Binding Sites for Abundant Nuclear Factors Modulate RNA Polymerase I-dependent Enhancer Function in Saccharomyces cerevisiae*

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The 190-base pair (bp) RNA enhancer within the intergenic spacer sequences of Saccharomyces cerevisiae rRNA cistrons activates synthesis of the 35S–rRNA precursor about 20-fold in vivo (Mestel, R., Yip, M., Holland, J. P., Wang, E., Kang, J., and Holland, M. J. (1989) Mol. Cell. Biol. 9, 1243–1254). We now report identification and analysis of transcriptional activities mediated by three cis-acting sites within a 90-bp portion of the rDNA enhancer designated the modulator region. In vivo, these sequences mediated termination of transcription by RNA polymerase I and potentiated the activity of the rDNA enhancer element. Two trans-acting factors, REB1 and ABF2, bind independently to sites within the modulator region (Morrow, B. E., Johson, S. P., and Warner, J. R. (1989) J. Biol. Chem. 264, 9061–9068). We show that REB2 is identical to the ABF1 protein. Site-directed mutagenesis of REB1 and ABF1 binding sites demonstrated uncoupling of RNA polymerase I-dependent termination from transcriptional activation in vivo. We conclude that REB1 and ABF1 are required for RNA polymerase I-dependent termination and enhancer function, respectively. Since REB1 and ABF1 proteins also regulate expression of class II genes and other nuclear functions, our results suggest further similarities between RNA polymerase I and II regulatory mechanisms. Two rDNA enhancers flanking a rDNA minigene stimulated RNA polymerase I transcription in a multiplicative fashion. Deletion mapping analysis showed that similar cis-acting sequences were required for enhancer function when positioned upstream or downstream from a rDNA minigene.

Eucaryotic cells contain three nuclear RNA polymerases that transcribe three distinct classes of genes (1). Despite clear differences, fundamental similarities unite transcription by the three polymerases, reflecting shared features of the reactions they catalyze (1, 2). Of the 10–15 subunit polypeptides constituting each of the yeast RNA polymerases, five small subunits are common to all three enzymes and two additional subunits are common to RNA polymerases I and III (2). The two largest subunits of each polymerase are unique, but share significant sequence similarity among the three forms of polymerase (1, 2). The parallels in regulatory mechanisms extend to their core transcription factors. All three nuclear transcription systems require the TATA-binding protein (3–8).

Activation or repression of RNA polymerase II transcription requires additional cis-acting elements, referred to as enhancers or silencers, respectively (9, 10). For RNA polymerase I, cis-acting sequences within the intergenic spacer region between tandemly repeated cistrons activate or enhance transcription from a gene promoter (reviewed in Refs. 11 and 12). In Xenopus laevis (11, 13), Drosophila (14), and rodents (15–17), these enhancer elements are imperfect duplications of all or a portion of the gene promoter. Several lines of evidence show that these latter enhancer elements are binding sites for basal transcription factors that interact with the gene promoter (11, 12).

In yeast, initiation of rRNA synthesis from a 35S–rRNA gene promoter is activated about 20-fold by a 190-bp RNA enhancer element which lies within the spacer region immediately downstream from the 25S rRNA coding sequences of an upstream cistron and approximately 2.2 kilobase pairs upstream from the gene promoter of a downstream cistron (18, 19). The sequences essential for yeast RNA enhancer activity overlap the 22-bp spacer promoter, previously shown to be sufficient to support RNA polymerase I-dependent transcription initiation in vitro (19–21). Thus, as has been shown for enhancer elements in X. laevis, it is likely that the yeast rDNA enhancer element requires sequences that bind basal transcription factors. Interestingly, additional sequences upstream of the spacer promoter potentiate the activity of the yeast rDNA enhancer element. Although these upstream sequences by themselves do not activate RNA polymerase I transcription, maximal rDNA enhancer activity requires this modulator region in addition to the spacer promoter region (19). Finally, sequences near the 5′ boundary of the enhancer element mediate RNA polymerase I-dependent termination of transcription (19). Thus, the 190-bp yeast RNA enhancer element is a complex element that mediates both enhancement of rRNA synthesis and transcription termination.

In this report, identification and characterization of the cis-acting sequences and trans-acting factors that modulate rDNA enhancer activity, as well as RNA polymerase I-dependent transcription termination, are presented.

