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Expression of yeast 5S RNA is independent of the rDNA enhancer region

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ABSTRACT

In the yeast *Saccharomyces cerevisiae*, each of the tandemly repeated ribosomal RNA genes carries a 5S gene within the 'non-transcribed' spacer region. These 5S RNA genes lie between the rDNA enhancer and the promoter of rRNA transcription. Since there is roughly equimolar synthesis of 5S RNA and the 35S rRNA precursor transcript we asked whether the enhancer plays a role in regulating the transcription of 5S RNA. A marked 5S gene was inserted into plasmids designed to test rDNA enhancer function. The enhancer failed to stimulate 5S RNA synthesis even though it stimulated transcription of a distal rRNA test gene greater than 10-fold. This failure is consistent with a model of enhancer function that proposes specific interactions between the enhancer and the 35S rRNA promoter via a looping out of the intervening 5S RNA gene.

INTRODUCTION

In *Saccharomyces cerevisiae* ribosomal RNA is encoded by a tandem array of approximately 200 genes situated on chromosome XII. Each repeat can be transcribed by RNA polymerase I to yield a ~7 kb ribosomal RNA precursor (35S) that is processed to generate the 25S, 18S and 5.8S ribosomal RNAs (1,2). Transcription of the 35S rRNA is influenced by the rDNA enhancer element that lies near the end of each transcription unit and stimulates transcription of both upstream and downstream units (3,4).

Unlike other eukaryotes, each *S. cerevisiae* rDNA repeat also contains a 5S RNA transcription unit (5). These 5S RNA genes are located within the 'spacer' DNA situated between the rDNA enhancer region and the start of 35S transcription (see Figure 1). The 5S RNA gene is transcribed by RNA polymerase III from the opposite DNA strand compared to the 35S molecule (5). In other eukaryotes, the 5S RNA genes are dispersed throughout the genome. Their transcription is not, generally, coordinately regulated with that of the larger ribosomal RNA species. The location of the 5S genes in *S. cerevisiae* suggests the possibility that there is coordinated transcription of the 35S and 5S RNAs in this organism. In particular, because the 5S gene lies between the enhancer and the promoter of 35S RNA (see Figure 1), do the sequences governing expression of the 35S transcript act on the 5S gene as well?

Here we report that there is, indeed, balanced expression of the 5S and 35S rRNA species, but that expression of 5S RNA is independent of the rDNA enhancer.

MATERIALS AND METHODS

Yeast Strains and Techniques

The *Saccharomyces cerevisiae* strains *(MATa ura3-1 leu2-3,112 trp1-D1 ade2-1 his3-11 can1-100)* were derived from W303-1A (obtained from R. Rothstein, Columbia University). Yeast transformation was performed as described by Ito et al. (6), and DNA isolation was performed as described in (7).

Preparation of RNA

Total RNA was prepared from logarithmically growing cells as described previously (3). Small RNA species (5S) were identified by fractionating 5μg of heat denatured total cellular RNA on 8% polyacrylamide/urea gels in TBE. Following electrophoresis the gels were equilibrated in 25mM NaPO4 and electroblotted to NYTRAN nylon membranes (0.2 μ pore size). RNA was fixed to the membranes by baking at 80° for 45 minutes. Filters were prehybridized and hybridized in 5× SSPE; 2× Denhardt's; 0.2% SDS; 100μg/ml salmon sperm DNA at 37°. Hybridization probes were oligonucleotides specific for either the Wild-type 5S RNA or the mutant 5S* molecule, labelled with [α^{32}P]-ATP by polynucleotide kinase. The blots were washed twice at room temperature and then twice at 55°. Large RNA species (T7rRNA-minigene transcripts) were identified by fractionating 5μg of total RNA on 1.5% agarose gels as described previously (8).

Preparation of polysomes

Total cellular ribosomes and polysomes were prepared as described previously (9).

