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Tripartite Upstream Promoter Element Essential for Expression of *Saccharomyces cerevisiae* Ribosomal Protein Genes

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To initiate a genetic analysis of yeast ribosomal protein gene promoters, we have constructed a gene fusion between the yeast ribosomal protein gene *RP39A* and the *Escherichia coli lacZ* gene. This gene fusion contains approximately 1,030 nucleotides of the 5′ flanking region and the first 49 1/3 codons of *RP39A* fused in frame to a large 3′ end fragment of *lacZ*. Whether it is introduced into yeast cells on a moderately high-copy-number plasmid, or integrated into the yeast genome at the *RP39A* locus, this *RP39A-lacZ* gene directs the synthesis of a hybrid transcript which encodes β-galactosidase activity. Deletions in the 5′ flanking region of *RP39A-lacZ* were constructed by linker insertion and BAL 31 mutagenesis. The expression of the mutant genes in yeast cells was assayed by measuring *RP39A-lacZ* mRNA and β-galactosidase levels. By these means we have shown that the sequences between nucleotides −256 and −170 upstream of *RP39A* are essential for expression of this gene. Three sequence motifs, HOMOL1, RPG, and a T-rich region, which were found in that order 5′ → 3′ upstream of most yeast ribosomal protein genes, were present within this interval. We found that substitution of the *CYC1-lacZ* upstream activation site with the fragment from nucleotides −298 to −172 upsteam of *RP39A*, containing the HOMOL1–RPG–T-rich motif in that 5′ → 3′ orientation, fully restored expression of the *CYC1-lacZ* gene. The essentiality of HOMOL1, the RPG sequence, and the T-rich region for wild-type levels of expression of *RP39A*, the conserved location and order of these sequence motifs in yeast ribosomal protein genes, and the ability of a DNA fragment carrying these three sequence elements to substitute for the upstream activation site regions of *CYC1* indicate that these three oligonucleotides may be essential to the transcription of yeast ribosomal protein genes.

A significant fraction of macromolecular synthesis in yeast cells is devoted to the biosynthesis of ribosomes. The 70 to 75 yeast ribosomal proteins and four ribosomal RNAs are produced in equimolar quantities (17, 20, 81). Accumulating evidence suggests that this balanced synthesis of ribosomal components in exponentially growing yeast cells may be regulated at many levels, including transcription, mRNA processing, mRNA turnover, translation, and ribosomal protein stability (13, 20, 21, 39–42, 63, 66, 69, 80–82; J. Woolford, unpublished data). Experiments thus far indicate that the rates of synthesis, half-lives, and steady-state levels of transcripts homologous to cloned ribosomal protein genes are approximately equal (40, 41; Woolford, unpublished data). To investigate the mechanisms leading to this balanced accumulation of ribosomal protein mRNAs, we have begun a study of ribosomal protein gene promoter elements.

Classical genetic analysis of yeast antibiotic resistance loci which encode ribosomal proteins and molecular genetic studies with more than 20 cloned yeast ribosomal protein genes have shown that ribosomal protein genes are generally scattered throughout the yeast genome (1, 4, 5, 14–17, 43, 84, 85). It is therefore reasonable to hypothesize that coordinate transcription of these scattered genes might be mediated by structurally similar promoters of approximately equal strength, present within or adjacent to each ribosomal protein gene.

In vivo analysis of cloned genes mutagenized in vitro indicates that, in *Saccharomyces cerevisiae*, the transcription of protein-encoding genes is generally dependent upon two regions within the sequences 5′ of the genes: (i) a downstream promoter element containing a TATA consensus sequence, located 20 to 100 nucleotides 5′ to the transcription initiation site; and (ii) one or more upstream promoter or activation sites (UASs) 50 to 500 nucleotides upstream of the gene (23). In addition, regulatory sequences, which are responsible for activating or inactivating transcription in response to certain physiological signals, are found within several hundred nucleotides upstream of many yeast genes (23).

Several families of coordinately transcribed yeast genes, including those involved in amino acid biosynthesis (32, 53), galactose utilization (19, 35, 83), and the expression of specific mating types (55), each contain their own common short oligonucleotide sequences upstream of their respective coregulated genes. Genetic and biochemical studies of the above gene sets suggest that the oligonucleotide sequences flanking them are necessary for the regulation of expression of the genes and that this regulation is mediated by the interaction of the polypeptide products of certain other regulatory loci with these sequences (19, 25, 27, 29, 33, 35, 53, 55, 59, 72–74, 83). Computer analyses of the 5′-flanking sequences of all available cloned yeast ribosomal protein genes have led to the identification of a number of short sequence motifs which are present 5′ to most yeast ribosomal protein genes (48, 79). These consensus sequence elements are of variable lengths and sequence complexities and are generally found at variable distances from the coding regions of the ribosomal protein genes. The most conserved and complex of the consensus sequences identified in these studies are: HOMOL1 (consensus, AACATCC/TG/ATA/GCA) (79) and RPG (consensus, ACCCATACATT/CT/A) (48); each is found upstream of 15 of the 20 sequenced yeast ribosomal protein genes (see Fig. 7).

To address the question of whether these or other sequence motifs are involved in the transcription of yeast ribosomal protein genes, we have initiated a genetic analysis

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of the promoter of the *RP39A* gene. *RP39A* is one of two duplicated genes which encode rp39 of *S. cerevisiae* (84). Both genes are transcribed, as determined by S1 mapping experiments with gene-specific probes and by the isolation and sequencing of cDNAs specific for each gene (Woolford, unpublished data). Since the *RP39A* gene does not have a readily assayable phenotype, we constructed a functional gene fusion between *RP39A* and the *Escherichia coli lacZ* gene. To identify regions upstream of the *RP39A* gene necessary for its expression, we constructed a series of linker insertion and deletion mutations within the 5′ flanking region of the *RP39A-lacZ* gene. These mutant genes were transformed into yeast cells, and their relative levels of expression were assayed by measuring β-galactosidase activity and the steady-state levels of *RP39A-lacZ* homologous mRNA. By this approach, we found that three oligonucleotide sequences are necessary for *RP39A* transcription: the HOMOL1 and RPG sequences previously identified by computer search and a third motif, a T-rich region. All three of these oligonucleotides are present in a defined order 120 to 450 nucleotides upstream of most of the sequenced ribosomal protein genes (see Fig. 7). We found that insertion of *RP39A* DNA containing these three oligonucleotide sequences into the 5′ flanking region of a yeast *CYC1-lacZ* hybrid gene which had been inactivated by removal of its UAS was sufficient to restore expression of this gene in vivo. These results indicate that the *RP39A* tripartite oligonucleotide sequence motif functions as an upstream promoter element for the yeast ribosomal protein genes.

MATERIALS AND METHODS

Strains, Plasmids, and Media. *E. coli* LG90 [F$^-$ Δ(*lac-proXIII*)] (26), obtained from L. Guarente, and HB101 (6) and *S. cerevisiae* JL8 (*cryl leu2-3 2-112 ura3-52 trpl*) (J. Larkin, Ph.D. thesis, Carnegie-Mellon University, 1985), obtained from J. Larkin, were used in these studies. Bacterial cultures were grown in LB (0.5% NaCl, 0.5% yeast extract, 1.0% tryptone) and, when appropriate for plasmid selection, 50 μg of ampicillin per ml. Growth of *S. cerevisiae* was at 30°C in YEPD (1.0% yeast extract, 2.0% peptone, 2.0% glucose) or in defined dropout medium with 2.0% glucose as the carbon source (36).

Enzymes, Chemicals, and Materials. T4 DNA ligase, [α-^{32}P]dCTP, and [γ-^{32}P]ATP were obtained from New England Nuclear Corp. Restriction endonucleases, including *Xho*I, nuclease BAL 31, calf alkaline phosphatase, and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) were purchased from Boehringer-Mannheim Biochemicals. *Xho*I was obtained from New England Biolabs and Bethesda Research Laboratories. *Xho*I linkers were obtained from New England Biolabs. DNase I and *o*-nitrophenyl-β-D-galactopyranoside were purchased from Sigma Chemical Co. DNase-free bovine serum albumin was obtained from Bethesda Research Laboratories. DEAE-nitrocellulose (NA45) was purchased from Schleicher & Schuell, Inc.

β-Galactosidase activity assays. β-galactosidase activity in transformed *E. coli* LG90 was detected by plate assays with the chromogenic substrate X-gal (26). β-galactosidase plate assays of yeast transformants were performed on solid dropout medium buffered with 0.1 M KH_2PO_4 and titrated to pH 6.6 with 5.0 M KOH (modification of the method of Rose et al. [67]). X-gal was added to the medium to a final concentration of 20 to 200 μg/ml. Liquid assays were performed by cell permeabilization (22), except that cultures were grown in defined dropout medium to an optical density at 610 nm of 0.10 to 0.30. All assays were performed in duplicate; 0.5 to 1.5 ml of culture was used for each assay. Three to 10 independent transformants were assayed for each deletion construct. Variation was usually less than 20%.

DNA and RNA isolation. Large quantities of purified plasmid DNAs were prepared as described previously (84). The miniprep procedure of Birnboim and Doly (3) was used to prepare microgram quantities of plasmid DNAs. Yeast genomic DNAs were prepared by a modification of the method of Davis et al. (10) as described by Last et al. (44). Yeast RNA was prepared from transformed strains grown on the appropriate selective dropout medium to a cell density of 1.5×10^7 to 4.0×10^7/ml, by a scaled-down version (Larkin, Ph.D. thesis) of the method of Hereford and Rosbash (31). Whenever possible, samples for DNA and RNA isolation and for β-galactosidase activity assays were withdrawn from the same cultures. DNA restriction fragments were isolated from preparative agarose gels by binding to and subsequent elution from activated strips of DEAE-nitrocellulose as described previously (44).