EXPERIMENTAL PROCEDURES

Materials—Oligonucleotides were provided by the DNA Synthesis Group, Cetus Corp., Emeryville, CA; Dr. J. Brueen, State University of New York; Dr. P. Luciw and the Protein Structure Laboratory, University of California, Davis. The sequences of oligonucleotides used in this study are presented in Table I. Polyclonal antibody directed against REB1 (22) was a generous gift from Dr. Jon Warner and Dr. Bernice Morrow, Albert Einstein College of Medicine. Monoclonal antibody

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1 The abbreviations used are: bp, base pair(s); PCR, polymerase chain reaction; UAS, upstream activation sequences.
Strain, Growth Conditions, and Yeast Transformation—Saccharomyces cerevisiae strain S173-6B (a leu2–3 leu2–112 his3-1 trpl-289 ura3-52), provided by Dr. F. Sherman, University of Rochester, Rochester, NY, was used for all of the in vivo analyses described here. Plasmids were prepared from S. cerevisiae strain S173-6B, which is identical to strain S173-6B except that it carries a null mutation in the PEP4 structural gene with insertion of a functional HIS3 gene. Yeast strains were grown at 30°C in YP medium supplemented with 2% glucose. Yeast were transformed by the alkali cation procedure with the addition of denatured salmon sperm DNA (24). Yeast transformants were grown at 30°C in YEP medium containing 0.67% yeast nitrogen base (Difco Laboratories), 2% glucose, and 1% casamino acids supplemented with 20 μg/ml each of leucine and histidine, as well as 40 μg/ml tryptophan. Transformants were selected by their ability to grow in the absence of uracil in the growth medium and screened for mitochondrial function.

Construction of Yeast DNA Mini-genes—The construction of the low copy number CEN plasmid prib1 containing a yeast rDNA minigene was described previously (19). The plib1 rDNA minigene contains an rDNA enhancer element fused to the 3S-rRNA gene promoter, heterologous pBR322 reporter sequences, and the 3S-rRNA 3′ terminal processing site (Fig. 1B). Plasmid prib2 was derived from plib1 by replacement of the 3S-rRNA 3′ terminal processing site with a second copy of the rDNA enhancer element, designated the test enhancer (Fig. 1B).

Plasmid prib3 was constructed as follows. Plasmid prib1 +290/ +131 (19) was digested with BamHI to remove the fragment containing the 3S-rRNA 3′ terminal processing site, repaired with the Klenow fragment of DNA polymerase I, and religated with insertion of a PvuI linker (Collaborative Research). The unique EcoRI and Sall sites flanking the enhancer element upstream from the 3S-rRNA gene promoter were then destroyed by repair with the Klenow fragment of DNA polymerase I and mung bean nuclease, respectively, to generate plasmid pribPvuI. A functional wild-type enhancer element was derived from plasmid prib1 +290/ +131, which contains rDNA enhancer sequences, by digestion of an EcoRI site at position +161 to a Sall linker site at position +29 relative to the HindIII site (position +1) in the enhancer element (19). Hybridization with EcoRI and Sall followed by repair with the Klenow fragment of DNA polymerase I.

The wild-type enhancer element fragment was blunt-ended ligated into the test enhancer site at the unique PvuI site in plasmid pribPvuI. Unique EcoRI and Sall sites flanking the test enhancer were regenerated as a consequence of ligation in plasmids prib2 wild-type (WT) and prib2 wild-type reverse orientation (WT reverse). Derivatives of prib2 containing deletion mutations within the test enhancer element were generated by EcoRI/Sall enhancer fragment swaps between the test enhancer in prib2 WT and prib2 WT reverse and mutant enhancer elements previously isolated in prib1 (19). Test enhancer carrying base substitutions in plasmids prib2 mutant (M) and prib2 mutant (M′) were generated by PCR (polymerase chain reaction) as described below and used to replace the test enhancer in prib2.

The rDNA minigene in plasmid prib3 was constructed by ligating an EcoRI fragment corresponding to the 3S-rRNA 3′ terminal processing site into the unique EcoRI site in plasmid prib2.

