2F. In this construct the initiation codon originates from the cycJ gene (in-frame fusion). The upstream activation sites of the cycJ gene are located on the Xhol-Xhol fragment (29). Deletion of this fragment and subsequent insertion of an L25-upstream DNA fragment (PstI-NsI; see Fig. 2) results in fusion gene construct G, which is under control of the L25-UAS but uses the cycJ promoter and transcription initiation sites. Alternatively, in the Xhol site a synthetic UASgalK element was inserted, separated from the cycJ - transcription initiation region by a λ-DNA fragment (Fig. 2H; see below).

Carbon-source upshift affects transcription

We first estimated the steady state concentrations of the hybrid transcripts from fusion genes B and G in response to an ethanol-glucose upshift. As can be concluded from the results of Northern hybridizations presented in Fig. 3 the induction patterns of the transcript levels of both genes parallels the coordinate increase of the rp-mRNA concentrations. Since the fusion transcript B contains only a few L25-derived nucleotides at its 5'-end, whereas transcript G encompasses exclusively non-ribosomal sequences, the observed upshift effect on mRNA levels cannot be due to a specific change of rp-mRNA stability. Recently, Donovan and Pearson (28) arrived at the same conclusion using a S16A-lacZ fusion gene. Under similar nutritional upshift conditions fusion transcript F decreases significantly since the UAScyc1 is subject to catabolite repression - results not shown (29).

The results presented in Fig. 3, in addition, allow the conclusion that upstream sequences, and not promoter or transcription start sites, are required for the transcriptional upshift. For, the L25-upstream DNA region extending from the NsI-site at -120 up to the M13-derived PstI-site, is able to transfer the carbon-source upshift effect on transcription onto the cycJ-galK fusion gene (Fig. 2G). It is conceivable, therefore, that the conserved nucleotide elements RPG1 and RPG2 which in cis activate transcription of the downstream gene, are specifically involved in the up-regulation of transcription.

A single RPG-box is sufficient for the transcriptional upshift

In general, the rp-gene upstream regions harbor two adjacent UASrp elements (10). We wondered whether under low physiological demands one box might be functional while at increased growth rate both boxes are required for transcription activation. In that case, a single box would be insufficient to mediate the observed carbon-source upshift effect. Analysis of the transcript levels from fusion gene B in comparison with fusion gene C demonstrates that this
assumption is invalid (see Fig. 3). Even in the presence of one box, - the gene-distal one being deleted (Fig. 2C) -, the upshift effect on transcription of the L25-ga/K fusion gene is manifest. However, it appears that the transcription of the hybrid gene C shows a somewhat delayed response to the carbon-source upshift. This phenomenon might reflect cooperativity in vivo of the adjacent UAS$_{t}$ elements as previously suggested (30).

Most yeast ribosomal protein genes occur in two copies on the haploid genome. In some cases the duplicate genes are expressed to a significantly different extent. For instance, the steady state amount of transcripts from the rp28-1 gene is estimated to be roughly 6 times as
high as the cellular level of mRNA transcribed from the rp28-2 gene (see Fig. 4). Most likely, this difference in transcript levels is due to a considerable difference in activating effect of the respective UASrp genes. Gel retardation assays and footprinting analysis using the yeast protein TUF (13) revealed that the rp28-1 gene carries two adjacent TUF-binding sites both having a high affinity for the protein in vitro, whereas rp28-2 harbors a single, weak TUF-binding DNA element (14). The result of the primer extension experiment presented in Fig. 4 shows that, despite this difference in gene activity, transcription of both gene copies responds proportionally to the carbon-source upshift, concomitantly with the expression of other rp-genes. These data confirm the abovementioned conclusion that a single UASrp element is sufficient to mediate the nutritional regulation.

The synthetic RPG-box confers carbon-source upshift response

Fusion gene D (see Fig. 2) has previously shown to be transcriptionally inactive (12). Transcription activity can be restored, however, by insertion of a synthetic oligomer carrying the RPG-consensus sequence into the PstI-site (Fig. 2; Refs. 12,30). Northern analysis of the transcript levels present immediately after an ethanol-glucose shift (see Fig. 5, blot D) indicates that, in addition to the transcription activity itself, also the upshift effect is restored by a synthetic box. One may argue, however, that insertion of the synthetic RPG element at the natural position of the L25 upstream sequence repairs a larger essential nucleotide element. In particular, its combination with a T-rich region (Py-stretch; see Fig. 2), commonly located 3' to the UASrp elements and suggested to be an integral part of a tripartite UAS-element (11), might account for the medium upshift effect. In that case the UASrp would be required but not