DNA methods. Restriction endonuclease digestions and ligation reactions were generally performed as described previously (44, 84). However, DNA fragments having cohesive ends generated by digestion with *Xho*I generally ligated at very low efficiencies relative to other types of fragments. We found that DNA fragments prepared by brief (40-min) digestions with *Xho*I at enzyme concentrations of 1 U per μg of DNA and ligated in 10- to 20-μl reaction volumes (as opposed to 100-μl reaction volumes) with 0.4 Weiss units of T4 DNA ligase were joined at significantly increased efficiencies. Cohesive ends were filled in with DNA polymerase I holoenzyme or Klenow fragment and dNTPs as described previously (52). Synthetic restriction-site linkers were ligated to blunt-ended DNAs as described by Maniatis et al. (52) with linkers in 50:1 to 500:1 (linker-to-DNA) molar excess.

Construction of *RP39A-lacZ* fusion vectors. A plasmid containing a functional *RP39A-lacZ* gene, fused in frame within the 50th codon of the *RP39A* gene, was constructed (Fig. 1) by joining a fragment containing approximately 1,030 nucleotides upstream of *RP39A*, the *RP39A* transcription and translation initiation sites, and 49 1/3 codons of *RP39A* (79) to a fragment from the 3′ end of the *E. coli lacZ* gene from plasmid pLG400. pLG400 was digested with *Bam*HI, and the ends were made flush by treatment with DNA polymerase I Klenow fragment. A 1.2-kilobase (kb) *Hae*III fragment of *RP39A* was prepared from plasmid pY10-78 (84) and blunt end ligated into the filled-in *Bam*HI site of plasmid pLG400. The ligation mixture was used to transform *E. coli* LG90 to ampicillin resistance. Transformants were screened for β-galactosidase activity on X-gal plates, and miniprep DNAs were prepared and analyzed from those colonies which were blue by this assay. Complete filling in of a *Bam*HI site and subsequent ligation to a *Hae*III site should result in the regeneration of a *Bam*HI site at the position of the original *Bam*HI site in pLG400. That the orientation of insertion of the *RP39A* fragment was correct was determined by using the unique *Bgl*II site as an internal reference point. One plasmid, pMR1, met both of these criteria. To construct the *E. coli*-yeast shuttle vector pMR10, containing *RP39A-lacZ*, plasmid YEp24 (7) was first linearized by digestion with *Sal*I and then partially digested with *Pst*I. The 4.7-kb *Sal*I-*Pst*I fragment was gel purified and ligated to the gel-purified large *Sal*I-*Pst*I fragment of pMR1. *E. coli* and *S.*

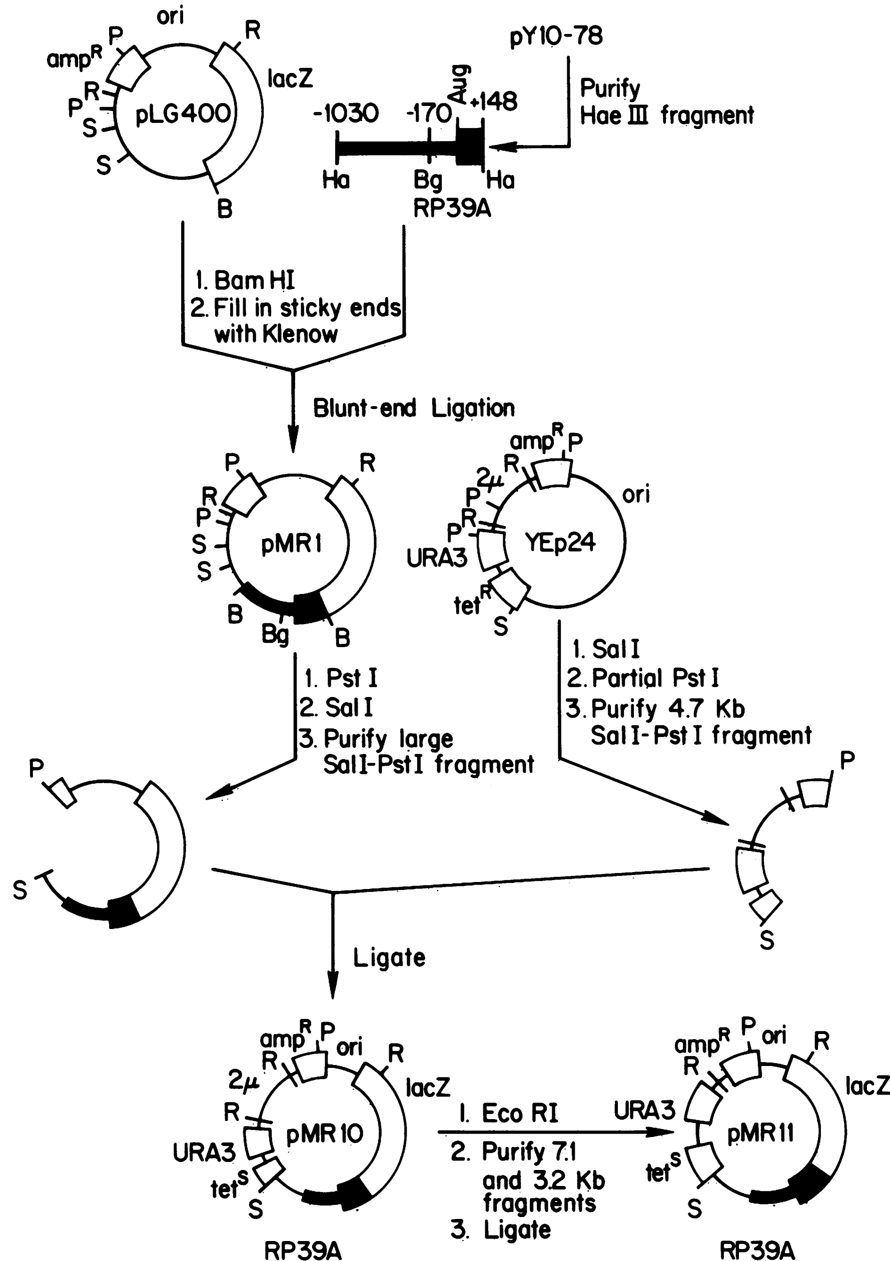

FIG. 1. Construction of an *RP39A-lacZ* hybrid gene on episomal and integrating plasmids. The 1.2-kb *HaeIII* fragment containing 1,030 bp of *RP39A* 5′ flanking region and 148 bp of *RP39A* coding sequences was obtained by partial digestion with *HaeIII* of the 3-kb *BamHI* fragment from plasmid pY10−78, containing the *RP39A* gene (84). Symbols: ▬, *RP39A* 5′ flanking sequences; ■, *RP39A* coding sequences; □, various different genes as indicated. Restriction sites: B, *BamHI*; Bg, *BglII*; Ha, *HaeIII*; P, *PstI*; R, *EcoRI*; S, *SalI*.

cerevisiae cells transformed with pMR10 were blue on X-gal plates.

An integrating form of pMR10, called pMR11, was constructed from pMR10 by deleting the *EcoRI* fragment containing the yeast 2 μm origin of DNA replication (75). pMR10 was digested with *EcoRI*, and the 7.1- and 3.2-kb *EcoRI* fragments were gel purified and ligated together. Miniprep DNAs were prepared and analyzed from those bacterial transformants which were blue on X-gal plates.

Random linker insertion mutagenesis. To maximize the frequency of random linker insertion into *RP39A*, the pMR10 *RP39A BamHI* fragment was first recloned into the *BamHI* site of pBR322 (76) to produce pBR1078B. In this smaller construct, the *RP39A BamHI* fragment was a more frequent target for linearization by DNase I. *XhoI* linker insertions into pBR1078B were generated by the method of Heffron et al. (30) as modified by Tatchell et al. (77). The efficiency of linker insertion was 80 to 90%. Approximately 25% of the linker insertions identified were found to be located in the *RP39A BamHI* fragment. Each of these linker insertions was mapped relative to the *RP39A* initiation codon by additional restriction analysis.

To assay the effects of selected linker insertions upon the expression of *RP39A-lacZ* in *S. cerevisiae* cells and to

generate substrates for deletion mutagenesis, the mutagenized *RP39A BamHI* fragments of interest were fused in frame to *lacZ* by cloning the selected mutant *RP39A BamHI* fragments into the *BamHI* site of pMR10Δ39A, a plasmid derived from pMR10. pMR10Δ39A lacks the 1.2-kb *RP39A BamHI* fragment and was constructed from pMR10 in two steps. pMR10 was linearized by partial digestion with *BamHI*, the 5′ ends were filled in with DNA polymerase I Klenow fragment, and the molecules were recircularized by ligation. After transformation, miniprep DNAs were screened for the loss of only the *BamHI* site in the Tc^s gene fragment. This plasmid was called pMR10B. To completely delete the *RP39A* fragment, pMR10B was digested to completion with *BamHI*, and the large *BamHI* fragment was recircularized to produce pMR10Δ39A. The structure of each new fusion plasmid was verified by restriction analysis. Each linker insertion-containing fusion plasmid is designated pMR10-X, where X represents the position of the linker insertion in the *RP39A* 5′ flanking region with respect to the initiation codon.