Construction of Base Substitution Mutations in the rDNA Enhancer Element—Base substitution mutations were incorporated into the rDNA enhancer element by PCR with plib1 +290/ +131 as template and are denoted by lowercase type and the "m" suffix (Fig. 1D). Mutations at Site 1 and Site 3 (Fig. 1D) were generated by PCR using clockwise mutagenic primers (muta1 and muta3, respectively) and an EcoRI primer (border of the enhancer element to sequences downstream from Site 1 or Site 3) and a clockwisely primer complementary to heterologous pBR322 reporter sequences located downstream from the 3S-rRNA gene promoter (pBR322H3ccw, 23b). For the site2m mutation, the four primer method (25) was used (Ribo21, pBR322RIccw, 23b; pBR322H3ccw, 24b). Plasmid PCR products were digested with EcoRI and HindIII and the mutant enhancer elements (EcoRI/Sall fragments) were used to replace the test enhancer elements in prib1 and prib2, respectively.

Analysis of 3S-rRNA Minigene Expression—In vivo expression of rDNA minigenes was monitored by Northern (RNA) blotting with 32P-labeled, single-stranded RNA probes complementary to pBR322 reporter sequences within the minigene transcripts. High specific activity RNA probes were prepared by SP6 RNA polymerase transcription according to the manufacturer's protocol (Promega). Plasmid pSP-322HB containing the 346-bp HindIII/BamHI fragment from pBR322 was used to generate probe complementary to the reporter sequences within minigene transcripts (19). As an internal control, a second probe complementary to 790 bases of the coding sequences of the yeast GCR1 gene was hybridized simultaneously or in parallel to an identical panel. The template for GCR1 probe was PvuII-digested pSP-GCR1, which contains the 2193-bp EcoRI/Sad fragment from GCR1 (26) cloned into pSP-RM3 vector (Promega). Total RNAs from yeast transformants carrying each plasmid was isolated, electrophoresed, and transferred to Nytran membranes (Schleicher & Schuell, Inc.) as described previously (19). Hybridization was performed in buffer containing: 50 μM sodium phosphate, pH 6.5, 0.5 μM NaCl, 1 mM EDTA, 2.5 × Denhardt's solution, 250 μg/ml salmon sperm DNA, and 50% formamide (0.1 ml/cm2) at 55°C for 2–5 h. For hybridization, prehybridization solution was added (0.08 ml/cm2, 55°C), which contained 106P-labeled RNA probe (5–10 × 10^6 cpm/ml). After hybridization at 55°C for 18 h, blots were washed in buffer containing: 20 μM sodium phosphate, pH 6.5, 50 μM NaCl, 1 mM EDTA, and 0.1% SDS (four changes at room temperature and three changes at 69°C, 20 min each).

Preparation of S100 Protein Extracts—Whole cell extracts (S100) were prepared essentially as described previously (27) except that MgCl2 was omitted from the final dialysis buffer. S. cerevisiae strain S173-6B, pep4, was cultured in YP media containing 2% glucose to early log phase (A560nm = 1.0–2.0). Cells from 12 liters of culture were harvested by centrifugation at 3000 rpm (5 min) in an HS-4 rotor ( Sorval) at 4°C. Cell pellets were resuspended in cold buffer (50 mM Tris, pH 7.9, 5 mM MgCl2, 0.2 mM EDTA, 2.5 mM phenylmethylsulfonyl fluoride, 1 mM MgSO4, and 20% glycerol) to a final volume of 45 ml. The cell suspension was frozen by dripping into liquid nitrogen and stored at −80°C.

To prepare S100 extracts, 25–50 g of frozen cell suspension was broken in an Eaton press. All subsequent procedures are performed at 4°C. Disrupted cells were suspended in an equal volume of extract buffer containing: 50 mM Tris-HCl, pH 7.9, 10 mM MgCl2, 2 mM dithiothreitol, 25% sucrose, and 20% glycerol. The suspension was adjusted to 10% of saturation by dropwise addition of saturated ammonium sulfate, stirred for 20 min, and centrifuged at 48,000 rpm in a Beckman Type 70Ti rotor for 3 h. Solid ammonium sulfate (0.4 g/ml) was added to the supernatant to pH 7.4 by dropwise addition of 1 N NaOH and the precipitate collected by centrifugation at 48,000 rpm in a Beckman Type 70Ti rotor for 30 min. The precipitate was resuspended in 5–10 ml of buffer containing: 50 mM Tris-HCl, pH 7.4, 6 mM MgCl2, 0.2 mM EDTA, 1 mM dithiothreitol, and 15% glycerol. The suspension was dialyzed against two changes (1 liter each) over 12 h of the same buffer without MgCl2. Insoluble material was removed by centrifugation for 1 h at 9000 ×g at 4°C. The supernatant was quick-frozen by dripping into liquid nitrogen and stored at −80°C. No loss of DNA binding activity of any of the factors described here was detected in extracts stored up to 6 months.

