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Sequences within the Spacer Region of Yeast rRNA Cistrons That Stimulate 35S rRNA Synthesis In Vivo Mediate RNA Polymerase I-Dependent Promoter and Terminator Activities

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Sequences within the spacer region of yeast rRNA cistrons stimulate synthesis of the major 35S rRNA precursor in vivo 10- to 30-fold (E. A. Elion and J. R. Warner, Cell 39:663–673, 1984). Spacer sequences that mediate this stimulatory activity are located approximately 2.2 kilobases upstream from sequences that encode the 5′ terminus of the 35S rRNA precursor. By utilizing a centromere-containing plasmid carrying a 35S rRNA minigene, a 160-base-pair region of spacer rDNA was identified by deletion mapping that is required for efficient stimulation of 35S rRNA synthesis in vivo. A 22-base-pair sequence, previously shown to support RNA polymerase I-dependent selective initiation of transcription in vitro, was located 15 base pairs upstream from the 3′ boundary of the stimulatory region. A 77-base pair region of spacer DNA that mediates transcriptional terminator activity in vivo was identified immediately downstream from the 5′ boundary of the stimulatory region. Deletion mutations extending downstream from the 5′ boundary of the 160-base-pair stimulatory region simultaneously interfere with terminator activity and stimulation of 35S rRNA synthesis from the minigene. The terminator region supported termination of transcripts initiated by RNA polymerase I in vivo. The organization of sequences that support terminator and promoter activities within the 160-base-pair stimulatory region is similar to the organization of rDNA gene promoters in higher organisms. Possible mechanisms for spacer-sequence-dependent stimulation of yeast 35S rRNA synthesis in vivo are discussed.

rRNA genes in the yeast *Saccharomyces cerevisiae* are tandemly repeated approximately 120 times in one cluster located on chromosome 12 (27). Each cistron contains the information for all of the mature rRNAs. RNA polymerase I is responsible for the synthesis of a 35S rRNA precursor that is processed to form the 18S, 5.8S, and 25S rRNAs. The 5S rRNA gene is located in the spacer region between 35S rRNA transcription units and is transcribed by RNA polymerase III. Synthesis of 35S rRNA has been studied in several laboratories using both in vitro (9, 11, 19, 23, 32, 33, 35, 36) and in vivo (1, 5, 15, 17, 19, 28) approaches. The 5′ terminus of 35S rRNA has been mapped in both *S. cerevisiae* (1, 17) and *Saccharomyces carlsbengensis* (19). RNA sequencing analysis showed that 35S rRNA molecules contain a 5′ triphosphate, suggesting that transcription is initiated with the 5′-terminal sequences of 35S rRNA (18, 26). Analysis of transcripts synthesized in isolated yeast nuclei in the presence of γ-sulfhydryl nucleoside triphosphates supports this conclusion (19, 23).

We showed previously that cloned yeast rDNA templates support RNA polymerase I-dependent selective initiation of transcription with a whole-cell extract (32). Transcription of this in vitro reaction initiates from a site 2.2 kilobases (kb) upstream from sequences that encode the 5′ terminus of 35S rRNA. Deletion mapping studies defined a 22-base-pair rDNA sequence as being both necessary and sufficient for selective initiation of transcription in vitro (33). The same in vitro reaction did not support detectable selective transcription from the 35S rRNA initiation site.

Synthesis of 35S rRNA has been studied in vivo by using autonomously replicating plasmids carrying 35S rRNA minigenes (5, 15, 28). The minigenes consist of a heterologous reporter sequence flanked by yeast rDNA sequences corresponding to the regions that encode the 5′ and 3′ termini of 35S rRNA. Minigene transcription is monitored by hybridization of specific probes to the heterologous reporter sequences in the minigene transcript. Nucleotide sequences extending 209 base pairs upstream from those that encode the 5′ terminus of 35S rRNA are sufficient to direct in vivo synthesis of a fusion transcript with the correct 5′ terminus (15). Elion and Warner (5) showed that additional sequences within the spacer region affect the relative abundance of fusion transcript synthesized from such a minigene. When all of the spacer sequences upstream from position −209, for example, are included in the minigene, synthesis of the fusion transcript is stimulated approximately 30-fold (5). A small region of spacer DNA located 2.2 kb upstream from sequences that encode the 5′ terminus of 35S rRNA was shown to be sufficient for a 20-fold stimulation of minigene transcription (5, 6). This segment of spacer DNA includes sequences that we showed to be sufficient to direct RNA polymerase I-dependent selective initiation of transcription in vitro (32, 33).

We report here a deletion map of spacer nucleotide sequences that are required for stimulation of 35S rRNA synthesis in vivo. We show that spacer sequences that are required for RNA polymerase I-dependent selective initiation of transcription in vitro and spacer sequences that modulate RNA polymerase I transcriptional termination in vivo are required for efficient stimulation of 35S rRNA synthesis in vivo.

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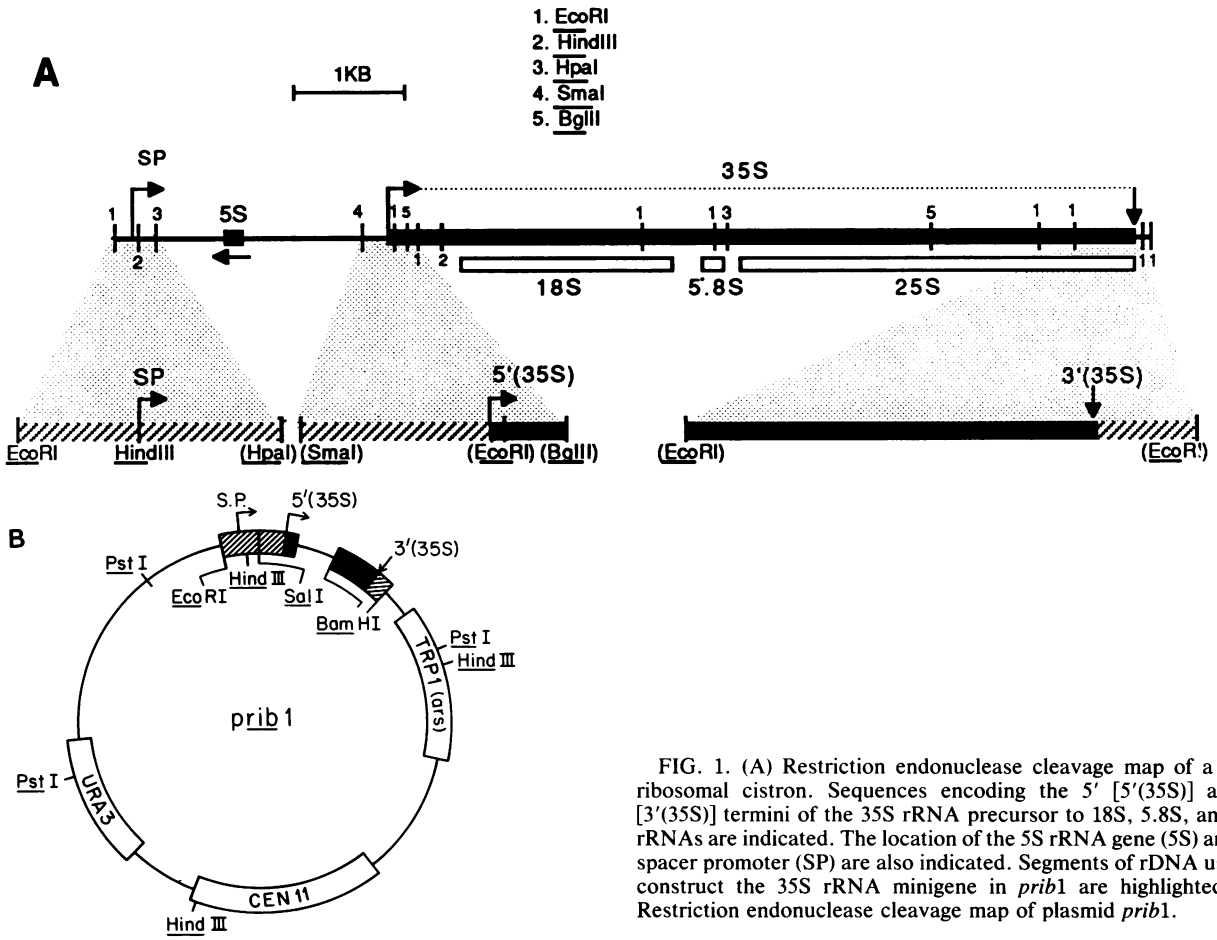

FIG. 1. (A) Restriction endonuclease cleavage map of a yeast ribosomal cistron. Sequences encoding the 5′ [5′(35S)] and 3′ [3′(35S)] termini of the 35S rRNA precursor to 18S, 5.8S, and 25S rRNAs are indicated. The location of the 5S rRNA gene (5S) and the spacer promoter (SP) are also indicated. Segments of rDNA used to construct the 35S rRNA minigene in *prib*1 are highlighted. (B) Restriction endonuclease cleavage map of plasmid *prib*1.