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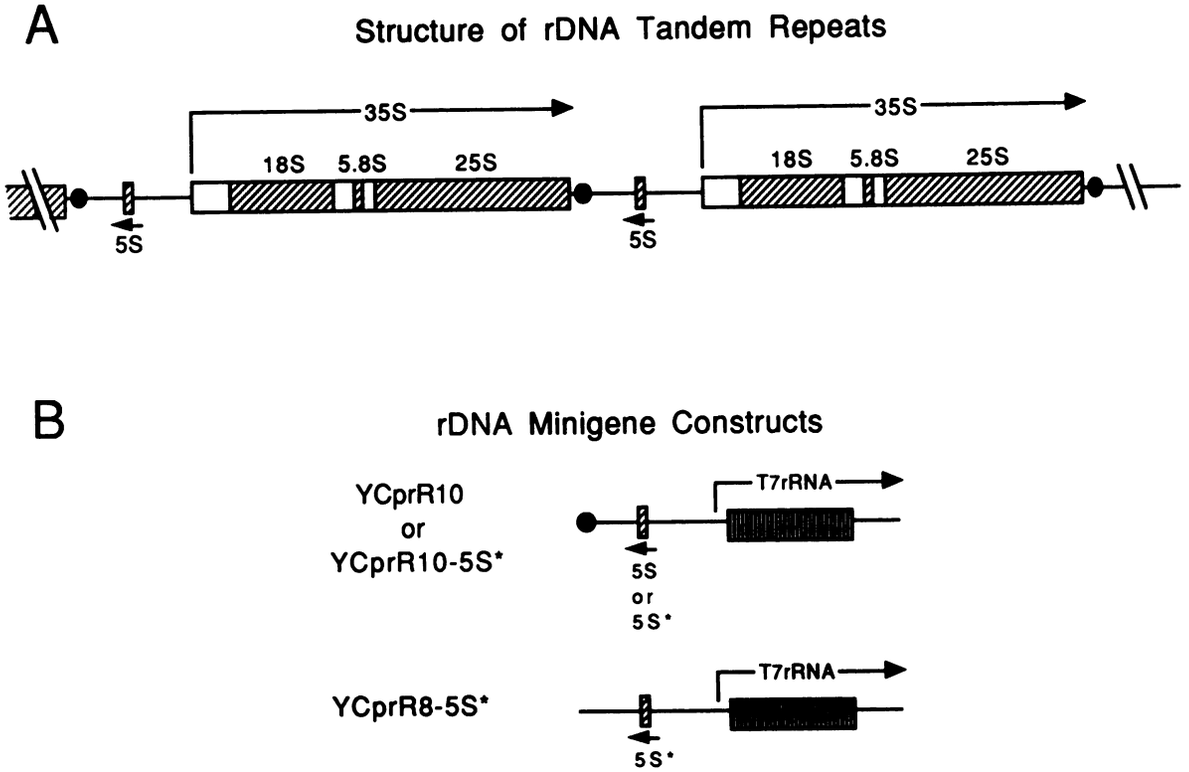

Figure 1. Structure of the *Saccharomyces cerevisiae* chromosome XII rDNA repeat and rDNA minigene constructs. (A) Chromosomal organization of the rDNA tandem repeats. The extent and orientation of the 5S and 35S transcription units are indicated as well as the locations of the final processing events leading to production of mature 18S, 5.8S and 25S species. (B) Structure of the T7-rDNA minigene vectors used in this study. 600 nucleotides from bacteriophage T7 have replaced nucleotides 124-6085 of the 35S rRNA transcription unit (see reference 3 for details). ●, rDNA enhancer; ▨, 5S or ribosomal RNA coding regions; −, ribosomal 'spacer' DNA; ▩, T7 DNA.

Isolation of ^{32}P-labelled RNA

Yeast were grown to mid-log phase ($1-1.5\times10^{-7}$ cells/ml) in rich medium depleted for phosphate (10), harvested and resuspended in fresh medium containing 125μCi/ml ^{32}P-orthophosphate (0 time). At various time points 1ml samples were removed and total RNA was prepared. 300,000 counts per minute of labeled RNA were fractionated on a 6% polyacrylamide denaturing gel as described above and the RNAs were visualized by autoradiography. The regions of the gel corresponding to the 5.8S, 5S, and tRNAs were excised and total counts per band were determined for each time point. After adjusting for background radioactivity and transcript size the ratio of 5.8S RNA to 5S RNA was determined.