BAL 31 deletion mutations from the *BglII* site at −172. Plasmid pMR10 was linearized by digestion with *BglII* and then treated with either 0.05 or 0.2 U of nuclease BAL 31 per 25 μg of plasmid DNA as described by Maniatis et al (52). Reactions were terminated at 1, 5, 10, and 20 min for 0.05-U digestions and at 1, 2.5, 5, 7.5, and 10 min for 0.2-U digestions by adding chilled EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*,-tetraacetic acid] to a final concentration of 20 mM. Approximate deletion sizes for each reaction time were determined by digesting a sample of each DNA with *BamHI* and sizing the products on a 1.0% agarose gel. The ends of the BAL 31-treated DNAs were then rendered flush by treatment with DNA polymerase I Klenow fragment. *XhoI* linkers were ligated to the blunt-ended plasmid DNAs (52), and then these plasmid DNAs were recircularized and used to transform *E. coli* to ampicillin resistance. Miniprep plasmid DNAs were digested with *BamHI* and *XhoI*, and the digestion products were sized on 2.0% agarose gels to categorize deletions. In this study, all pMR10 derivatives possessing deletions within the *RP39A* gene 5′ flanking region are designated Δ−X/−Y, where −X and −Y are the deletion endpoints.

Linker insertion deletion mutations. Additional deletion mutations of three sorts were constructed in the 5′ flanking region of *RP39A-lacZ*: (i) between sites of two different linker insertions, (ii) between the *BglII* site at −172 and an endpoint of a BAL 31 deletion from −172, and (iii) between one linker insertion site and an endpoint of a BAL 31 deletion from −172 (see above for details). Each set of deletions was made by ligating the small *XhoI-SalI* or *XhoI-XbaI* fragments of those plasmids having *XhoI* linkers inserted at a variety of positions upstream of *RP39A* to the large *XhoI-SalI* or *XhoI-XbaI* vector fragments, respectively, of those plasmids having *XhoI* linkers inserted at positions nearer to the *RP39A* initiation codon (Fig. 2). To facilitate the construction of those deletions having an endpoint at the *BglII* site at position −172, we first constructed plasmid pMR10−170. In this construct, the unique *BglII* site was replaced by an *XhoI* linker. DNA sequence analysis of this mutation revealed that the filling in of the *BglII* site at position −172 had been incomplete and that the *XhoI* linker had ligated to the plasmid DNA at position −170. Therefore, each of the deletions directed in the 5′ or 3′ directions from the *BglII* site had an endpoint at −170.

BAL 31 deletion mutations from −299. Plasmid pMR10−305 (*XhoI* linker inserted at −305) was linearized by digestion with *XhoI* and then digested with BAL 31, and the products were analyzed and processed as described above. Miniprep DNAs from these transformants were analyzed by restriction analysis, and the effects of some size-selected bidirectional deletions upon gene expression in *S. cerevisiae* were measured. At this time, we determined by sequence analysis that plasmid pMR10−305 carried a duplication of the region between −266 and −305 such that this region was repeated immediately upstream of the linker at −305. Duplications like that found in plasmid pMR10−305 are not uncommon in cases of linker insertion mutagenesis and presumably occur during the treatment of DNase I-linearized DNAs with DNA polymerase I (Tatchell et al.

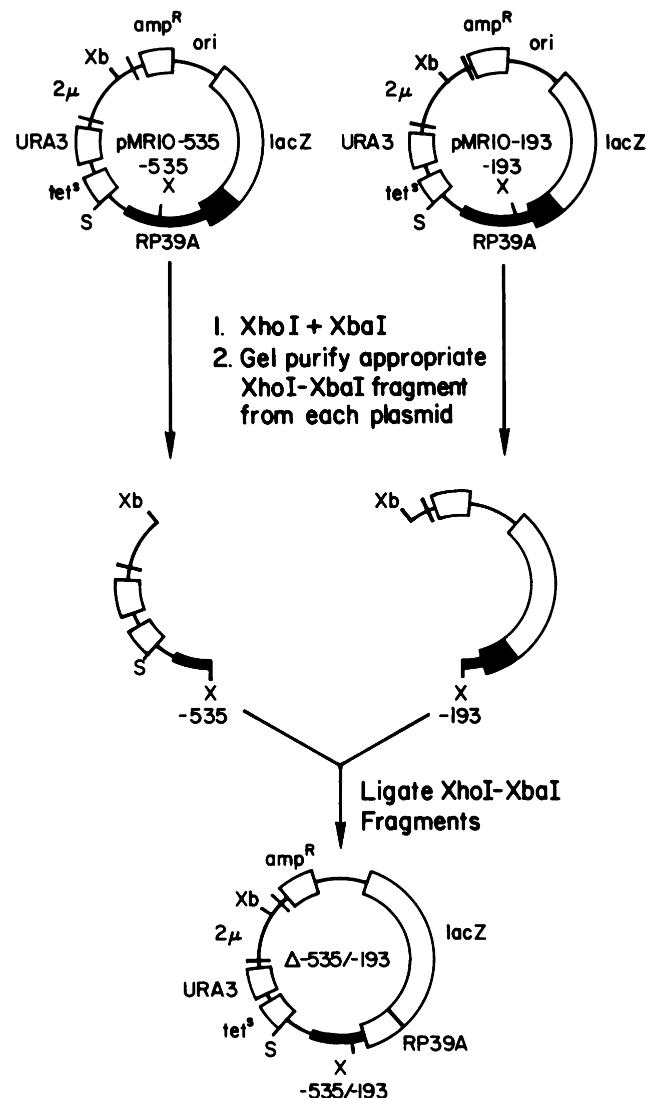

FIG. 2. Construction of deletion mutations in the 5′ flanking sequences of *RP39A-lacZ*. Deletion mutations were constructed by ligating pairs of fragments from different pMR10 derivatives containing *XhoI* sites created by linker insertion or BAL 31 exonuclease mutagenesis. The example shown above creates a deletion between linker insertions at nucleotides −535 and −193. For further details, see Materials and Methods. Restriction sites: X, *XhoI*; Xb, *XbaI*. Other restriction sites and symbols are as indicated in the legend to Fig. 1.

[77]). Therefore, we chose the linker insertion *Xho*I site of plasmid pMR10−299 to serve as a common 5′ end for these deletions. (The 5′-directed *Xho*I-*Xba*I fragment of Δ−240/−193 was used to obtain the *Xho*I 5′ end of Δ−240/−232.) Deletions having a common end at −299 (and Δ−240/−232) were constructed by using the same strategy (Fig. 2) as that described in the previous section on linker insertion deletion mutations. The variable ends of the deletions extending in the 3′ direction from −299 were selected from those BAL 31 bidirectional deletions, generated from the *Xho*I site in pMR10−305, which were already known to have interesting phenotypes. To determine whether multiple *Xho*I linkers were present at any one position in any of the constructs described here or in the three previous sections, plasmid DNAs were digested with *Stu*I (West et al. [83]). Only two plasmids, Δ−240/−144 and Δ−240/−193, were found to have two or more *Xho*I linkers by this assay.

Construction of *RP39A-CYC1* hybrid promoter fusions. DNA fragments upstream of *RP39A* extending from nucleotide −1030 to −170, −240, or −299 were joined to the *Xho*I site at nucleotide −247 upstream of the initiation codon of the *CYC1-lacZ* gene of plasmid pLG669Z (12, 28). Plasmids pMR10−170, Δ−240/−193, and pMR10−299 were digested with *Xba*I and *Xho*I, and the small *Xho*I-*Xba*I fragments were ligated in each case to the large *Xho*I-*Xba*I fragment of pLG669Z (Fig. 3A).

To specifically replace the UAS region of *CYC1* with a smaller fragment containing the putative *RP39A* upstream promoter element (Fig. 3B), plasmid pLG669Z was digested with *Xho*I, and the *Xho*I ends of the large fragment were converted to *Bgl*II sticky ends by linker ligation. A *Bgl*II linker was ligated to the filled-in *Xho*I linker insertion site 299 base pairs (bp) upstream of *RP39A-lacZ* in plasmid pMR10−299, by similar means. Digestion of this DNA with *Bgl*II yielded a 135-bp *Bgl*II fragment (including *Xho*I and *Bgl*II linkers) extending from nucleotides −298 to −172 upstream of *RP39A*. This fragment was ligated to the *Bgl*II-linkered large fragment derived from pLG669Z to produce plasmids pMR672A and pMR672B. As determined by restriction mapping, the *RP39A* fragment in pMR672A had the same orientation with respect to *lacZ* as in pMR10−299; in pMR672B, the orientation of the *RP39A* fragment was reversed. As controls, we constructed two plasmids lacking the *CYC1-lacZ* UAS region. Plasmids pMR670 and pMR671 were derived from pLG669Z by removing the *Xho*I fragment from −247 to −700 (pMR670) and by ligating *Bgl*II linkers to the resulting *Xho*I site (pMR671).

Bacterial and yeast transformation. Ligated plasmid DNAs were transformed into *E. coli* LG90 or HB101 or both by the method of Davis et al. (9). *S. cerevisiae* JL8 was transformed by the lithium acetate method of Ito et al. (34) with 2 to 5 μg of episomal plasmid DNAs. Integation of plasmid pMR11 was directed to the *RP39A* locus by the method of Orr-Weaver et al. (62); i.e., 5 to 10 μg of plasmid pMR11 was first linearized at the unique *Bgl*II site before transformation. The stability of integrative tranformants was determined by the method of Last et al. (44).