MATERIALS AND METHODS

Materials. [α-^{32}P]dATP (800 Ci/mmol), [α-^{32}P]CTP (410 Ci/mmol), and [α-^{32}P]UTP (410 Ci/mmol) were purchased from Amersham Corp. Restriction endonucleases were obtained from New England BioLabs, Inc., Bethesda Research Laboratories, Inc., or International Biotechnologies, Inc. Avian myeloblastosis virus reverse transcriptase and DNA polymerase I Klenow fragment were purchased from Bethesda Research Laboratories. T4 DNA ligase was obtained from P-L Biochemicals, Inc. *Sal*I and *Eco*RI restriction site linkers were from Collaborative Research Inc. S1 nuclease was from Boehringer Mannheim Biochemicals, and polynucleotide kinase was from Pharmacia Fine Chemicals. Exonuclease III and the pBR322 *Hin*dIII site primer were purchased from New England BioLabs. SP6 RNA polymerase and the cloning vectors pSP64 and pSP65 were obtained from Promega Biotec, Inc.

Strains, growth conditions, and yeast transformation. *S. cerevisiae* S173-6B (a *leu2-3 leu2-112 his3-1 trp1-289 ura3-52*), provided by F. Sherman, University of Rochester, Rochester, N.Y., was used for all of the analyses described in this report. Yeast cells were grown at 30°C in a minimal medium containing 0.67% yeast nitrogen base without amino acids (Difco Laboratories) and 2% glucose supplemented with 20 μg each of leucine, histidine, tryptophan, and uracil per ml where indicated. Yeast transformations were performed by using the alkali cation procedure of Ito et al. (13). Transformations were selected based on their ability to grow in the absence of uracil in the growth medium.

Construction of plasmids carrying a 35S rRNA minigene. Plasmid *prib*1 was generated from two separate plasmid constructions. The first plasmid construction involved blunt-end ligation of DNA fragments necessary for selection and autonomous replication in yeast cells into unique restriction sites in pBR322. A 1.3-kb fragment of yeast DNA containing the *TRP1* gene and an autonomous replication sequence was ligated into the *Sal*I site of pBR322 followed by ligation of a 1.6-kb DNA fragment containing the centromere for chromosome 11 (*CEN11*) into the *Bal*I site. These two segments of DNA permit mitotically stable autonomous replication of the plasmid in yeast cells at a low copy number. Finally, a 1.1-kb fragment of DNA containing a functional *URA3* gene was ligated into the *Pvu*II site of pBR322 to permit selection of cells carrying the plasmid after transformation of a *ura3* recipient yeast cell. The second plasmid construction involved replacement of the region between the *Eco*RI and *Hin*dIII sites in pBR322 with a truncated version of a yeast 35S rRNA promoter. Yeast rDNA sequences extending from one *Eco*RI site 160 base pairs upstream from sequences required from in vitro transcription (referred to as the spacer promoter) to a *Bgl*II site approximately 100 base pairs downstream from sequences that encode the 5′ terminus of 35S rRNA was ligated between the *Eco*RI and *Hin*dIII site of pBR322 (Fig. 1). Spacer sequences between unique *Hpa*I and *Sma*I sites in this segment of rDNA were removed, and a unique *Sal*I linker was inserted at the *Hpa*I-*Sma*I junction. An *Eco*RI site located approximately 50 base pairs downstream from sequences that encode the 5′ terminus of 35S

rRNA was destroyed by repair with Klenow fragment, leaving a unique *Eco*RI site at the junction of pBR322 and yeast rDNA sequences. A DNA fragment extending from the *Eco*RI site in this latter plasmid downstream to the unique *Bam*HI site in pBR322 was ligated to a complementary fragment extending from the unique *Eco*RI site in pBR322 in the plasmid containing the *TRP1*, *CEN11*, and *URA3* elements upstream to the unique *Bam*HI site. The final step in the construction involved the addition of *Bam*HI linkers to the termini of a 600-base-pair *Eco*RI fragment of rDNA that includes sequences encoding the 3′ terminus of 35S rRNA (Fig. 1) and ligation of this fragment of DNA into the unique *Bam*HI site of the plasmid to generate plasmid *prib*1 (Fig. 1).

The 35S rRNA minigene in *prib*1 contains a 350-base-pair pBR322 reporter sequence flanked by yeast rDNA sequences that encode the 5′- and 3′-terminal sequences of 35S rRNA. Yeast spacer rDNA sequences that were shown previously to stimulate 35S rRNA synthesis (5, 6) and to support RNA polymerase I-dependent transcription in vitro (32, 33) are located immediately upstream from the sequences which encode the 5′ terminus of 35S rRNA on a unique *Eco*RI-*Sal*I DNA fragment. Generation of *prib*1 derivatives carrying deletion mutations within the spacer DNA sequences and derivatives carrying spacer DNA sequences from different alleles of the yeast ribosomal cistron was effected by *Eco*RI-*Sal*I fragment exchanges as described below.

A *prib*1 derivative carrying spacer sequences in the reverse orientation relative to the 35S rRNA minigene was generated by digestion of *prib*I with *Eco*RI and *Sal*I, repair with Klenow fragment, and religation.

Plasmid *prib*2 is identical to *prib*1, except that the *Bam*HI fragment in *prib*1 containing sequences that encode the 3′ terminus of 35S rRNA was replaced in *prib*2 with an *Eco*RI-*Sal*I fragment containing the spacer promoter. In plasmid *prib*2, this second copy of the *Eco*RI-*Sal*I fragment carries a deletion mutation extending from position −161 to position −122.

Construction of deletion mutations within the spacer-promoter region of *prib*1. Deletion mutations within the 292-base-pair *Eco*RI-*Sal*I fragment of spacer rDNA in *prib*1 were constructed as previously described (33). Spacer rDNA sequences are numbered relative to the *Hin*dIII cleavage site (position +1) within the spacer sequences. Mutagenesis was performed initially by using a plasmid designated *prib*105R. Plasmid *prib*105R carries the *Eco*RI-*Hpa*I fragment of rDNA that supports RNA polymerase I-dependent selective in vitro transcription in vitro (33). For convenience, a *Sal*I linker was inserted into the *Hpa*I site to generate the 292-base-pair *Eco*RI-*Sal*I fragment carried in *prib*1. Two collections of deletion mutations were constructed with *prib*105R by using exonuclease III and S1 nuclease as previously described (33). One collection extended from the *Eco*RI site, and the other extended from the *Sal*I site. All deletion mutations from the *Eco*RI site contained an *Eco*RI linker at their respective end points, and those extending from the *Sal*I site contained a *Sal*I linker. Consequently, an *Eco*RI-*Sal*I fragment could be recovered from a *prib*105R plasmid carrying a deletion mutation extending from the *Eco*RI site or the *Sal*I site and then ligated into the unique *Eco*RI-*Sal*I sites of *prib*1. In this manner the 5′ and 3′ boundaries of sequences within the 292-base-pair *Eco*RI-*Sal*I fragment, which are required to efficiently stimulate 35S rRNA synthesis, could be mapped in vivo by using *prib*1 plasmids carrying a series of deletion mutations extending from the *Eco*RI site and a series of deletion mutations extending from the *Sal*I site, respectively. Many of the deletion mutants as well as the internal deletions from positions −11 to +21 and from positions −15 to +12 were as previously described for mapping sequences required for RNA polymerase I-dependent in vitro transcription from this fragment of rDNA spacer DNA (33). Additional mutants reported here were recovered from the *prib*105R-based collection, and their nucleotide sequences were determined by the dideoxy sequencing method (30).