**Fig. 2. Gene constructs used to analyze the carbon-source upshift effect on transcription.**

The upper panel (A-E) shows constructed fusion genes composed of various parts of the L25-upstream region and the galK marker gene (cloned in vector YCpR6A12H). In A the complete L25-upstream region is present including the conserved sequence elements RPG1 and RPG2, a T-rich region (Py-stretch) and the major transcription initiation site (iT: *.) of the L25-gene (22). ATG originates from the galK-gene (fusion in the leader). The constructs were made using a M13-cloning step; M13-DNA is indicated by //////. Deletions have been performed by Bal31-treatment from the KpnI site, followed by cutting with HincII (adjacent to PstI in the M13-multicloning region) and religation (12). In B a deletion mutant is delineated still containing both RPG-elements. In C a deletion mutant is shown carrying only RPG2. In D a deletion-mutant is shown in which both RPG-boxes have been removed; only the Py-stretch is present. In E also the Py-stretch is deleted. In the M13-derived PstI-site synthetic oligonucleotides containing the RPG-consensus sequence were inserted (D and E).

The lower panel (F-H) shows fusion constructs composed of the cycP-upstream region and the galK marker gene (cloned in vector YCpR1-1). In F the complete cycP-upstream region is present including the Xhol-Xhol fragment carrying the upstream activation sites UAS1 and UAS2 (29). iT indicates the heterogeneous transcription start site of the cycP-gene. In this construct ATG is derived from the cycP-gene (in-frame fusion). In G the Xhol-Xhol fragment is removed and substituted by the Pst-I-NsiI generated fragment carrying the upstream sequences of the L25-gene (cf. A). In H, a similar deletion mutant a synthetic oligonucleotide bearing the RPG-consensus sequence is inserted at a position separated from the Xhol-site by a 254 nucleotides long fragment of lDNA. In this way, in gene constructs G and H the distance of the UASrp to the cycP transcription initiation sites is about the same.
Fig. 3. Effect of carbon-source upshift on rp-gene transcription.
Northern analysis of transcripts from fusion genes B, C and G - cf. Fig. 2 - was performed using gene-specific probes in a similar way as described in the legend to Fig. 1. Again, average values for transcript levels were obtained by densitometric scanning of several autoradiograms.

sufficient for the nutritional upshift. Therefore, we also inserted a synthetic element in a fusion gene carrying a deletion extending to a position downstream of the T-stretch (Fig. 2E) and analysed the respective hybrid mRNA concentration after the shift. Fig. 5 (blot E) shows that also in this construct, though giving rise to a lower transcriptional activity (consistent with previous observations; cf. Ref. 12), the medium upshift effect is manifest.

Finally, insertion of a synthetic box in a heterologous environment was examined. We have previously shown (30) that a synthetic RPG-element, substituting the Xhol-Xhol fragment
Fig. 4. Differential expression of duplicate copy genes for rp28.

To determine the relative amounts of rp28-1 mRNA vs. rp28-2 mRNA a primer extension experiment was performed as described in Materials and Methods. RNA was isolated from yeast cells grown in ethanol-based medium (0) as well as after shifting to glucose (10, 20, 30, 60 and 120 min.), annealed with a rp28 gene-specific primer and used as templates for reverse transcription. Sequence differences in the respective 5'-untranslated leader regions allowed the detection of gene copy specific extension products. At the left a dilution series is included (ranging from 0.12 pmol to 0.30 pmol primer with 6 μg total RNA) demonstrating that the probe was present in sufficient excess. The first lane contains only the primer, the last lane represents a primer extension experiment without dideoxy nucleotides.

containing the UAS_cyc1 is able to activate transcription of a cyc1-galK fusion gene (cf. Fig. 2). However, glucose addition to an ethanol-grown culture of the respective transformants did not evoke the expected increase of the cellular concentration of the fusion transcript (result not shown). As mentioned above, the cyc1-gene is subject to catabolite repression. The glucose-susceptible sites were demonstrated to coincide with the UAScyc1 (29) which has been deleted from the above-mentioned gene construct. However, the cyc1-upstream region immediately downstream of the XhoI site turned out to contain an additional repression site, most likely the CCGCC motif (31), which probably interfered with the regulation of transcription activation by the synthetic RPG-oligomer. Since expression of fusion gene G did display the glucose-stimulatory effect on transcription (see Fig. 3) we decided to enlarge the
distance between the presumed generic repression site and the synthetic RPG-element by insertion of a fragment of spacer DNA (see Fig. 2H). As can be concluded from the result of Northern analysis shown in Fig. 5 (blot H), the UAS

In conclusion, though the context of the UAS_{rpg} elements may contribute to its ultimate effect, the results described in this paper clearly indicate that the rp-gene-specific increase in transcription in response to a carbon-source upshift is mediated through the UAS_{rpg}. Since these cis-acting DNA elements represent binding sites for trans-acting factors (TUF; Refs.
13,14), the molecular events underlying the effects of a nutritional upshift may actually seize upon the amount or the activity of these factors.

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REFERENCES

18. Riezman, H., pers. comm.