Oligonucleotide directed mutagenesis of the 5S RNA gene

The plasmid substrate for oligonucleotide directed mutagenesis of the 5S gene was single stranded pEMBL(+)/rR1.7. This vector contains a subclone of the rDNA locus containing the T7rRNA-minigene described in reference (3) cloned into the vector pEMBL8(+). 100 pmole of the mutagenic oligonucleotide (JW72 = CCTGAGTTTTGAGCTCGCGTATGC) was incubated in linker-kinase buffer containing 50mM DTT, 0.5mMATP and 5 units of T4 Polynucleotide Kinase at 37° for 30 minutes and then heated to 80° for 10 minutes to stop the reaction. 10 pmole each of kinased JW72 and phosphorylated universal primer (which hybridizes to the opposite side of the vector, requiring the extension reaction to proceed only half the distance of the plasmid) were hybridized to 1μg of the (+) strand of pEMBL(+)/rR1.7 in 10mM Tris, pH8; 10mM $MgCl_2$. Primers were extended in 1mM dNTPs; 50μM ATP; 10mM DTT by incubating with 5 units of Klenow fragment and 1 unit of T4 DNA Ligase at 14° for 4 hrs. After transformation into *E. coli* strain C600, mutant genes were identified by colony hybridization to ^{32}P-kinased JW72 at 30° followed by washing in 2× SSPE/.1% SDS at T_m −7° (T_m = 2° (A+T) + 4° (G+C)). A control blot carried wild-type pEMBL(+)rR1.7 and had absolutely no signal under these conditions; experimental blots revealed about 20% of the colonies as positive signals. Several putative mutants were picked, colony purified and retested in the same way as above. Plasmid DNA was prepared from several positive clones. Since mutant molecules would contain a new and unique Sac I site introduced by JW72, the plasmids were digested with Cla I + Sac I and screened for the appropriate restriction pattern. One positive mutant was subjected to double strand sequence analysis (using *Sequenase*, United States Biochemicals Corp.) to confirm the sequence integrity of the new mutant 5S gene (hereafter termed 5S*).

Plasmid constructions

The DNA containing the 5S* gene from pEMBL(+)rR1/7-5S* was subcloned into the yeast episomal single copy vectors YCprR8 and YCprR10 (3). These plasmids are derived from YCp50, and contain an ARS, a centromere, and the *URA3* gene, as well as an rRNA minigene. YCprR10 includes the rRNA enhancer; YCprR8 does not. The integrity of these constructs was verified by colony hybridization, restriction, Southern and sequence analyses.