Analysis of expression of the 35S rRNA minigene in *prib*1. Expression of the 35S rRNA minigene was monitored by Northern (RNA) blotting with a single-stranded RNA probe complementary to the pBR322 reporter sequences within the minigene. Yeast transformants carrying *prib*1 and its derivatives were grown in 10-ml cultures and harvested in the early log phase (A_{660}, 0.5 to 1.0). Cells were collected by centrifugation and suspended in 0.5 ml of NNES buffer (50 mM sodium acetate [pH 5.0], 100 mM NaCl, 10 mM EDTA, and 0.5% sodium dodecyl sulfate) plus 0.5 ml of NNES-buffered phenol at 60°C. Cells were disrupted with glass beads by four 30-s cycles of vortexing and heating at 60°C. Hot NNES buffer and phenol (60°C, 2.5 ml each) were added and vortexed. The extract was cooled rapidly in a dry ice-acetone bath and centrifuged at 2,000 rpm for 15 min at 4°C in a Sorvall SS34 rotor. The aqueous phase was extracted with an equal volume of NNES-buffered phenol, extracted with ether to remove residual phenol, adjusted to 0.3 M NaCl, and precipitated with 2 volumes of 95% ethanol at −20°C.

Samples of 5 μg of total cellular RNA were electrophoresed in 1% agarose gels in the presence of formaldehyde as previously described (32). RNA was transferred to Nytran membranes (Schleicher & Schuell & Co.) according to the protocol of the manufacturer.

Two single-stranded RNA probes were used for Northern blotting analysis. One was generated by SP6 RNA polymerase-dependent transcription of a pSP65 vector (Promega Biotec) carrying the 346-base-pair *Hin*dIII-*Bam*HI fragment from pBR322 in the presence of [α-^{32}P]CTP. This probe hybridized to transcripts derived from the pBR322 reporter sequences in the 35S rRNA minigene. The second probe was generated by transcription of a pSP65 vector carrying a 536-base-pair *Hin*dIII fragment from the coding region of the yeast enolase gene *ENO2* (12). This latter probe hybridized with mRNA synthesized from the two yeast enolase genes and served as an internal control. Hybridization reactions (10 ml at 55°C) contained 50 mM sodium phosphate (pH 6.5), 0.8 M NaCl, 1 mM EDTA, 0.05% polyvinylpyrrolidone, 250 mg of depurinated salmon sperm DNA per ml, 500 μg of total yeast cellular RNA per ml, 50% deionized formamide, and a 13:1 ratio of pBR322-specific probe (1×10^7 cpm) to enolase-specific probe (8×10^5 cpm). After autoradiography, the relative proportion of enolase mRNA to 35S rRNA minigene transcript was determined with a soft laser densitometer. Total RNA isolated from three to six independent transformants was analyzed for each mutant derivative of *prib*1.

Transcripts synthesized from *prib*1 sequences located immediately upstream and downstream from the 35S rRNA minigene were detected by Northern blotting with probes generated by transcription of pSP65 and pSP64 vectors carrying the 746-base-pair *Pst*I-*Eco*RI fragment from pBR322 and the 276-base-pair *Bam*HI-*Sal*I fragment from pBR322, respectively.

Mapping of the 5′ terminus of 35S rRNA minigene transcripts. Total yeast RNA was isolated from a 1-liter culture

of a transformant carrying *prib*1 as previously described (32). Total RNA was fractionated by sucrose density gradient centrifugation (32), and fractions enriched in the 35S rRNA minigene transcript were pooled and ethanol precipitated. The 5′ terminus of the minigene transcript was mapped by primer extension by using a primer (5′ d[GCAATTTAACTGTGAT]) that is complementary to pBR322 sequences in the minigene transcript. Samples (10 pmol) of the primer, labeled with [γ-^{32}P]ATP and polynucleotide kinase (6.5×10^6 cpm/pmol), were hybridized with 40 μg of RNA enriched from the minigene transcript in a 14-μl reaction containing 10 mM Tris (pH 7.4), 160 mM NaCl, and 10 mM $MgCl_2$. Annealing was for 5 min at 67°C followed by 25 min at 42°C. The primer extension reaction (30 μl) contained the primer-RNA duplex in a reaction containing 100 mM Tris (pH 8.3), 75 mM KCl, 15 mM $MgCl_2$, 10 mM dithiothreitol, 800 μM each dATP, dGTP, dCTP, and dTTP, 45 μM actinomycin D, and 2.5 U of avian myeloblastosis virus reverse transcriptase. The reaction was performed for 30 min at 42°C and terminated by the addition of 5 μl of 50 mM EDTA and incubation at 70°C for 10 min.

The primer extension product was analyzed on an 8% polyacrylamide DNA sequencing gel alongside a dideoxy sequencing reaction generated with the same 16-base primer and an M13 clone containing the *Sal*I-*Bam*HI fragment from *prib*1.

In vitro transcription of *prib*1 and mutant derivatives of *prib*1. Plasmid templates were limit digested with *Bam*HI and then transcribed with a yeast whole-cell extract as previously described (32). Transcripts were analyzed by autoradiography after electrophoresis on 3% polyacrylamide gel as previously described (33).

RESULTS

Construction and characterization of plasmids carrying a yeast 35S rRNA minigene. To study yeast rDNA spacer sequences involved in stimulating 35S rRNA synthesis in vivo, a plasmid designated *prib*1 (Fig. 1) was constructed. This plasmid contains a 35S rRNA minigene composed of a pBR322 reporter sequence flanked by yeast rDNA sequences that encode the 5′ and 3′ termini of 35S rRNA. Results from other laboratories have established that these latter rDNA sequences are sufficient to direct synthesis of a 35S rRNA fusion transcript with the correct 35S rRNA 5′ and 3′ termini in vivo (5, 6, 14). Located immediately upstream from sequences that encode the 5′ terminus of 35S rRNA in *prib*1 is a segment of yeast rDNA spacer sequence (Fig. 1). This fragment of spacer rDNA includes sequences that support RNA polymerase I-dependent selective transcription in vitro (32, 33) and sequences that are required to stimulate 35S rRNA fusion transcript synthesis in vivo (5, 15, 28). Plasmid *prib*1 also carries the centromere region from chromosome 11 (*CEN11*), the autonomous replication sequences near *TRP1*, and a selectable *URA3* gene (Fig. 1B).

We previously identified four alleles of yeast rDNA that differ in nucleotide sequence within the region of spacer DNA contained in *prib*1 (33). These alleles could also be distinguished on the basis of their transcriptional properties. Three alleles, designated type I, supported efficient selective transcription in vitro, whereas the fourth, designated type II, was transcribed at less than 10% the efficiency observed with type I alleles (33). To test the ability of different alleles to stimulate 35S rRNA synthesis in vivo, spacer rDNA sequences extending from an *Eco*RI site to an *Hpa*I site (Fig. 1) from a type I allele (*prib*105 [33]) and a type II allele (*prib*20/DFY15 [33]) were ligated between the unique *Eco*RI and *Sal*I sites in *prib*1. In each case, the spacer sequences were in the correct orientation relative the 35S rDNA minigene. By utilizing a single-stranded RNA probe that is complementary to the pBR322 reporter sequences in the minigene, the minigene-encoded 35S fusion transcripts were measured relative to endogenous enolase mRNA by Northern blotting analysis of total cellular RNA isolated from transformants carrying the *prib*1 derivatives. Plasmids containing type I or II spacer sequences directed synthesis of similar amounts of a transcript of the expected size for the 35S fusion transcript (Fig. 2A). Furthermore, the amount of 35S fusion transcript synthesized from the minigenes containing type I or II spacer sequences was 17-fold higher than observed for a minigene in which spacer rDNA sequences extending from the *Eco*RI site to the *Hin*dIII site (deletion