Nucleic acid electrophoresis, transfer, and hybridization. DNAs and RNAs were sized on 1.0 and 1.2% agarose gels, respectively, transferred to nitrocellulose, and hybridized to in vitro-labeled DNA probes essentially as described previously (44).

Determination of plasmid copy number. Plasmid copy numbers in yeast strains transformed with pMR11 or pMR10 deletion derivatives were determined by Southern blot analysis of *Eco*RI-digested genomic DNAs (7, 44, 62, 71).

DNA sequencing. DNA sequence analysis of selected promoter deletions was performed by the method of Maxam and Gilbert (54). Plasmid DNAs were end labeled at the unique *Bgl*II or *Xho*I sites with [γ-^{32}P]ATP and T4 polynucleotide kinase. Sequence ladders were resolved on 20, 8, or 6% polyacrylamide–50% (wt/vol) urea gels. For the 20% gels, the urea concentration was reduced to 7 M.

RESULTS

Construction of a hybrid *RP39A-lacZ* gene. To facilitate assaying the expression of the yeast *RP39A* gene, we constructed plasmid pMR10, in which approximately 1,030 nucleotides of DNA upstream of *RP39A* and the first 49 1/3 codons of *RP39A* were ligated in frame to the *E. coli lacZ* gene (Fig. 1). The 5′ end of the *RP39A* gene fragment of this construct extended through one end of another unidentified yeast gene (M. Rotenberg, unpublished data). Since the DNA sequences in the interval between the upstream gene and *RP39A* most likely contain the entire promoter region for *RP39A*, we expected this *RP39A-lacZ* fusion construct to be expressed in yeast cells. Consistent with these expectations, yeast cells transformed with pMR10 produced blue colonies on X-gal plates, and extracts of these transformed cells contained both β-galactosidase activity (Table 1) and hybrid mRNA molecules homologous to both *RP39A* 5′ and *lacZ* 3′ sequences (see Fig. 6 and data not shown).

To integrate the hybrid *RP39A-lacZ* gene into the yeast genome, we first constructed plasmid pMR11 (Fig. 1). This construct was identical to pMR10, except that the yeast 2 μm origin of DNA replication had been deleted (75). Integration was directed to the *RP39A* chromosomal locus by the method of Orr-Weaver et al. (62). Plasmid pMR11 was linearized within the *RP39A* sequences by digestion with *Bgl*II and then used to transform *S. cerevisiae* JL8, selecting for complementation of the *ura3-52* allele. As determined by Southern blot analysis (Fig. 4) (7, 44, 62, 71), multiple tandem integrations of *RP39A-lacZ* at the *RP39A* locus were common. The steady-state levels of fusion-specific mRNA encoded by *RP39A-lacZ* were directly proportional to the dosage of the hybrid gene (data not shown). Similarly, the level of β-galactosidase activity measured in transformed yeast cells was directly proportional to the dosage of *RP39A-lacZ* (Table 1). This suggests that the *RP39A* promoter is equally active whether it is plasmid borne or integrated as one or more copies into the yeast genome at the *RP39A* locus.

Linker insertion mutations. The *RP39A-lacZ* gene fusion was used as a substrate for the construction and characterization of mutations in the *RP39A* 5′ flanking region. Our purpose was to identify and define those sequences upstream of the TATA box (23) region which are essential for *RP39A* transcription. As a preliminary step in the construction of deletion mutations, we first generated a set of random *Xho*I linker insertion mutations throughout the DNA upstream of *RP39A-lacZ* in pMR10. Restriction digest analysis of approximately 200 miniprep DNAs resulted in the identification of 48 plasmids in which *Xho*I linkers had been inserted into the 1.2-kb fragment containing sequences from nucleotide −1030 upstream of *RP39A* to nucleotide +148. The distribution of *Xho*I linkers throughout this fragment was essentially random (data not shown). Mutant alleles of the hybrid gene were reconstructed in pMR10 by using these mutagenized *RP39A* fragments (see Materials and Methods), and these mutant *RP39A-lacZ* genes were then used to transform *S. cerevisiae* JL8. We determined the effects of 12 of these linker insertion mutations, which mapped between

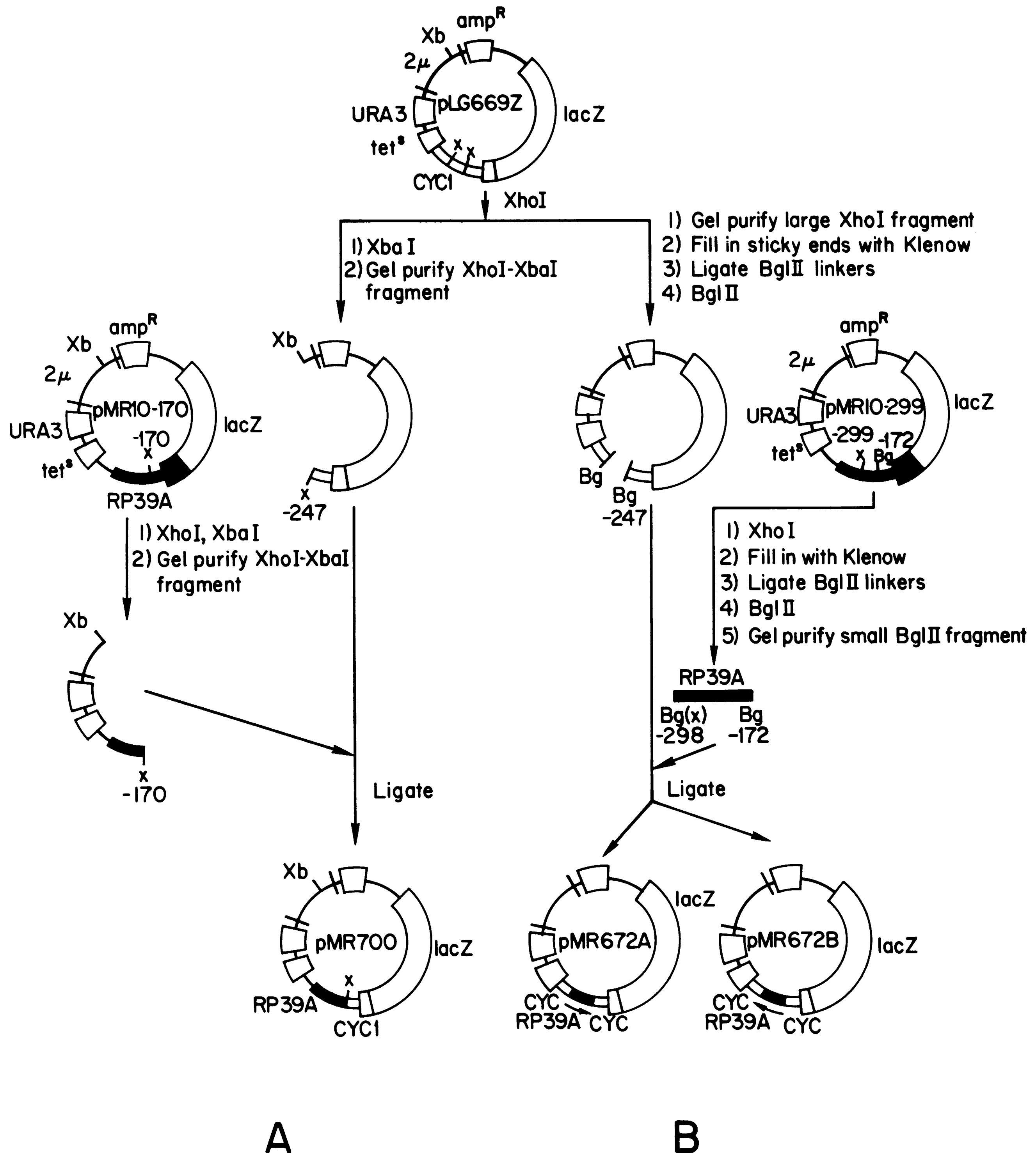

FIG. 3. Construction of *RP39A-CYC1* hybrid promoter fusions. (A) *CYC1* DNA upstream of the *XhoI* site at nucleotide −247 was substituted with *RP39A* DNA from nucleotides −1030 to −299, −240, or −170. The pLG669Z *XhoI-XbaI* fragment carrying the *CYC1-lacZ* gene was ligated to the *XhoI-XbaI* fragment of plasmids pMR10−170, Δ−240/−193, and pMR10−299. Shown above is the construction with plasmid pMR10−170. (B) The *CYC1* UAS region was specifically replaced with a fragment containing the putative *RP39A* upstream promoter element (−172 to −298). Plasmids carrying the *RP39A* fragment in either orientation with respect to *CYC1-lacZ* were obtained (pMR672A or pMR672B). The relative positions of the yeast ribosomal protein gene consensus sequences HOMOL1, RPG, and T-rich region in all of the above constructs are shown in Fig. 8. All symbols and restriction sites are as described in the legends to Fig. 1 and 2. Details of these constructions are described in the text.

nucleotides −535 and +100, upon expression of β-galactosidase from *RP39A-lacZ* in yeast cells. With one exception, only those linker insertions which mapped to the transcribed region of *RP39A-lacZ* had major effects on expression of the hybrid gene (data not shown). The exception, pMR10−234, reduced β-galactosidase levels to approximately 17% of wild type (Fig. 5). DNA sequence analysis of this mutation revealed that it was a deletion of the region between −234 and −199 nucleotides upstream of the *RP39A* translational initiation codon. This 34-nucleotide region included both the RPG consensus sequence and a large portion of the T-rich region (Fig. 5a and b; see below). No other linker insertions mapped to the HOMOL1 or RPG consensus sequences. Two linker insertions within the T-rich region, at positions −193 and −170, each only very minimally affected gene expression (data not shown). The effects of linker insertions which mapped to positions upstream of −535 were not determined, as it is very unlikely that such insertions would affect gene expression (see below).