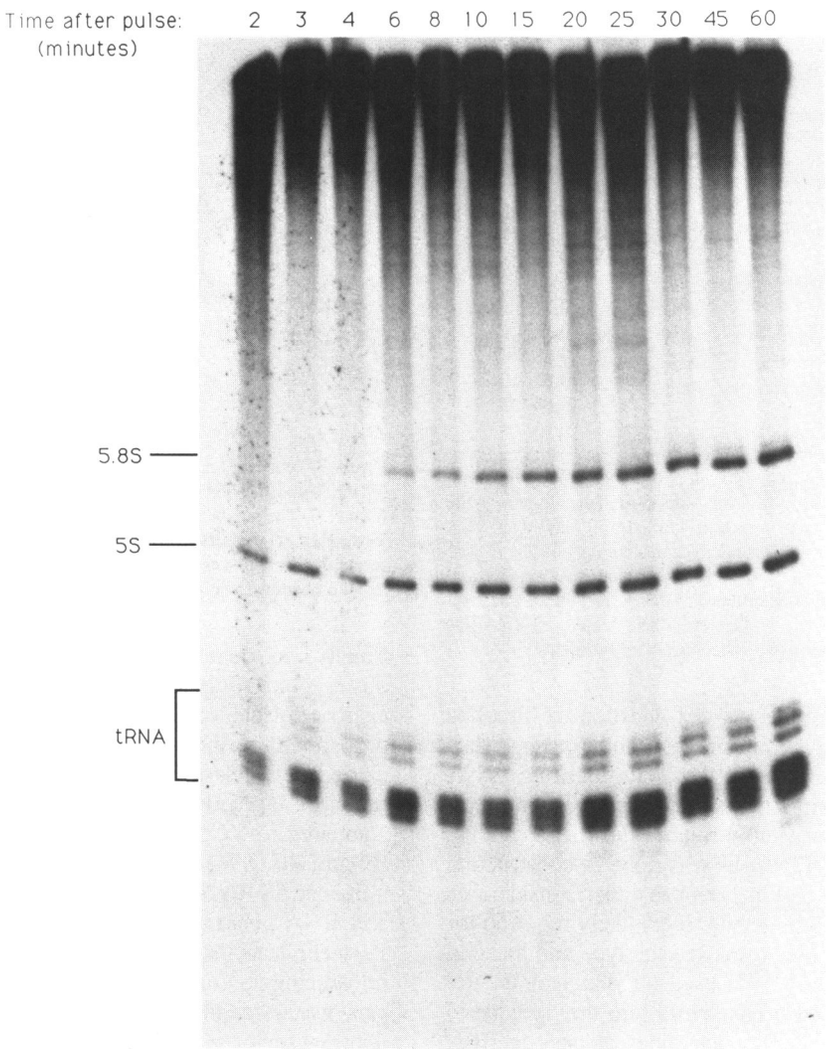

Figure 2. Comparative analysis of newly synthesized rRNA species. A mid-log culture growing in phosphate depleted medium was administered a pulse of ^{32}P-orthophosphate at time = 0, and samples were removed at the indicated times for RNA preparation and fractionation on a 6% polyacrylamide denaturing gel. The identities of the 5S, 5.8S and tRNA species are indicated. Quantitation of the radioactivity contained on this gel is presented in Experiment 1 of Table 1.

RESULTS AND DISCUSSION

Relative expression of 5.8S and 5S rRNAs

The uncharacteristic physical linkage of the 5S and 35S ribosomal RNA transcription units in *Saccharomyces cerevisiae* suggested that the expression of these genes might be regulated via a common mechanism. We, therefore, sought to determine whether the transcription of the 35S and the 5S RNAs is, indeed, equimolar. The 35S rRNA primary transcript is rapidly processed to yield the mature 25S, 18S and 5.8S ribosomal components. In normally growing cells this processing is nearly 100% efficient (1). We compared the accumulation of radioactivity following a pulse of radiolabelled precursor in 5.8S RNA with that in 5S RNA. These two gene products (as well as the 25S and 18S) are required in equimolar ratios within the ribosome, and steady state levels of these species do not deviate significantly from a 1:1 ratio. Wild type yeast were grown in rich medium depleted for phosphate (10). At time=0 125 μCi/ml ^{32}P-orthophosphate was added to the culture. Samples were removed at regular intervals and the levels of newly synthesized 5.8S and 5S RNAs were compared (Figure 2). The amount of radioactivity contained within each band was quantitated. After deducting background and normalizing for size (tRNA synthesis serving as an internal control) the ratio of newly synthesized 5.8S RNA (158 nucleotides) to 5S RNA (121 nucleotides) was determined (Table 1). At early time points the ratio was <0.3 due to a lag in appearance of mature 5.8S RNA, reflecting the time required to process the mature species from the larger 35S precursor (1). Thereafter, the ratio rose to 0.9 where it remained unchanged for up to 60 minutes. In a second experiment similar kinetics were observed but the final ratio leveled off at 0.75. These results indicate that no substantial excess of of 5S RNA transcription occurs, supporting the notion that there is a mechanism coordinating the syntheses of the two ribosomal RNA transcription units. An integrated regulatory mechanism coordinating the activity of Pol I and Pol III could insure the production of these rRNA species in equimolar ratios as is required in ribosomes.