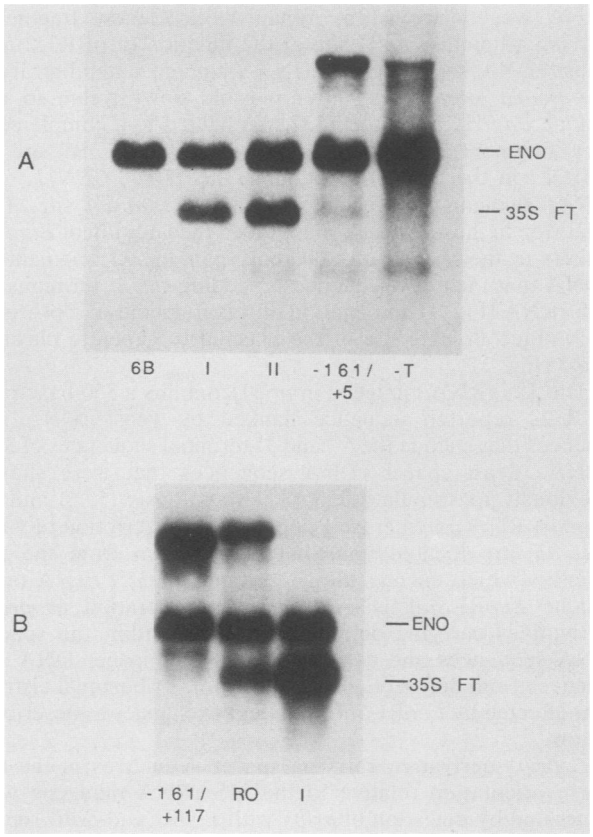

FIG. 2. Northern blotting analysis of total cellular RNA isolated from strains carrying derivatives of *prib*1. The position of a 35S rRNA fusion transcript (35S FT) detected with a hybridization probe corresponding to the pBR322 reporter sequences within the 35S rRNA minigene is indicated, as is the position of enolase mRNA (ENO), which served as an internal control. (A) Lanes correspond to total cellular RNA isolated from strains carrying the following plasmids: 6B, the recipient yeast strain without a plasmid; I, *prib*1 containing spacer sequences from a type I rRNA cistron; II, *prib*1 containing spacer sequences from a type II rRNA cistron; −161/+5, *prib*1 containing a deletion of spacer sequences extending from positions −161 to +5; −T, *prib*1 containing a deletion of rDNA sequences than encode the 3′ terminus of 35S rRNA. (B) Lanes correspond to total cellular RNA isolated from strains carrying the following plasmids: −161/+117, *prib*1 containing a deletion of spacer sequences extending from positions −161 to +117; RO, *prib*1 containing type I spacer sequences in the reverse orientation; I, *prib*1 containing type I spacer sequences in the natural orientation.

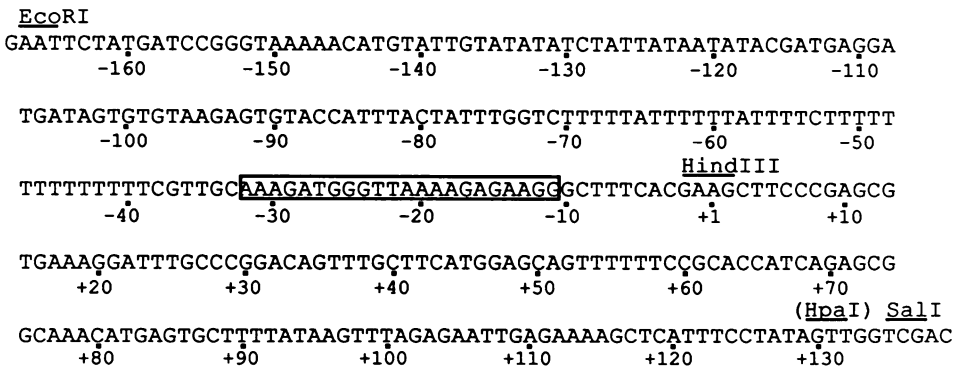

FIG. 3. Nucleotide sequence of the spacer region of *prib*1 from a type I rRNA cistron. The sequence is numbered relative to the *Hin*dIII cleavage site at position +1. Sequences shown previously (33) to be sufficient for RNA polymerase I-dependent selective initiation of transcription in vitro (the spacer promoter) are indicated in the boxed region of the sequence.

from positions −161 to +5) were deleted. Elion and Warner (5) showed that these latter sequences are required to stimulate 35S rRNA transcription in vivo. The level of stimulation of 35S fusion transcript synthesis by spacer sequences in *prib*1 was similar to that reported by Elion and Warner (5, 6) for their minigene constructs.

Synthesis of a discrete 35S fusion transcript depended on the presence of rDNA sequences that encode the 3′ terminus of 35S rRNA. Deletion of these latter sequences from *prib*1 resulted in the synthesis of transcripts that are larger than the 35S fusion transcript synthesized from the complete minigene (Fig. 2A). Reversing the orientation of type I spacer rDNA sequences relative to the 35S rRNA minigene in *prib*1 caused a fivefold reduction in stimulation of fusion 35S transcript synthesis (Fig. 2B).

For the deletion mapping studies reported below, *prib*1 containing type I spacer sequences derived from *prib*105 (33) was employed. The nucleotide sequence of the spacer region extending from *Eco*RI to *Hpa*I in this allele was previously determined (33) and is shown in Fig. 3. A *Sal*I linker was inserted into the *Hpa*I site to generate *prib*1 containing unique *Eco*RI and *Sal*I sites flanking their spacer sequences (Fig. 1).

The 5′ terminus of the 35S fusion transcript was mapped to verify that it is the same as that published for 35S rRNA (1, 17, 19). Total cellular RNA was isolated from a strain carrying *prib*1 and fractionated by sucrose density gradient centrifugation. RNA present in fractions enriched for the 35S fusion transcript was used as a template for avian myeloblastosis virus reverse transcriptase in the presence of a primer that is complementary to pBR322 sequences in the fusion transcript. A single primer extension product of the correct molecular weight was observed (Fig. 4). RNA isolated from a strain lacking *prib*1 did not serve as a template for this primer extension product (Fig. 4). These data confirmed that the minigene in *prib*1 directed synthesis of a transcript with the same 5′ terminus as native 35S rRNA.

Mapping of the 5′ boundary of rDNA spacer sequences that stimulate 35S rRNA synthesis. Deletion mutations extending from the *Eco*RI site in *prib*1 toward the *Sal*I site were generated as described in Materials and Methods. The endpoints of the deletion mutations relative to the *Hin*dIII site at position +1 were determined by DNA sequencing (Fig. 5). Plasmid *prib*1 derivatives containing each of the deletion mutations were cleaved with *Bam*HI and transcribed by using a yeast-whole extract as described previously (32). Plasmid templates containing deletions extending from the *Eco*RI site up to position −32 directed the synthesis of a transcript initiated at the spacer promoter and truncated at the *Bam*HI cleavage site (Fig. 6A). Deletions extending to position +5 and beyond did not direct synthesis of this

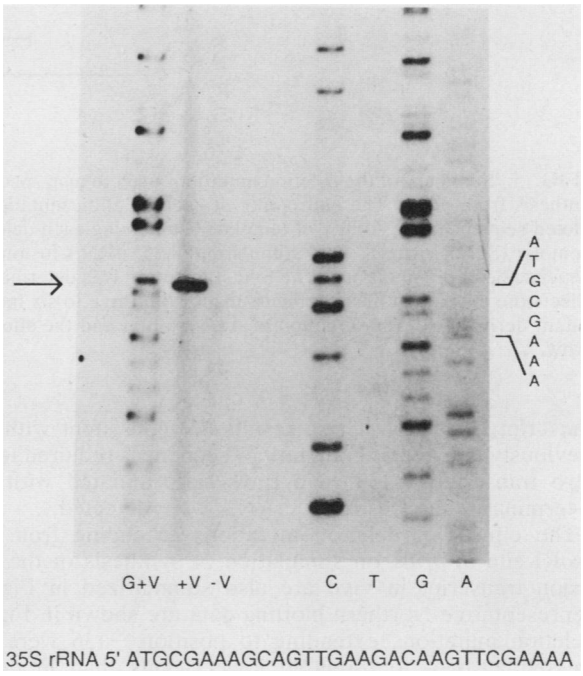

FIG. 4. Mapping of the 5′ terminus of the 35S rRNA fusion transcript synthesized from the minigene in *prib*1. the 35S fusion transcript was partially purified by sucrose density gradient centrifugation and used as a template for primer extension with avian myeloblastosis virus reverse transcriptase and an oligonucleotide primer complementary to pBR322 sequences in the 35S rRNA minigene as described in Materials and Methods. Lanes contained RNA isolated from a strain containing *prib*1 (+V) and RNA isolated from the recipient yeast strain before transformation with *prib*1 (−V). The arrow indicates the position of the primer extension product. The 5′ terminus of the primer extension product was determined relative to a set of dideoxy sequencing reactions performed with the 35S rRNA minigene from *prib*1 and the same oligonucleotide primer used for primer extension of the 35S rRNA fusion transcript. The primer extension product was mixed with the dideoxy G sequencing reaction (G+V) to confirm the 5′ terminus of the 35S rRNA fusion transcript. The 5′ terminus of the primer extension product corresponds exactly to the 5′ terminus of 35S rRNA shown below the autoradiogram.