Defining the 5′ boundary of the *RP39A* upstream promoter element. To determine the minimum 5′ flanking sequences necessary for expression of *RP39A*, we constructed a series of deletions in pMR10 extending toward the *RP39A-lacZ* gene from an *Xho*I linker insertion at nucleotide −535 (see Materials and Methods and the legend to Fig. 2 for details). Each plasmid construct contained the *RP39A* 5′ flanking DNA between positions −1030 and −535. Deletions extending from −535 to either −435 or −305 (Δ−535/−435 or Δ−535/−305) had only a minimal effect on expression of β-galactosidase or mRNA from *RP39A-lacZ*, but deletions Δ−535/−199, Δ−535/−193, and Δ−535/−170 completely abolished expression of the gene fusion (Fig. 5b and 6). Southern blots of *Eco*RI-digested yeast genomic DNAs probed with the *RP39A Bam*HI fragment of plasmid pBR1078B indicate that the decrease in expression due to these deletions, or the deletions described below, was not due to dramatic changes in plasmid copy number (data not shown). Therefore, these deletion mutations demonstrate than an upstream promoter for *RP39A* lies between nucleotides −305 and −199. This interval contains both the HOMOL1 and the RPG consensus sequences (Fig. 5 and 7).

HOMOL1 and RPG consensus sequences are necessary for *RP39A-lacZ* expression. To further define the 5′ boundary of the *RP39A* upstream promoter element, as well as to further address the roles of the HOMOL1 and the RPG sequences in promoting *RP39A-lacZ* expression, we constructed another series of deletions extending toward the gene from a linker insertion site at nucleotide −299. Our strategy for these constructions is shown in the example in Fig. 2 and is described in Materials and Methods. Deletions extending toward the gene from −299 had minimal effects upon steady-state levels of *RP39A-lacZ* homologous mRNA and β-galactosidase expression unless the deletions extended beyond nucleotide −257, i.e., into or beyond the HOMOL1 sequence (Fig. 5b and 6). Deletion Δ−299/−246, which enters the HOMOL1 sequence, caused a precipitous decrease in *RP39A-lacZ* expression to 10 to 15% of wild-type levels. This indicates that the HOMOL1 sequence motif is particularly crucial to upstream promoter element function. Deletions Δ−299/−243 and Δ−299/−238, both of which extend beyond the HOMOL1 sequence, resulted in a further decrease in β-galactosidase levels to 6 to 8% of wild-type levels. Mutation Δ−299/−232, which deletes both HOMOL1 and the 5′ one-third of the RPG sequence motif, completely eliminated gene expression. This suggests that the RPG sequence motif is also important to upstream promoter element function.

To determine with greater precision which *RP39A* 5′ flanking sequences downstream of nucleotide −299 are necessary for *RP39A-lacZ* expression, we constructed a series of deletions between pairs of linker insertions within the interval −299 to −193 (Fig. 2). In addition, deletions were constructed in pMR10 that extended in one or both directions from the *Bgl*II site at position −172 with nuclease BAL 31. No *RP39A-lacZ* mRNA or β-galactosidase activity was detectable in cells transformed with deletions Δ−266/−199, Δ−266/−193, or Δ−240/−144. Deletion Δ−234/−170 reduced expression of the *RP39A-lacZ* gene to less than 1% of the wild-type level (Fig. 5b and 6). However,

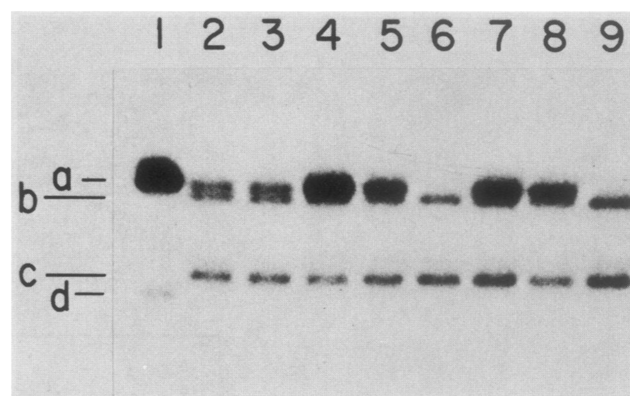

FIG. 4. Multiple tandem integrations of *RP39A-lacZ* at the *RP39A* locus. DNA was extracted from *S. cerevisiae* JL8 transformed with pMR10 or *Bgl*II-linearized pMR11 DNAs, digested with *Eco*RI, and subjected to Southern blot analysis with a ^{32}P-labeled probe containing the *RP39A* gene. In lane 1, band a results from plasmid pMR10. In lanes 2 through 9, band a results from tandem integration of pMR11. The intensity of band a is proportional to the number of tandem integrations. In lanes 2 through 9, band b results from single integrations of *Bgl*II-linearized pMR11. Band c is the chromosomal copy of *RP39A* in strain JL8 transformed with *Bgl*II-cut pMR11, and band d is the wild-type copy of *RP39A* in strain JL8 transformed with pMR10. DNA samples loaded in lanes 1 through 9 were isolated from transformants 1 through 9, respectively, described in Table 1.

TABLE 1. Expression of β-galactosidase from episomal and integrated *RP39A-lacZ* hybrid genes

Plasmid	Transformant	Copies of *RP39A-lacZ* per cell[a]	Relative β-galactosidase activity
pMR10[b]	1	30–35	100.0
pMR11[c]	2	2	8.7
	3	2	8.6
	4	6–7	19.2
	5	3	7.3
	6	1	2.6
	7	4	12.9
	8	3	12.6
	9	1	3.5

[a] The copy number and site of integration were determined by Southern blot analysis as shown in Figure 4.

[b] pMR10 contains a yeast 2μm origin of DNA replication and is episomal.

[c] pMR11 contains no yeast 2μm sequences and is an integrating plasmid. The plasmid was linearized by digestion with *Bgl*II such that integration was directed to the chromosomal *RP39A* locus (see the text).

(a)

-340 -330 -320 -310 -300 -290 -280 -270 -260 -250 -240 -230
TAAAATGCAGCAACATACATATGTTGAGTTGTATAGACATCTATATATAACAAGCACAGAACCGTCTAATTGGTATTTTTCAGGACATTTTAAACATCCGTACAACGAGAACCCATACATTACTTT
HOMOL1 RPG

-220 -210 -200 -190 -180 -170 -160 -150 -140 -130 -120 -110 -100
TTTTAATATTCTTTTTGTTTTCATCGCCTTCTTTTTATTTTTATCCGAAGATCTTTTGGAACCCGCTCTGCGAATAGCGAAGCAGGATACCAAAGTGAAACTTGGACATAACTCATCATTAAAGAAGTAT
T-RICH REGION

-90 -80 -70 -60 -50 -40 -30 -20 -10 10
ACTGTTAAGAGAGGCATTCATTTCGTGTATTATAACGTTTAGCATCAGTTACCCTTGAAAGCCCAACATATACAAAAATACGCGTCCAAG ATG TCT ACT AAA GCC CAA AAC CCT ATG CGT
MET Ser Thr Lys Ala Gln Asn Pro Met Arg

(b)