5S RNA synthesis is independent of the 35S enhancer element

The 5S gene lies between the enhancer and the promoter of 35S rRNA transcription (3, 5). Does the enhancer also influence the

TABLE 1: Coordinated Synthesis of 5S RNA and 5.8S rRNA

Time after Pulse	Corrected Ratio of 5.8S to 5S RNA	
	Experiment 1	Experiment 2
2 min.	0.28	0.25
3 min.	0.28	0.31
4 min.	0.47	0.31
6 min.	0.49	0.32
8 min.	0.56	0.36
10 min.	0.75	0.48
15 min.	0.77	0.71
20 min.	0.95	0.66
25 min.	0.86	0.68
30 min.	0.91	0.67
45 min.	0.89	0.64
60 min.	0.97	0.75

Wild-type yeast cultures were grown to mid-log in phosphate depleted medium. At time=0 a pulse of ^{32}P-orthophosphate was added to the culture. At the indicated intervals, 1 ml. samples were removed, chilled to 0° and total RNA was prepared and fractionated on a 6% polyacrylamide denaturing gel. The regions of the gel corresponding to the 5.8S, 5S, tRNAs and background areas were excised and total radioactivity in each band was determined. After correction for transcript size and background, the ratio of newly synthesized 5.8S rRNA to 5S RNA was calculated. The data shown represent two independent experiments.

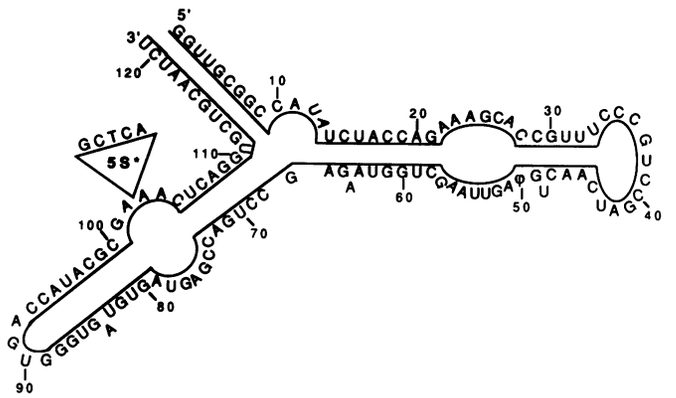

Figure 3. Sequence and predicted secondary structure of *Saccharomyces cerevisiae* 5S RNA and the 5S* mutant. The predicted secondary structure was adapted from (13). The numbers indicate base pairs counting from the 5′ end of the mature 5S species. The insert indicates the location of the addition of 5 nucleotides to create the mutant species 5S*. The symbol ϕ at position 50 is the pseudouracil residue characteristic of this position in 5S RNA species.

transcription of 5S RNA? To answer this question we first had to create a 5S gene producing a transcript that can be distinguished from the other 5S transcripts in the cell. Because RNA Polymerase III transcription units contain internal promoter sequences (11) one could not simply replace part of the coding sequence with heterologous DNA, as was done in constructing the T7rRNA-minigenes (3). Furthermore, the considerable secondary structure of the 5S molecule, most likely required for transcript stability and function, restricted the type and location of mutation that could be used. We used oligonucleotide site-directed mutagenesis to insert 5 base pairs into the 5S gene to yield a transcript with five extra nucleotides at position 102, referred to a 5S* (Figure 3). This position was chosen in hopes that the insertion would neither interrupt the Pol III internal control region (positions 57−99) nor greatly alter secondary stem-loop structures (12, 13). Sequence analysis showed that the 5 bp insertion is the only difference between 5S* and the wild-type 5S genes. The two 5S RNA species can be separated on a polyacrylamide denaturing gel and can be further distinguished using an oligonucleotide probe that hybridizes, under appropriate conditions, only to the mutant molecule carrying the insertion.

To determine whether the rDNA enhancer has an effect on 5S transcription, the 5S* gene was used to replace the wild-type 5S gene in the yeast vectors YCprR10 and YCprR8, yielding YCprR10-5S* and YCprR8-5S* (Figure 1B). These plasmids contain subclones of the rDNA repeat sequences with and without the rDNA enhancer, respectively (3). YCprR8-5S*, YCprR10-5S*, and YCprR10 were transformed into wild-type yeast and total RNA was prepared and subjected to Northern analysis as described in Materials and Methods. Hybridization with the oligonucleotide probe JW72, specific for the 5S* species, (Figure 4A) shows that equal amounts of 5S* product are produced from either 5S* plasmid, indicating that the presence of the rDNA enhancer has no effect on 5S* RNA synthesis. No signal was obtained from RNA produced in the YCprR10-bearing strain, demonstrating the specificity of the probe for the 5S* transcript. To verify that equal amounts of RNA were loaded in each lane, the same filter was hybridized with a probe that is capable of pairing with the wild-type species (Figure 4B).