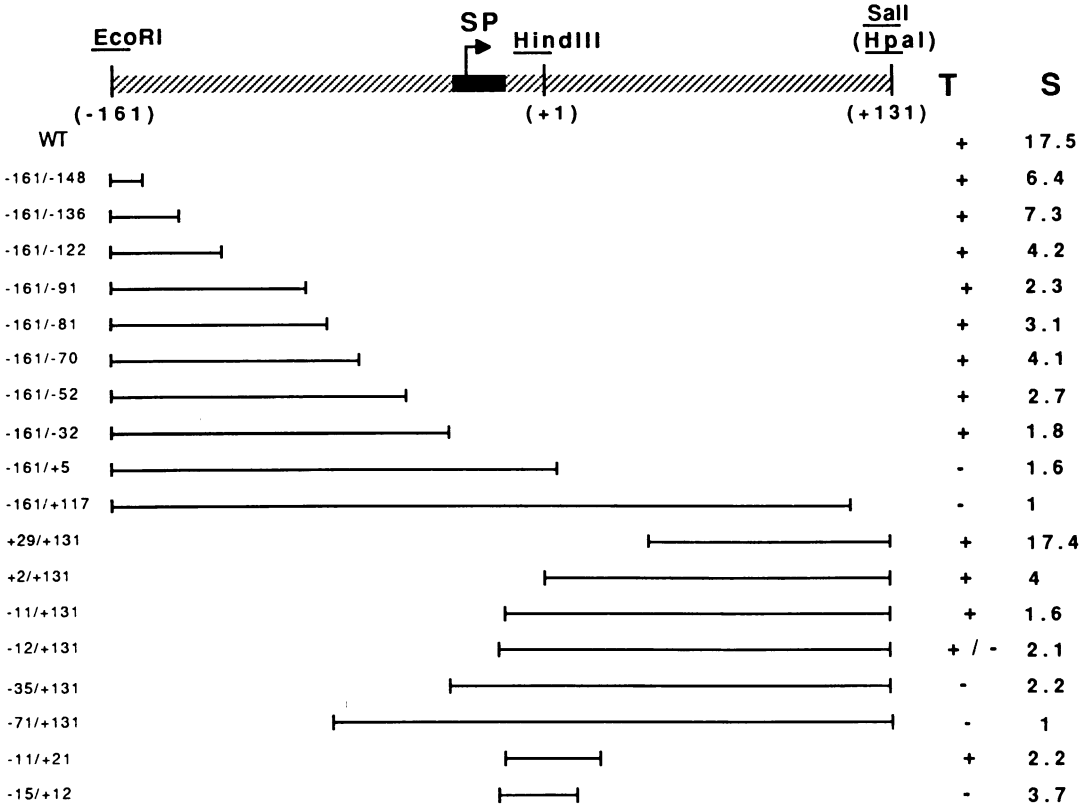

FIG. 5. Summary of the deletion mutations used to map spacer sequences that are required for efficient stimulation of 35S fusion transcript synthesis from *prib*1. The end points of each deletion mutation, determined by DNA sequencing, are indicated. The bars correspond to deleted sequences. T, Ability of templates containing each deletion mutation to support selective initiation of transcription from the spacer promoter (SP) in vitro. S, Fold stimulation of 35S rRNA fusion transcript from the minigene in *prib*1 plasmids containing each of the spacer sequence deletion mutations. The fold stimulation was determined by Northern blotting analysis as described in Materials and Methods and reflects the average of measurements made with three to six independent transformants obtained after transformation with each of the *prib*1 mutant derivatives. The direction of transcription and the site of initiation of transcription from the spacer promoter are indicated by the arrow.

transcript (Fig. 6A). These results are consistent with the previously mapped 5′ boundary of sequences required for in vitro transcription (33). No transcripts initiated with the 5′-terminal sequences of 35S rRNA were detected.

The effects of deletion mutations extending from the *Eco*RI site in *prib*1 on stimulation of synthesis of the 35S fusion transcript in vivo are also summarized in Fig. 5. Representative Northern blotting data are shown in Fig. 7. Deletion mutations extending to position −136 were expressed at two- to threefold lower levels than in *prib*1. Synthesis of the 35S fusion transcript from plasmids carrying a deletion mutation extending to position −122 and beyond was further reduced. Stimulation of 35S fusion transcript synthesis was almost completely abolished when the deletion mutation extended to position −32 and beyond. These observations suggested that virtually all of the spacer rDNA sequences extending downstream from the *Eco*RI site in *prib*1 are required for maximal stimulation of 35S fusion transcript synthesis in vivo.

Interestingly, plasmids carrying deletion mutations extending from the *Eco*RI site in *prib*1 to position −122 and beyond directed the synthesis of a large amount of a high-molecular-weight (HMW) transcript (3.2 kb) not observed with *prib*1 (Fig. 7). Plasmids carrying deletion mutations extending to positions −148 and −136 directed the synthesis of smaller amounts of the HMW transcript. The plasmid derivative containing the reverse orientation of the rDNA spacer sequence also directed synthesis of the HMW transcript (Fig. 2B). To further characterize the HMW transcript, Northern blotting analyses were performed with a hybridization probe complementary to pBR322 sequences within the 35S rRNA minigene as well as probes complementary to pBR322 sequences in *prib*1 located immediately upstream and downstream from the 35S rRNA minigene. The HMW transcript hybridized with the 35S rRNA minigene reporter probe (Fig. 8A) and with a probe complementary to pBR322 sequences extending from the *Eco*RI site in *prib*1 upstream to the *Pst*I site (Fig. 8B). The HMW transcript did not hybridize with a probe complementary to pBR322 sequences located immediately downstream from the 35S rRNA minigene (Fig. 8C). These observations suggested that the HMW transcript was generated by readthrough of a transcript initiated upstream from the rDNA spacer sequences in *prib*1 and that rDNA spacer sequences immediately downstream from the *Eco*RI site in *prib*1 were required to terminate this transcript in an orientation-dependent manner. This conclusion was further supported by the observation that the steady-state level of a 1.75-kb transcript that hybridized to the probe complementary to pBR322 sequences located upstream from the 35S rRNA minigene in *prib*1 was dramatically reduced commensurate with the appearance of the HMW transcript (3.2 kb) (Fig. 8B). The difference in the apparent size of these transcripts was consistent with readthrough of the 1.75-kb

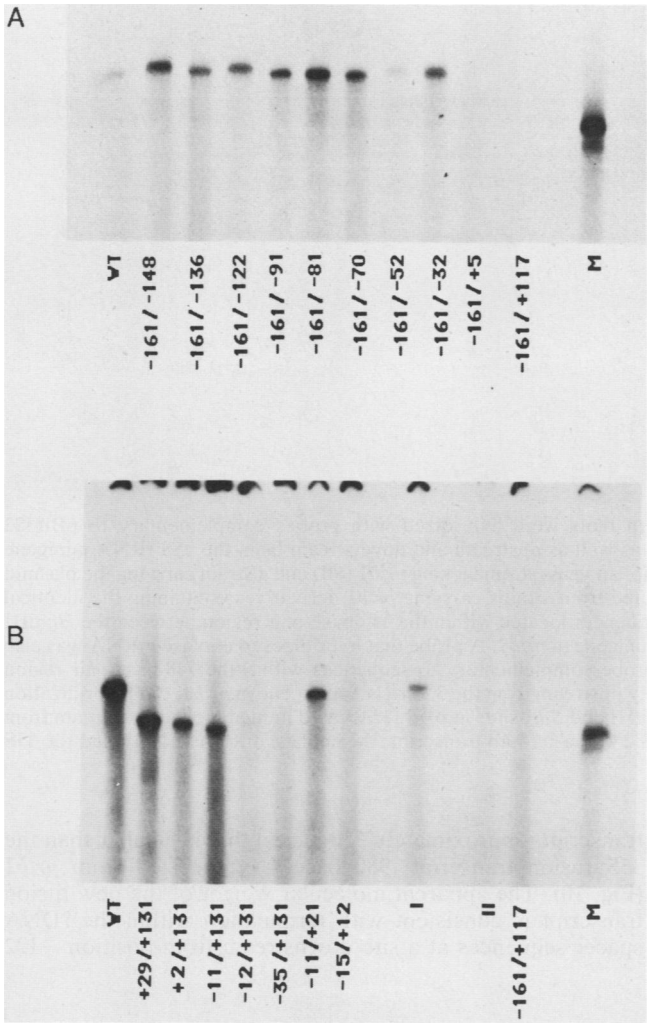

FIG. 6. In vitro transcription assays of *prib*1 and its derivatives. Plasmid DNAs were limit digested with *Bam*HI and transcribed with a yeast whole-cell extract as described in Materials and Methods. (A) Plasmid *prib*1 (WT) and a series of *prib*1 derivatives containing the indicated deletion mutations extending from the *Eco*RI site at position −161. (B) plasmid *prib*1 (WT) and *prib*1 derivatives containing the indicated deletion mutations. Plasmid *prib*1 containing spacer sequences from a type I rRNA cistron (I) and a type II rRNA cistron (II) are also shown. M indicates lanes containing marker transcripts.