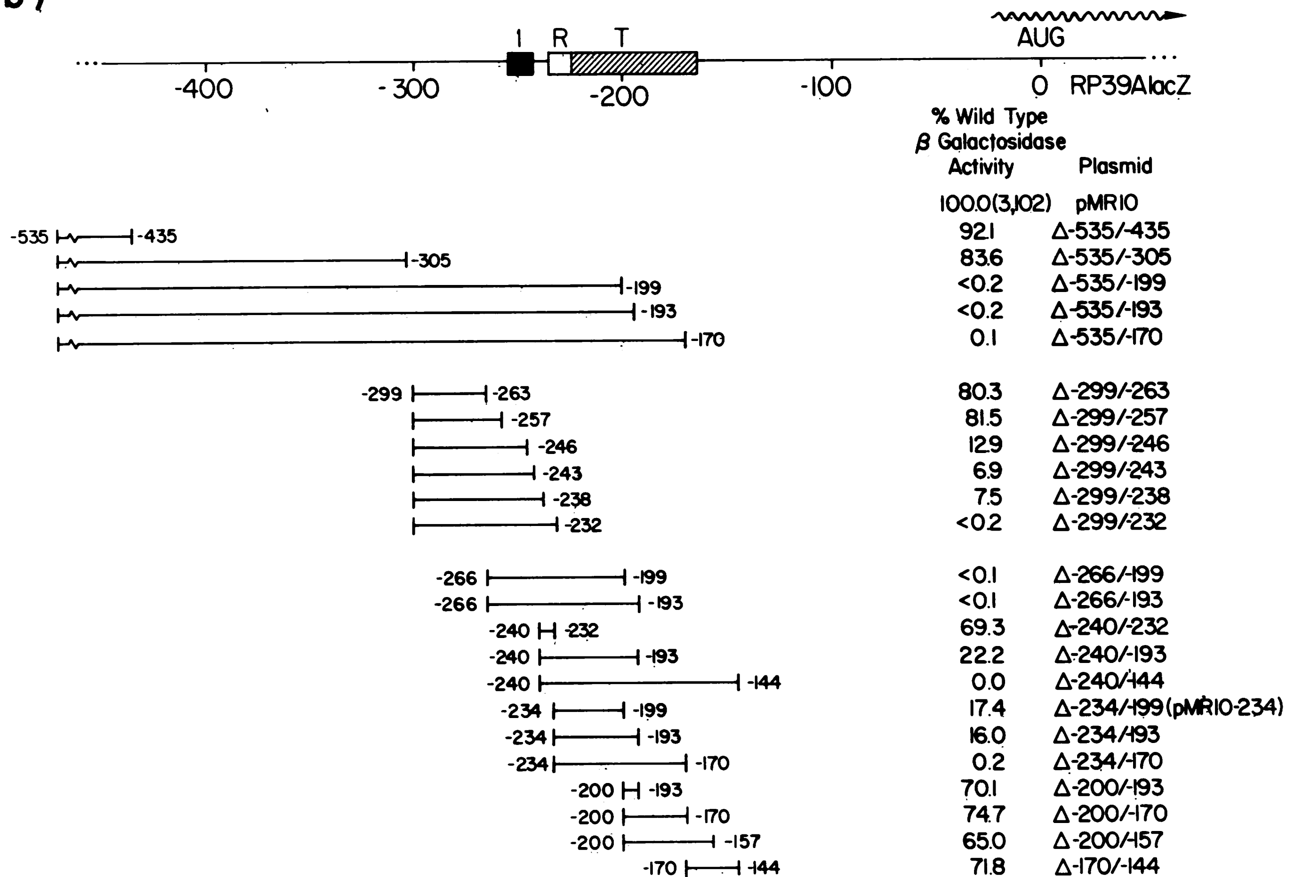

FIG. 5. Expression of *RP39A-lacZ* gene fusions containing deletions in the 5′ flanking region. (a) The nucleotide sequence of the 5′ flanking region of wild-type *RP39A* is shown. The three consensus sequences HOMOL1, RPG, and the T-rich region are underlined and labeled. A putative TATA box is underlined. The positions of 5′ ends of wild-type *RP39A* major and minor transcripts, as determined by S1 nuclease mapping (Woolford, unpublished data), are designated by dark and light arrows, respectively. The first 10 codons of the *RP39A* gene are shown. (b) Structure and expression of deletion mutations upstream of *RP39A-lacZ*. Deletions were constructed as described in Materials and Methods and are indicated by solid lines. The positions of endpoints of each deletion were determined by DNA sequencing and are indicated with respect to the position of the *PR39A-lacZ* AUG initiation codon. The three consensus sequences HOMOL1 (■), RPG (□), and the T-rich region (▨) are indicated. The *RP39A-lacZ* transcript is designated by a wavy line. The relative β-galactosidase activities of the deletion mutants are indicated as percentages of that of the wild-type *RP39A-lacZ* fusion (pMR10). The absolute level of expression of the wild-type *RP39A-lacZ* fusion (pMR10) is shown in parentheses (β-galactosidase activities were determined as described previously [22]).

the mutations Δ−240/−193, Δ−234/−199 (linker insertion mutation pMR10−234), and Δ−234/−193 reduced expression of *RP39A-lacZ* to between only 22 and 16% of the wild-type levels. This difference between the effects of mutations Δ−240/−193 and Δ−266/−193, for example, indicates that there is likely an important promoter element situated between positions −266 and −240, which includes the HOMOL1 sequence. This is corroborated by the observation that Δ−299/−246 was expressed at levels nearly 10-fold lower (10 to 15% of the wild-type level) than Δ−299/−257 (see above). However, the absence of expression from Δ−240/−144 and the very low levels of β-galactosidase expressed from Δ−234/−170 indicate that sequences necessary for *RP39A* expression also lie in the

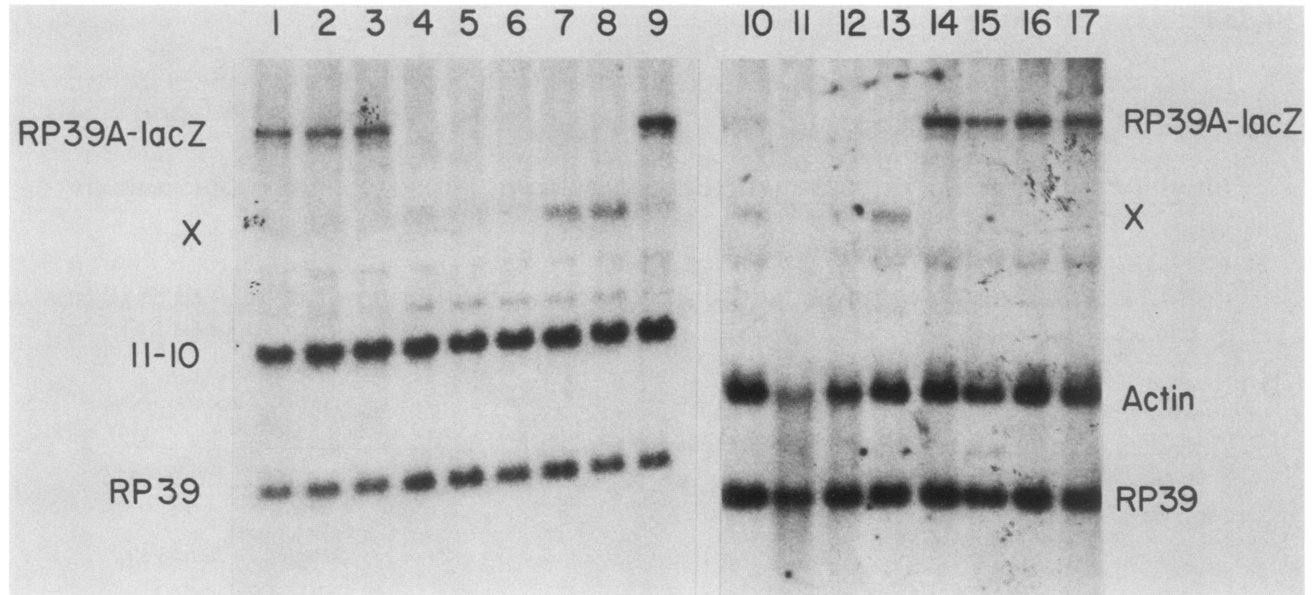

FIG. 6. Levels of transcripts from wild-type and promoter deletion derivatives of the *RP39A-lacZ* gene. Total RNA was extracted from *S. cerevisiae* JL8 transformed with pMR10 and its deletion derivatives (Fig. 5b). Samples (10 μg) of each of these RNAs were separated by denaturing gel electrophoresis, blotted to nitrocellulose, and hybridized to either ^{32}P-labeled *RP39A* DNA and plasmid 11-10 (lanes 1 through 9) or ^{32}P-labeled *RP39A* and *ACT1* DNAs (lanes 10 through 17). Plasmid pY11-10 (84) carries an insert homologous to an abundant yeast transcript. The *ACT1* DNA is a 1.65-kb *Bam*HI-*Hin*dIII fragment of plasmid pYactl (60) which is homologous to the yeast actin mRNA. Lanes: 1, pMR10; 2, Δ−535/−435; 3, Δ−535/−305; 4, Δ−535/−199; 5, Δ−535/−193; 6, Δ−535/−170; 7, Δ−266/−199; 8, Δ−266/−193; 9, pMR10; 10, Δ−240/−193; 11, Δ−234/−199; 12, Δ−234/−193; 13, Δ−234/−170; 14, Δ−200/−193; 15, Δ−200/−170; 16, Δ−200/−157; 17, Δ−170/−144. Band X is a transcript which is homologous to both *RP39A* and *lacZ* sequences and appears only when the wild-type *RP39A* upstream promoter element is inactivated.

interval between −240 and −144, which includes the RPG sequence.

A T-rich sequence 3′ to the HOMOL1 and RPG consensus sequences is important for expression of *RP39A-lacZ*. Comparison of β-galactosidase levels expressed from Δ−240/−193 (22%), Δ−234/−199 (17%), and Δ−200/−170 (75%) with those expressed from Δ−240/−144 (0%) and Δ−234/−170 (<1%) indicates that simultaneous deletion of the intervals −234 to −199 and −199 to −170 had a greater effect upon *RP39A-lacZ* gene expression than did deletion of either interval alone (Fig. 5b and 6). Furthermore, deletion of the intervals −200 to −193, −200 to −157, or −170 to −144 alone also had only a minor effect on *RP39A-lacZ* expression (Fig. 5b and 6). These results suggest that at least a portion of the interval from −240 to −170 is likely to consist of some type of repeated element, such that a significant percentage of the repeats may be deleted without eliminating gene expression or that the interval contains structurally dissimilar elements, each of which is capable of partially promoting gene expression, or both. Inspection of the sequences within the interval from −240 to −170 (Fig. 5a) revealed that, in addition to the single RPG consensus at −236 to −225, there was an adjacent, very T-rich region. Thirty-three of the 55 nucleotides between the 3′ end of the RPG element (−225) and nucleotide −170 are dTMPs. Deletion of either a portion of the RPG sequence (Δ−240/−232) or the 3′ half of this T-rich region (Δ−200 series) had a small but reproducible effect on *RP39A-lacZ* expression, but deletion of most of the RPG sequence and the 5′ half (or all) of the T-rich region (e.g., Δ−240/−193 or Δ−234/−170) greatly reduced gene expression. Thus, it is very likely that these sequences function together to promote expression of *RP39A*. Analysis of the 5′ flanking sequences of 19 other cloned yeast ribosomal protein genes revealed the presence of T-rich regions which were similarly located upstream of all but two of these genes (Fig. 7).