Finally, to demonstrate that the enhancer is functional in these constructs, RNA was fractionated on a 1.5% agarose/formaldehyde gel, transferred to nitrocellulose, and probed with a riboprobe homologous to the T7rRNA-minigene carried on the plasmid. As was observed previously, T7 transcript levels were greatly increased in YCprR10 and YCprR10-5S* as compared to YCprR8-5S*, the construct lacking the enhancer (Figure 4C). We conclude that the rDNA enhancer does not influence 5S RNA expression, in contrast to its effect on a distal 35S RNA gene.

To eliminate the possibility that plasmid derived sequences were influencing 5S* expression in response to the enhancer, the 5S* gene was tested in a construct in which it lies between two rRNA minigenes integrated either at the *URA3* locus or within the rDNA repeat. Once again, two such constructs were used, one with and one without the enhancer upstream of the 5S* gene. Within a factor of 2, equal amounts of 5S* gene product were produced from all constructs regardless of the presence of the enhancer or of the locus of insertion (data not shown). We conclude from all of these analyses that coordinate expression of the 5S and 35S rRNA genes in yeast is not mediated via the activity of the rDNA enhancer region.

Is the Mutant 5S* Functional?

To determine whether the 5S* gene product is functional, ribosomes were prepared from the same strains analyzed above, and the ribosomal RNA was fractionated as described. No 5S* RNA was ever detected in the ribosomal RNA (data not shown). We conclude that although the 5S* gene is transcriptionally active, the gene product is not efficiently incorporated into ribosomes. Although numerous predictions of the two-dimensional structure of 5S RNA exist (13), the three dimensional structure, particularly in the region in which we made the insertion (14), is not sufficiently established to predict how an alteration would affect the interaction of 5S* with the ribosome.

Our results indicate that the steady state level of the 5S* molecule is at least 5-times less than we would expect if it were present in equimolar amounts with wild-type 5S. Because analysis of mature ribosomes revealed that the 5S* species is not efficiently incorporated into ribosomes, its decreased abundance within the cell may be due to rapid degradation. This possibility does not