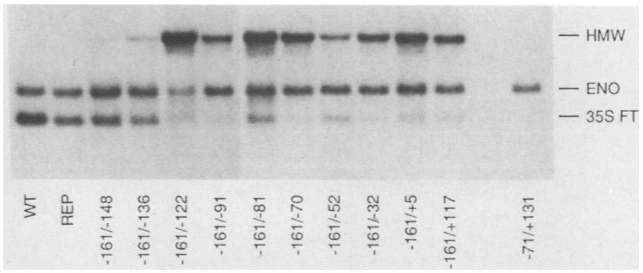

FIG. 7. Mapping of the 5′ boundary of spacer sequences in *prib*1 required for efficient stimulation of 35S rRNA fusion transcript synthesis. Northern blots were hybridized with a probe complementary to the pBR322 reporter sequences within the minigene in *prib*1, and a probe that hybridizes to enolase mRNA which served as the internal control. The positions of the 35S rRNA fusion transcript (35S FT) and a HMW readthrough transcript detected with the pBR322 reporter sequence probe are indicated as is the position of enolase mRNA (ENO). The lanes correspond to total cellular RNA isolated from strains carrying the following *prib*1 derivatives: WT, *prib*1 containing no deletion mutation; REP, *prib*1 in which the unique *Eco*RI site at the junction of pBR322 vector sequences and rDNA spacer sequences was repaired with the Klenow fragment of *Escherichia coli* DNA polymerase I. The remaining lanes correspond to total cellular RNA isolated from strains carrying *prib*1 derivatives containing the indicated deletion mutations.

transcript to a site at or near the 3′ end of the 35S rRNA minigene. The significance of these latter observations for spacer rDNA-dependent stimulation of 35S rRNA synthesis is described below.

Mapping of the 3′ boundary of rDNA spacer sequences that stimulate 35S rRNA synthesis. To map the 3′ boundary of sequences that stimulate 35S rRNA synthesis, a series of deletion mutations extending from the *Sal*I site in *prib*1 toward the *Eco*RI site was generated as described in Materials and Methods. The endpoints of these deletion mutations are summarized in Fig. 5. Plasmids carrying each deletion mutation were cleaved with *Bam*HI and tested for their ability to support selective initiation of transcription in vitro. Plasmids carrying deletion mutations extending from the *Sal*I site at position +131 up to position −11 directed the synthesis of discrete transcripts of the expected size (Fig. 6B). The template efficiency of a plasmid carrying a deletion mutation extending to position −12 was reduced to 10% of that observed for the plasmid carrying a deletion extending to position −11. No detectable transcripts were synthesized from plasmids carrying deletion mutations extending beyond position −12. These results are in agreement with the previously mapped 3′ boundary of sequences required for in vitro transcription (33). Plasmid *prib*1 containing spacer rDNA sequences from a type II allele did not direct the synthesis of a detectable transcript in vitro (Fig. 6B).

The effects of deletion mutations extending from the *Sal*I site in *prib*1 toward the *Eco*RI site on stimulation of synthesis of the 35S fusion transcript in vivo are summarized in Fig. 5. Representative Northern blotting data for each of the *prib*1 mutants are shown in Fig. 9. Synthesis of the 35S fusion transcript was stimulated 17-fold from a *prib*1 mutant carrying a deletion mutation extending from position +131 to position +29. Synthesis of the 35S fusion transcript was stimulated fourfold from a mutant plasmid carrying a deletion mutation extending to position +2, whereas stimulation was twofold or less when the deletion mutations extended to position −11 and beyond. These observations positioned the 3′ boundary of spacer rDNA sequences required to stimulate 35S fusion transcript synthesis at or near the *Hin*dIII site at position +1. Increases in the steady-state levels of the HMW transcript observed with *prib*1 plasmids carrying deletion mutations extending from the *Eco*RI site (Fig. 7) were not observed with *prib*1 plasmids carrying deletions extending from the *Sal*I site at position +131 up to position −71 (Fig. 7). These observations suggest that rDNA spacer sequences required for termination of a readthrough transcript that gives rise to the HMW transcript reside within a 77-base-pair region between positions −148 and −71.

Sequences within the spacer region of rDNA modulate termination of transcripts initiated by RNA polymerase I. The results described above suggested that spacer rDNA sequences between positions −148 and −71 in *prib*1 that were required for efficient stimulation of 35S fusion transcript synthesis also modulated transcription termination of a transcript that gave rise to the HMW transcript. The nuclear

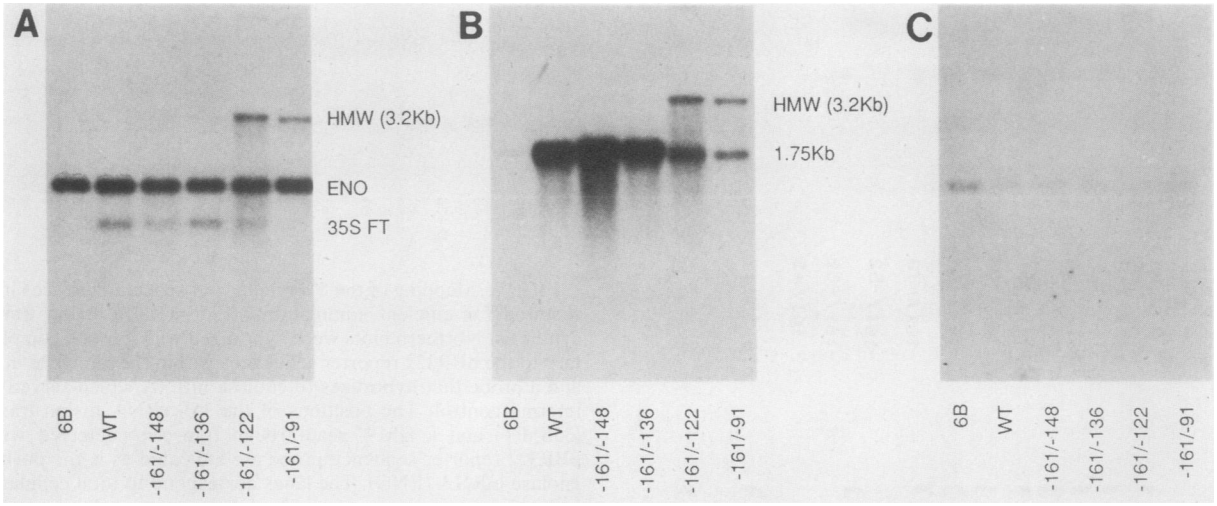

FIG. 8. Characterization of a HMW transcript from *prib*1. Northern blots were hybridized with probes complementary to pBR322 sequences located within the reporter region of the 35S rRNA minigene as well as upstream and downstream from the 35S rRNA minigene in *prib*1. The lanes correspond to total cellular RNA isolated from the recipient yeast strain lacking *prib*1 (6B) and a strain carrying the plasmid *prib*1 (WT). The remaining lanes correspond to total cellular RNA isolated from strains carrying *prib*1 derivatives containing the identical deletion mutations. (A) Hybridization with a probe complementary to sequences located within the 346-base-pair region between the *Hin*dIII and *Bam*HI sites of pBR322, which are contained within the 35S rRNA minigene in *prib*1. A probe that hybridizes to enolase mRNA was also included and served as an internal control. (B) Hybridization with a probe complementary to sequences within the 748-base-pair region between the *Pst*I and *Eco*RI sites in pBR322 which are located immediately upstream from the 35S rRNA minigene in *prib*1. (C) Hybridization with a probe complementary to the 276-base-pair region between the *Bam*HI and *Sal*I sites in pBR322 located immediately downstream from the 35S rRNA minigene in *prib*1. The positions of the HMW transcript (3.2 kb), a 1.75-kb transcript, the enolase mRNA (ENO), and the 35S fusion transcript (35S FT) are indicated.