Substitution of *CYC1* UAS function by the *RP39A* upstream promoter element. We wished to determine whether the *RP39A* upstream promoter element defined by the deletions described above was sufficient for promoting high levels of expression of another yeast gene which was lacking its upstream activation sequences. We were also interested in determining whether HOMOL1, RPG, and the T-rich region all had to be present in such a construct to obtain promoter function. We constructed promoter fusions in which all of the *RP39A* sequences between ca. −1030 and −299, −240, or −170 were substituted for the *CYC1* UAS region (24, 25) in a *CYC1-lacZ* gene fusion (28) (Fig. 3 and 8). All of the sequences normally positioned downstream of the *CYC1* UAS region were still present in each of these constructs. None of the sequences within the putative *RP39A* upstream promoter element were present in the first construct; only the HOMOL1 motif was present in the second construct; and HOMOL1, RPG, and the T-rich region were all present in the third construct. The effects of these promoter fusions on *CYC1-lacZ* expression in cells grown in glucose medium are shown in Fig. 8. The fusion containing nucleotides −1030 to −299 (pMR702) was not expressed, and that containing nucleotides −1030 to −240 (pMR701) produced only a small amount of β-galactosidase activity (1.8% of that of wild-type *RP39A-lacZ*; 8.1% of that of wild-type *CYC1-lacZ*). The promoter fusion containing the fragment from nucleotide −1030 to −170 (pMR700), in which all three *RP39A* upstream promoter sequence elements were present, directed

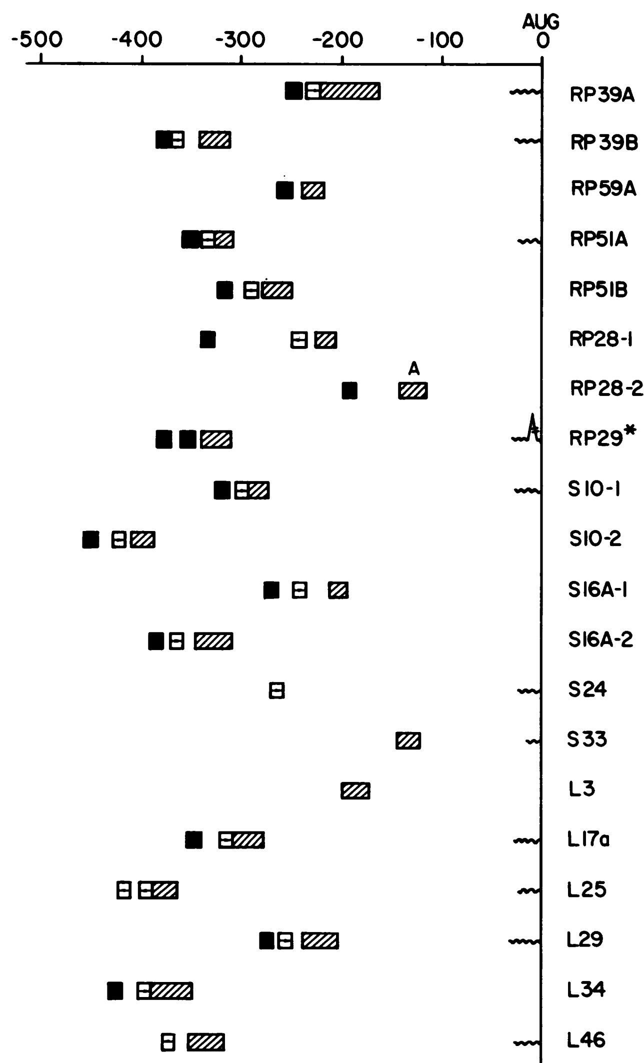

FIG. 7. Location of HOMOL1, RPG, and T-rich consensus sequences upstream of yeast ribosomal protein genes. The positions of the three yeast ribosomal protein gene consensus sequences HOMOL1 (AACATCC/TG/ATA/GCA), RPG (ACCCATACAT-T/CT/A), and the T-rich region are depicted with respect to the translational initiation sites of each ribosomal protein gene. Symbols: ■, HOMOL 1; ⊟, RPG; ▨, T-rich region; *, indication of a 458-nucleotide intron in the 5' leader of the *RP29* mRNA. In four cases the RPG sequence occurred in the opposite orientation with respect to HOMOL1 and the T-rich region (⊟). Sequence data were compiled only from published data (1, 37, 45–50, 56, 57, 68, 70, 79, 80). Approximate lengths of mRNA 5' leader regions, where known, are indicated by wavy lines.

the synthesis of a very high level of β-galactosidase activity which was nearly equivalent to that of the wild-type *RP39A-lacZ* construct.

We also replaced the *CYC1-lacZ* UAS with a fragment containing only the *RP39A* flanking DNA between nucleotides −298 and −172. Plasmid pMR672A, containing this fragment inserted upstream of *CYC1-lacZ* in the same 5'→3' orientation as it is found upstream of *RP39A*, directed the synthesis of high levels of β-galactosidase from the *CYC1-lacZ* gene. When this *RP39A* fragment was inserted upstream of *CYC1-lacZ* in the opposite orientation (pMR672B), the hybrid *CYC-lacZ* gene was not expressed in yeast cells. These results indicate that in one orientation, the *RP39A* upstream promoter element was sufficient to replace the *CYC1* UAS, i.e., to act in concert with the *CYC1* downstream transcription initiation signals in promoting *CYC1-lacZ* expression.

DISCUSSION

To identify the promoter elements of the ribosomal protein gene *RP39A*, we constructed an *RP39A-lacZ* fusion which included ca. 1,030 nucleotides of the 5'-flanking sequences and the first 49 1/3 codons of *RP39A*, fused in frame to a large 3' end fragment of the *E. coli lacZ* gene. Whether introduced into yeast cells on a moderately high-copy-number plasmid or integrated as one or multiple tandem copies into the yeast genome at the *RP39A* locus, this *RP39A-lacZ* gene fusion directed the synthesis of a hybrid mRNA containing *RP39A* 5' sequences and *lacZ* 3' sequences (Fig. 6) and also production of β-galactosidase activity, presumably arising from rp39A–β-galactosidase hybrid protein (Table 1).

We found that the levels of gene fusion-specific mRNA and β-galactosidase activity synthesized in yeast cells were directly proportional to the dosage of the hybrid gene (Table 1). This suggests that the *RP39A* promoter functions at equal efficiencies whether it is plasmid borne or integrated into the *S. cerevisiae* genome at the *RP39A* locus. Therefore, any *trans*-acting molecules involved in the synthesis of ribosomal proteins or, specifically, ribosomal protein 39A must not be rate limiting in cells containing extra plasmid-borne copies of the *RP39A* gene. This is not unexpected, given that there are approximately 70 different ribosomal protein genes, most of which are probably duplicated (17). The net increase in total ribosomal protein gene promoters in a haploid cell containing 30 episomal copies of *RP39A-lacZ* would then be only approximately 20% (from 140 copies to 170 copies).

Through a combination of in vitro linker insertion and BAL 31 deletion mutagenesis, using the *RP39A-lacZ* gene fusion as a substrate (Fig. 5b), we have shown that the sequences between nucleotides −256 and −170 define an upstream promoter element which is essential for wild-type levels of transcription of *RP39A*. The 12-nucleotide HOMOL1 yeast ribosomal protein gene consensus sequence lies at the 5' boundary of this region, and the 12-nucleotide RPG sequence was found 6 bp 3' to HOMOL1. The remainder of this 87-bp region is very T-rich. Of the 55 nucleotides which lie 3' to the RPG sequence, 33 are dTMPs (Fig. 5a). We found that similar T-rich regions which are 20 to 60 bp in length and 55 to 85% T residues, are common to the 5' flanking regions of all but two of the sequenced yeast ribosomal protein genes. Furthermore, HOMOL1, RPG, and the T-rich regions are present in the same order upstream of most sequenced yeast ribosomal protein genes. For every gene in which a HOMOL1 or RPG sequence (or both) was identified, RPG lay immediately downstream of HOMOL1 (48), and the T-rich region was located immediately 3' to these sequence motifs (Fig. 7). One of the two sequenced yeast ribosomal protein genes which lacks such a T-rich region, *RP28-2*, possesses a similarly positioned A-rich region. Therefore, *RP28-2* may be considered to differ in this respect from all but one of the other sequenced yeast ribosomal protein genes only in that the T-rich region is located on the opposite strand of DNA.