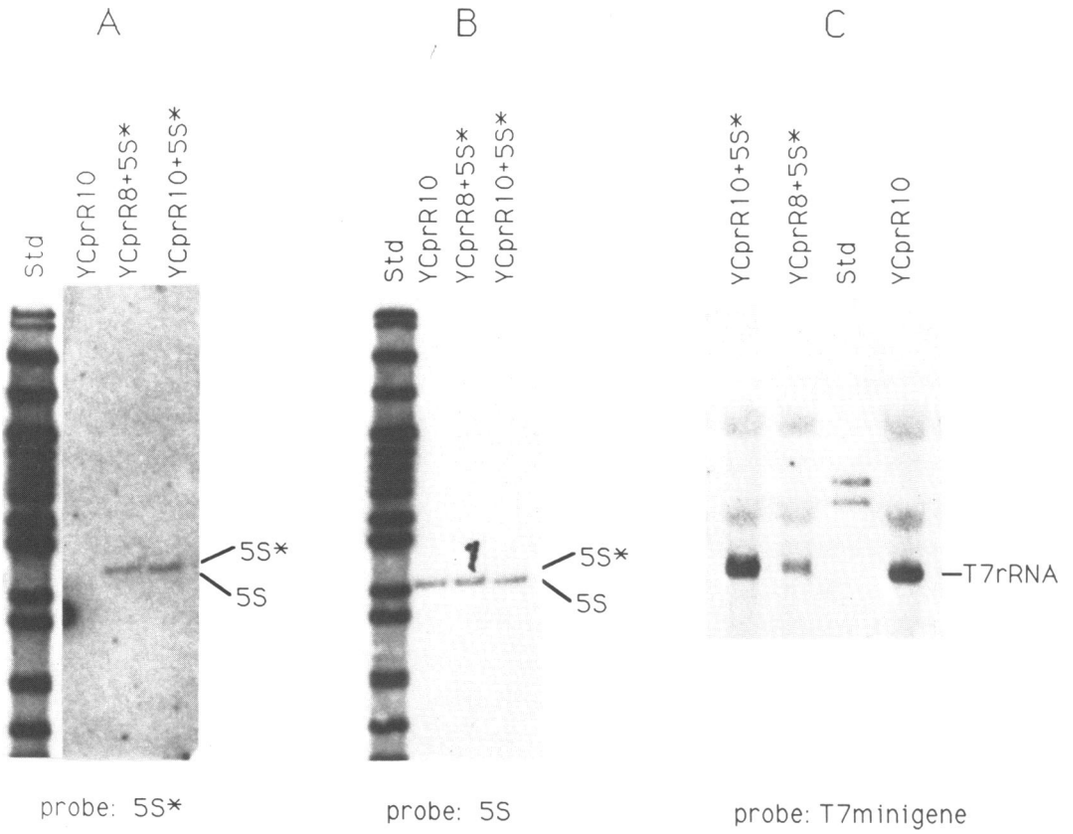

Figure 4. Expression of *Saccharomyces cerevisiae* 5S RNA is not influenced by the yeast rDNA enhancer. (A) Total RNA from cultures harboring the indicated plasmids were fractionated on an 8% polyacrylamide denaturing gel, eletroblotted to NYTRAN and probed with a ^{32}P-labelled oligonucleotide specific for the mutant 5S* RNA species. The standards are ^{32}P-labelled restriction fragments of DNA generated from an Msp I digest of pBR322. (B) This blot was stripped and then reprobed with a ^{32}P-labelled oligonucleotide homologous to the wild-type species. (C) Duplicate RNA samples were fractionated on a 1.5% agarose gel under denaturing conditions, blotted to NYTRAN and probed with a riboprobe homologous to the T7rDNA-minigene carried on these plasmids. The standards are ^{32}P-labelled restriction fragments of a Hind III digest of λ DNA.

alter our conclusion regarding the lack of effect of the enhancer, however, since 5S* transcribed from either of the plasmids (see Figure 4A) would be equally unstable.

What is the mechanism coordinating 5S and 35S transcription?

Our results rule out involvement of the rDNA enhancer in coordinating the transcription of 5S RNA with the larger ribosomal RNAs in yeast. Although this finding leaves us with no other obvious cis-acting control element, work from other laboratories suggest that synthesis of 5S RNA may be mediated via the availability of the transcription factor TFIIIA (15, 16). TFIIIA is required for the transcription of 5S RNA genes and has the ability to bind to both the 5S gene and the 5S RNA transcript (17). 5S transcription can be inhibited by the addition of excess 5S RNA, presumably due to competition for the binding of TFIIIA. The ribosomal protein YL3 (L5 is the mammalian homologue) also binds specifically to 5S RNA and competes with TFIIIA for binding, suggesting a model for the regulation of 5S synthesis based on the relative amounts of YL3 and TFIIIA bound to 5S RNA (16). According to this model TFIIIA is available to activate the synthesis of 5S RNA as long as no free 5S RNA (ie, 5S RNA not bound to YL3) is present. Excess production of 5S RNA would immediately lead to inhibition of 5S transcription. Ultimately, then, it would be the level of YL3 synthesis that regulates the expression of 5S RNA. This would make sense since YL3 expression is itself coordinately controlled with the syntheses of the other ribosomal proteins and with the larger rRNA species (16). This model, then, is consistent with our result that synthesis of 5S RNA is coordinated with the expression of the larger rRNA species. This mechanism is also consistent with our current model for the mode of action of the rDNA enhancer, which proposes specific interactions between the enhancer and the 35S rRNA promoter (4), excluding the spacer region containing the 5S gene.

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