RNA polymerase responsible for HMW transcript synthesis was not identified. To determine whether the spacer rDNA sequences were capable of directing termination of a transcript initiated by RNA polymerase I, the *Bam*HI fragment containing sequences encoding the 3′ terminus of 35S rRNA in *prib*1 (Fig. 1B) was replaced with a second copy of the rDNA spacer sequences in *prib*1 extending from the *Eco*RI site (position −161) to the *Sal*I site (position +131). These latter rDNA spacer sequences were inserted in the same orientation as the rDNA spacer sequences upstream from the 35S fusion transcript initiation site. The resulting plasmid carrying two insertions of the rDNA spacer sequences was designated *prib*2. *prib*2 directed the synthesis of a new fusion transcript (approximately 550 bases) that is smaller than the 35S fusion transcript (980 bases) synthesized from *prib*1 (Fig. 10). The apparent molecular weight of the new fusion transcript is consistent with termination within the rDNA spacer sequences at a site downstream from position −122

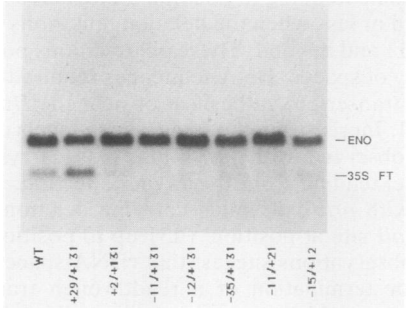

FIG. 9. Mapping of the 3′ boundary of spacer sequences in *prib*1 required for efficient stimulation of 35S rRNA fusion transcript synthesis. Northern blots were hybridized with a probe complementary to the pBR322 reporter sequences within the minigene in *prib*1 and a probe that hybridizes to enolase mRNA and served as the internal control. The positions of the 35S rRNA fusion transcript (35S FT) and enolase mRNA (ENO) are indicated. The lanes correspond to total cellular RNA isolated from *prib*1 (WT) and *prib*1 derivatives containing the indicated deletion mutations.

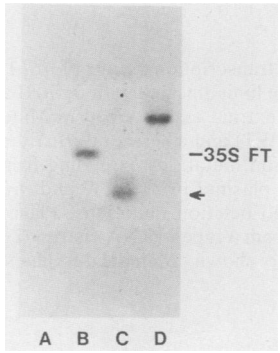

FIG. 10. Termination of transcripts initiated by RNA polymerase I at the 35S rRNA promoter by sequences within the *Eco*RI-*Sal*I fragment of yeast rDNA. Plasmid *prib*2 was generated from plasmid *prib*1 by replacement of the *Bam*HI fragment of rDNA containing sequences that encode the 3′ terminus of 35S rRNA in *prib*1 with a second copy of spacer sequences contained on the *Eco*RI-*Sal*I fragment of yeast rDNA as described in Materials and Methods. The Northern blot was hybridized with a probe complementary to pBR322 sequences within the minigenes in *prib*1 and *prib*2. Total cellular RNA was isolated from strains carrying the following plasmids: A, recipient yeast strain without a plasmid; B, plasmid *prib*1; C, plasmid *prib*2; D, plasmid *prib*2 containing a deletion mutation extending from positions −161 to −122 within the second copy of yeast rDNA spacer sequences. The arrow indicates the position of a 550-base transcript synthesized from plasmid *prib*2. The position of the 980-base transcript synthesized from plasmid *prib*1 (35S FT) is also indicated.

and upstream from sequences required for RNA polymerase I-dependent promoter activity in vivo. A *prib*2 plasmid carrying a deletion of sequences between positions −161 and −122 within the rDNA spacer sequences located downstream from the 35S fusion transcript initiation site did not direct the synthesis of the new fusion transcript (Fig. 10). Instead, a prominent transcript, terminated or processed within vector sequences beyond the rDNA spacer, was observed. These results suggested that the same sequences that terminated a readthrough transcript from *prib*1 that gave rise to the HMW transcript (Fig. 7) terminated a 35S fusion transcript initiated by RNA polymerase I in *prib*2.

DISCUSSION

Utilizing a centromere-containing plasmid carrying a 35S rRNA minigene, nucleotide sequences within the spacer region of yeast ribosomal cistrons that are required for efficient stimulation of 35S rRNA synthesis in vivo were identified by deletion mapping analysis. Elion and Warner (5, 6) previously reported that a fragment of spacer rDNA bounded by *Eco*RI and *Hin*dIII sites located 2.2 kb upstream from sequences that encode the 5′ terminus of 35S rRNA is sufficient for 10- to 20-fold stimulation of synthesis of 35S rRNA transcript in vivo. The deletion mapping analysis reported here showed that virtually the entire 160-base-pair *Eco*RI-*Hin*dIII fragment was required to maximally stimulate 35S rRNA minigene transcript synthesis in vivo. We previously showed (33) that yeast strains contain multiple ribosomal cistron alleles that differ in nucleotide sequence within the *Eco*RI-*Hin*dIII fragment required to stimulate 35S rRNA synthesis. These alleles were further distinguished on the basis of their ability to support efficient RNA polymerase I-dependent initiation of transcription in vitro. Type I cistrons served as templates for efficient RNA polymerase I-dependent transcription, whereas the template activity of type II cistrons was less than 10% that observed with type I cistrons (33). This difference in template activity was attributed to a single-base-pair difference between these alleles within the 22-base-pair spacer promoter (33). We show here that sequences that include the *Eco*RI-*Hin*dIII fragment from both type I and II cistrons stimulated 35S rRNA synthesis in vivo at the same level (17-fold). The nucleotide sequence of the type II allele tested here is identical to that reported by Elion and Warner (5, 6) and Swanson et al. (33). All of the deletion mapping studies in this report were performed with a type I allele.

Two activities that are likely to be relevant to the mechanism of spacer rRNA-dependent stimulation of 35S rRNA synthesis have been mapped within the 160-base-pair *Eco*RI-*Hin*dIII fragment. Nucleotide sequences located immediately downstream from the *Eco*RI site in *prib*1 were first identified on the basis of their ability to terminate readthrough transcripts initiated upstream from the *Eco*RI site. Subsequent experiments showed that these latter rDNA sequences support termination of transcripts initiated in vivo by RNA polymerase I at the 35S rRNA promoter. Removal of all or a portion of the sequences required for terminator activity in *prib*1 led to a three- to fivefold reduction in stimulation of 35S fusion transcript synthesis but did not abolish stimulation. These results suggest that sequences within the *Eco*RI-*Hin*dIII fragment that modulate transcription terminator activity are partially responsible for the stimulation of 35S fusion transcript synthesis from *prib*1.

Sequences that are required for RNA polymerase I-dependent initiation of transcription in vitro were previously mapped between positions −32 and −11 (33) within the *Eco*RI-*Hin*dIII fragment required for stimulation of 35S rRNA synthesis in vivo. Reexamination of the template activities in vitro of *prib*1 deviatives carrying specific deletion mutations revealed the same 22-base-pair spacer promoter element defined previously. Transcripts initiated from the spacer promoter in *prib*1 read through the 35S rRNA promoter region, and no transcripts initiated from the 35S rRNA promoter were detected in vitro. Deletion of *prib*1 sequences extending from the *Eco*RI site at position −161 to position −32 and beyond as well as deletion of sequences from the *Sal*I site at positions +131 to −11 and beyond resulted in almost complete loss of stimulation of 35S fusion transcript by spacer sequences. These results suggest that sequences within the *Eco*RI-*Hin*dIII fragment that include those required from RNA polymerase I-dependent initiation of transcription in vitro play a prominant role in stimulating 35S fusion transcript synthesis from *prib*1 in vivo.

The presence of RNA polymerase I-dependent promoters within the spacer region of ribosomal cistrons is not unique to *Saccharomyces* species. In species of *Drosophila* (20) and *Xenopus* (25) and in rats (2), functional promoter elements are present in rDNA spacer sequences. In *Xenopus laevis* (3) these promoter elements play a role in stimulating synthesis of the major cellular rRNA precursor in vivo. Although the mechanism underlying spacer-promoter-dependent stimulation of rRNA precursor synthesis is not well understood, it has been suggested that spacer-promoter elements are involved in sequestering RNA polymerase I and/or transcription factors for delivery to the major gene promoter (3, 25, 31).

Transcription terminators are also present within the spacer region of ribosomal cistrons from several species (4, 7, 8, 10, 16, 22, 24, 25, 34). In mice (7, 10) and *Xenopus* species (4, 24, 25), transcription terminators located immediately upstream from each respective gene promoter are required for maximal transcription from the gene promoters in vivo. It has been suggested that these latter terminators function to terminate transcriptions initiated from spacer promoters or readthrough transcriptions from the preceeding cistron in the tandem array in such a way as to permit reinitiation at the gene promoter without disassociation from the template (3, 4, 7, 10, 24, 25). This readthrough enhancement mechanism, first proposed by Tom Moss (25), and may play an important role in maintaining highly efficient synthesis of rRNA in eucaryotic cells.