When the positions of HOMOL1, RPG, and the T-rich region of *RP39A* were matched with the deletions constructed upstream of *RP39A-lacZ* (Fig. 5b), those *RP39A-*

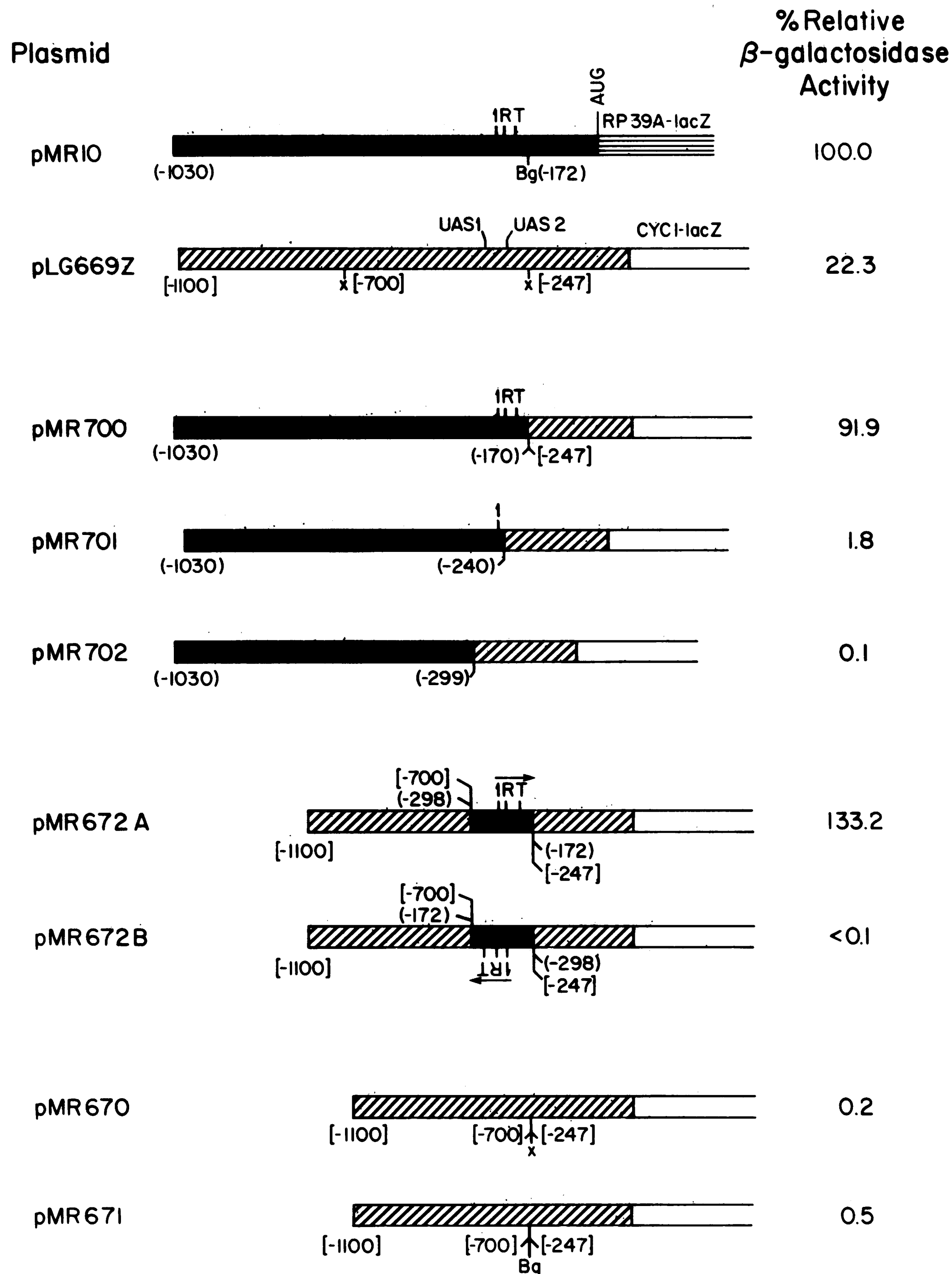

FIG. 8. Expression of hybrid *RP39A-CYC1* promoters. Construction of plasmids containing hybrid *RP39A-CYC1* promoters is described in the text and shown in Fig. 3. The positions of *CYC1* upstream activation sites (UAS1 and UAS2) and the *RP39A* consensus sequences HOMOL1 (1), RPG (R), and the T-rich region (T) are indicated. β-galactosidase activities for each promoter fusion construct in *S. cerevisiae* JL8 are presented as percentages of wild-type *RP39A-lacZ* expression. Numbers in parentheses indicate nucleotide positions within *RP39A* 5′ flanking DNA, and numbers in brackets refer to *CYC1* 5′ flanking sequences. Symbols: ≡, *RP39A-lacZ* coding sequences; □, *CYC1-lacZ* coding sequences; ■, *RP39A* 5′-flanking sequences; ▨, *CYC1* 5′ flanking sequences.

lacZ constructs from which HOMOL1 alone was deleted (Δ−299/−246, Δ−299/−243, or Δ−299/−238) directed the synthesis of 6 to 15% of wild-type levels of β-galactosidase activity. Specific deletion of the 5′ one-third of the RPG sequences (Δ−240/−232) resulted in only a 30% drop in *RP39A-lacZ* expression; however, deletion of both HOMOL1 and RPG (Δ−266/−199 or Δ−299/−232) completely abolished expression of *RP39A-lacZ*. Although deletion of the 3′ half of the T-rich region (Δ−200 series) had minimal effects on expression, deleting most of the RPG sequence and the 5′ half of the T-rich region (Δ−240/−193 or Δ−234/−193) caused a 78 to 84% decrease in β-galactosidase

activity. Deletion of most of the RPG sequence and all of the T-rich region (Δ−234/−170) reduced expression to less than 1.0% of wild-type. These results suggest that HOMOL1, RPG, and the T-rich region function together to promote expression of *RP39A*. It is also possible that these sequence elements play a role in the selection of appropriate transcriptional initiation sites and that deletion of these elements leads to the synthesis of aberrant, unstable, or untranslatable mRNAs. Results obtained by deletion analysis of the yeast *RP59A* gene demonstrate that HOMOL1 and T-rich region sequences are also essential for *RP59A* promoter function (J. Larkin, J. Thompson, and J. Woolford, manuscript in preparation). Unlike most ribosomal protein genes, *RP59A* lacks an RPG sequence (Fig. 7).

To address the question of whether the *RP39A* upstream promoter element is sufficient for promoting high levels of expression of another yeast gene which is lacking its upstream activation sequences, DNA fragments from the *RP39A* 5′ flanking region were substituted for the *CYC1* UAS in a *CYC1-lacZ* gene fusion (Fig. 3 and 8). Insertion of fragments containing the *RP39A* HOMOL1, RPG, and T-rich sequences upstream of *CYC1-lacZ* (pMR672A and pMR700) restored β-galactosidase expression to levels similar to that of wild-type *RP39A-lacZ*; however, HOMOL1 alone, when substituted for the *CYC1* UAS, only minimally restored *CYC1-lacZ* expression (pMR701). Expression of *CYC1-lacZ* was fully restored only when the HOMOL1–RPG–T-rich sequences were inserted in the same 5′→3′ orientation as that which occurs upstream of ribosomal protein genes, i.e., 5′–HOMOL1–RPG–T-rich–3′ (pMR672A versus pMR672B). This is in contrast to the situation observed for UASs 5′ to the yeast *GAL1* (35, 74), *CYC1* (24), and *HIS4* (33) genes, and for many eucaryotic enhancer sequences (38), which have been shown to function in either orientation. In our promoter fusion constructs, the spacing between the *RP39A* upstream promoter element and the downstream *CYC1* TATA sequences (in pMR672A and pMR700) is roughly the same as that found between the upstream promoter element sequences and the TATA sequence of the wild-type *RP39A* gene (pMR10) (12, 27). Our deletion mutations do not address the importance of the relative orientation or spacing or both of the HOMOL1, RPG, and T-rich sequences with respect to one another; however, we note that the RPG sequence was found to occur in either orientation with respect to the HOMOL1 and T-rich sequences (Fig. 7) and that spacing between the three sequences varied somewhat among the 20 ribosomal protein genes thus far examined.

A number of questions relating to the role of these consensus sequences in promoting yeast ribosomal protein gene expression remain to be answered. How does the tripartite upstream promoter element function? It is particularly striking that one of the promoter sequence elements is a T-rich region. Does a conformational change of this region facilitate the binding of RNA polymerase II or act as a signal for directing polymerase toward the region to be transcribed or both? Do one or more of the promoter sequence elements bind *trans*-acting transcriptional factors? Transcripts from the 70 ribosomal protein genes represent 15% of total steady-state mRNA transcribed by RNA polymerase II in yeast cells (84). Therefore, any *trans*-acting regulatory factors directly or indirectly involved in ribosomal protein gene transcription may represent a significant fraction of yeast transcriptional factors.

Why do many ribosomal protein genes possess both HOMOL1 and RPG sequences while some contain only one or the other? We noted that eight of the last nine nucleotides of HOMOL1 are homologous to eight of the first nine nucleotides of RPG. These two sequences may therefore be functionally redundant. Alternatively, it is possible that HOMOL1 or RPG sequences lie further upstream from some ribosomal protein genes than does the DNA thus far sequenced. If these genes are actually lacking the HOMOL1 or RPG sequence motif or both, do they share other sequence elements in common which may have promoter functions and which may aid in coordinating the expression of these genes with one another and with the other ribosomal protein genes? Is there some hierarchy in the role of the three consensus sequences in regulating ribosomal protein expression? For example, are any of these sequences particularly involved in the regulation of transcription of ribosomal protein genes during sporulation or after heat shock (41, 42, 64)?

Are any of the ribosomal protein gene upstream promoter elements shared with any other class of genes, particularly those whose gene products are also involved in the mechanisms of translation? Interestingly, the RPG sequence has recently been identified in the 5′ flanking region of the yeast gene encoding the translational elongation factor EF-1α (48, 61). T-rich regions may also play roles in promoting the expression of a variety of yeast genes in addition to the ribosomal protein genes. Inspection of the 5′ flanking sequences of 20 randomly selected non-ribosomal protein yeast genes has led to the identification of similar T-rich regions within 250 nucleotides upstream of the TATA sequences of 12 of these genes (2, 8, 11, 18, 32, 51, 58, 65, 72, 78, 86).

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