The organization of sequences within the *Eco*RI-*Hin*dIII fragment of yeast rDNA that mediate terminator and promoter activities is very similar to the gene promoters from mice (7, 10) and *Xenopus* species (4, 24, 25). These observations suggest that the mechanism of spacer-sequence-dependent stimulation of 35S rRNA synthesis in yeast cells is likely to be similar to the readthrough enhancement mechanism proposed for mice (7, 10) and *Xenopus* species (3, 4, 24, 25). It is unlikely that sequences within the *Eco*RI-*Hin*dIII spacer rDNA fragment represent the gene promoter, since the 35S rRNA precursor appears to possess a 5′ triphosphate indicative of a transcriptional initiation event (18, 26) and since 35S fusion transcript was synthesized from *prib*1 derivatives that lack the *Eco*RI-*Hin*dIII fragment. A more attractive hypothesis is that sequences within the *Eco*RI-*Hin*dIII fragment mediate termination and reinitiation of readthrough RNA polymerase I and perhaps de novo initiation of transcription by RNA polymerase I. These events would, in turn, increase the number of initiation events at the 35S rRNA downstream from the spacer

sequences. Alternatively, the termination sequences may function to prevent occlusion of the spacer promoter and/or a binding site for an important transcription factor. An essential feature of these hypotheses is that these spacer sequences contain a binding site for a transcription factor that is required for efficient reinitiation at the 35S rRNA promoter.

The data presented in this report show that sequences within the *Eco*RI-*Hin*dIII spacer fragment that modulate termination of readthrough transcripts as well as transcripts initiated from the 35S rRNA promoter are required for maximal stimulation of 35S fusion transcription synthesis from *prib*1. It is now clear that the 3′ terminus of mature 35S rRNA is generated by RNA processing rather than by transcription termination. Kempers-Veenstra et al. (14) showed that the 3′ terminus of mature 35SrRNA fusion transcripts synthesized from a 35S rRNA minigene extends 15 to 45 bases beyond the mature 35S rRNA 3′ terminus in strains carrying a mutation impaired for 3′ processing of 5S rRNA. They further showed that deletion of sequences encoding the 3′ terminus of 35S rRNA in the minigene resulted in the accumulation of 35S rRNA fusion transcripts extending 210 bases beyond the mature 35S rRNA 3′ terminus (14). The 3′ terminus of these latter transcripts is located downstream from sequences within the *Eco*RI-*Hin*dIII spacer fragment shown in this report to be required for terminator function and upstream from sequences required for in vitro promoter activity. This site is in agreement with the predicted 3′ terminus of 35S fusion transcriptions synthesized from *prib*2 containing the *Eco*RI-*Hin*dIII spacer sequences at the 3′ end of the 35S rRNA minigene. We have recently demonstrated that SP6 transcripts containing sequences that encode the 3′ terminus of 35S rRNA are processed in vitro by yeast extracts to generate the mature 35S rRNA 3′ terminus (Yip and Holland, unpublished results). Taken together, the data suggest that sequences within the *Eco*RI-*Hin*dIII fragment are responsible for termination of transcription by RNA polymerase I; however, they do not unequivocally rule out RNA processing followed by rapid degradation of the readthrough transcript.

It is clear that spacer sequences that are required to stimulate 35S rRNA synthesis in vivo are significantly larger than those required for spacer-promoter function in vitro. Differences in the dimensions of sequences required for RNA polymerase I promoter function in vivo and in vitro has been observed in other eucaryotic cells (31). In mice, humans, and *Xenopus* species, rDNA sequences extending approximately from positions −40 to +5 relative to the transcription initiation site are sufficient for RNA polymerase I-dependent selective initiation of transcription in vitro; however, sequences extending approximately from positions −160 to +5 are required for efficient initiation of transcription in vivo (31). It has been proposed that the sequences required for selective initiation of transcription in vitro represent an essential core promoter element that is separated from upstream sequences that are important for efficient initiation of transcription in vivo (31). It is possible that the 22-base-pair sequence within the *Eco*RI-*Hin*dIII spacer fragment represents a core promoter element that is sufficient for selective initiative of transcription in vitro but is also required for maximal stimulation of 35S rRNA synthesis in vivo.

Thus far we have not been able to detect transcripts initiated from the spacer promoter in vivo. S1 nuclease mapping experiments showed that most if not all of the spacer region of yeast rDNA cistrons is transcribed (32). However, because of the extremely low steady-state concentration of these transcripts and heterogeneity in the nucleotide sequence of the spacer regions among ribosomal cistrons in yeast strains (33), it has not been possible to unambiguously map the 5′ terminus of spacer transcripts to the spacer promoter. Development of an in vivo assay for the spacer promoter is an essential step toward testing a number of crucial aspects of the readthrough enhancement model in yeast. An in vivo assay for spacer-promoter activity is also essential for determining whether the differences in template activity observed in vitro for type I and II rDNA alleles are reflected in the ability of these spacer promoters to support reinitiation by readthrough RNA polymerases and/or de novo initiation of transcription by RNA polymerase I in vivo.

Reversing the orientation of the rDNA spacer sequences in *prib*1 results in a marked reduction in the stimulation of 35S fusion transcript; however, the three- to fourfold stimulatory activity that remains is significantly higher than that observed when the spacer sequences are completely deleted. This observation is difficult to reconcile with the orientation dependence of the terminator activity and with spacer-promoter activity, which would be expected to be orientation dependent. Elion and Warner (6) reported that the ability of the *Eco*RI-*Hin*dIII spacer sequences to stimulate 35S rRNA synthesis in the reverse orientation is highly dependent on adjacent nucleotide sequences. They observed up to 20-fold stimulation of 35S rRNA synthesis from some minigene constructs in which the *Eco*RI-*Hin*dIII spacer sequences were inserted in the opposite orientation relative to the 35S promoter (6). In each construction, the ability of the spacer sequences to stimulate 35S rRNA synthesis was apparently dependent on sequences adjacent to the *Eco*RI-*Hin*dIII fragment. Although the nature of the nucleotide sequence context dependence observed in their minigene constructs is unclear, the results suggest that the ability of yeast rDNA spacer sequences to stimulate 35S rRNA synthesis is not strictly orientation dependent. Similar results have been reported for *Xenopus* spacer sequences. The spacer regions of *Xenopus* ribosomal cistrons contain one or more spacer-promoter elements as well as numerous copies of 60/81-base-pair repeating elements (31). The 60/81-base-pair repeat element does not have promoter activity but is partially homologous to a 40-base-pair region within the gene promoter and the spacer promoters (21). Plasmids containing a *Xenopus* gene promoter adjacent to 60/81-base-pair repeat elements are transcribed preferentially relative to plasmids containing only the gene promoter in injected oocytes, and the observed preferential transcription is not dependent on the orientation of the 60/81-base-pair repeat elements relative to the gene promoter (21). Furthermore, the 60/81-base-pair repeat elements alone compete transcription from a plasmid carrying only the gene promoter when coinjected into oocytes (29). These observations suggest that the 60/81-base-pair elements bind a transcription factor that is necessary for efficient initiation of transcription from the gene promoter. They also suggest that binding of the putative transcription factor to the 60/81-base-pair repeat element stimulates transcription from an adjacent gene promoter in an orientation-independent manner. Apparently at odds with this hypothesis is the recent report that sequences within the *Xenopus* gene promoter that are homologous to the 60/81-base-pair element can be replaced with linker sequences without measurable effects on promoter activity (37). Although both the *Xenopus* spacer-promoter elements and the 60/81-base-pair elements can stimulate transcription

from the gene promoter, DeWinter and Moss (3) have shown that maximal stimulation of gene-promoter activity requires the spacer-promoter element in an orientation-dependent manner. It is possible that orientation-independent component of the stimulation of 35S rRNA synthesis by spacer sequences occurs by sequestration of an essential transcription factor in a manner analogous to that suggested for the 60/81-base-pair elements in *X. laevis*. If this is the case, the spacer-sequence requirements for stimulation of 35S rRNA synthesis in the opposite orientation would differ from those described in this report.

It is becoming clear that efficient rRNA synthesis from tandem arrays of ribosomal cistrons in eucaryotic cells involves the complex interplay of a gene promoter element with terminator and promoter elements within the spacer region of each cistron. Although the details of this transcriptional mechanism remains to be established, it seems likely that the essential feature of the mechanism will be conserved among different eucaryotes.

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