Protein concentration was determined by bichinchoninic acid assay (Pierce) using a bovine serum albumin standard. DNA binding activity was assayed by step elution of the S100 whole cell extract fractionated by Mono S HR/5 FPLC (Pharmacia) cation exchange chromatography as described previously (28) using a 50–500 mM KC1 linear gradient followed by step elution with 1.0 mM and 2.0 mM KC1. While Mono S fractions (1 ml) stored at −80°C showed no loss of activity, binding activities were reduced when aliquots were left on ice.

Preparation of DNA Probes—32P-Labeled oligonucleotide probes were generated as follows: 2 pmol of single-stranded oligonucleotides was 5′ end-labeled in a 10 μl reaction containing: 80 mM Tris-HCl, pH 7.6, 12 mM MgCl2, 6 mM dithiothreitol, 50 μCi of [γ-32P]ATP (Amersham Corp; >5000 Ci/mmol), and 10 units of T4 polynucleotide kinase (Pharmacia Biotech Inc.). Labeling reactions were performed at 37°C for 45 min. The products were heated to 80°C for 1 min. An excess (50%) of an unlabeled complementary oligonucleotide was annealed to generate a double-stranded probe, and free [γ-32P]ATP and single-stranded oligonucleotides were removed using a G-25 Quick Spin columns (Boehringer Mannheim). The specificity of the probe was determined by scintillation counting of an aliquot adsorbed on DE81 filter paper.

DNA probes corresponding to the modulator region of the rDNA enhancer element (EcoRI/FokI) were prepared by 35 rounds of PCR using oligonucleotide primers complementary to pBR322 sequences that flank the rDNA enhancer element (pBR322RIccw, 23b; pBR322H3ccw, 24b) in appropriate prib1 templates. After phenol extraction, the PCR product was precipitated from 1.2 μM ammonium acetate, 55% isopropanol. The PCR product was ligated-digested with EcoRI and FokI and then treated with calf intestinal phosphatase. To inactive calf intestinal phosphatase, the reaction was adjusted to 40
RESULTS

The rDNA Enhancer Contains Binding Sites for the trans-Acting Factors REB1 and ABF1—To better understand how the rDNA enhancer stimulates transcription, we investigated the roles of protein factors which bind cis-acting sequences that modulate rDNA enhancer function, previously determined by deletion analysis (19). Morrow et al. (34) showed that there are two DNA binding activities, designated REB1 and REB2, that bind to rDNA enhancer sequences between positions –161 and –93. J u et al. (32) subsequently cloned the gene encoding REB1. However, the protein(s) responsible for REB2 binding activity had not been conclusively identified. The REB1 and REB2 binding sites in the rDNA enhancer were designated Site 1 and Site 2, respectively (Fig. 1D). Prior deletion mapping analysis suggested that a third site, designated Site 3 (Fig. 1D), located between Site 1 and Site 2 is required for maximum enhancer activity in vivo (19).

To identify protein factors that bind to these sites, gel mobility shift assays were performed with Mono S-fractionated yeast whole cell extracts and DNA probes corresponding to enhancer sequences between the EcoRI site at position –161 and the FokI site at position –93 (Figs. 1D and 2). Double-stranded oligonucleotides corresponding to Sites 1, 2, and 3, designated Ribo1, Ribo2, and Ribo3 (Fig. 1, Table I) were synthesized and used in parallel gel mobility shift assays. Two previously described double-stranded oligonucleotide probes, HMRE and HMRB, which specifically bind the factors ABF1 and RAP1, respectively, were used to standardize Mono S chromatographic profiles (Ref. 28 and Fig. 2).

REB1 binding activity detected with the Ribo1 oligonucleotide appeared as a major activity peak in fraction 42 (Fig. 2). A similar pattern of REB1 binding was observed using an EcoRI/FokI probe corresponding to wild-type rDNA enhancer sequences extending from position –161 to –93 (wild-type modulator region). An EcoRI/FokI probe containing a triple base substitution mutation within the REB1 consensus binding site, designated site1m (Fig. 1, Table I), failed to bind REB1 (Fig. 2). To verify that the binding activity that interacts with Site 1 is REB1, gel mobility supershift assays were performed using the EcoRI/FokI probe, Mono S fraction 42, and a polyclonal antibody directed against REB1 (Fig. 3). The gel mobility shift complex was supershifted in the presence of the REB1 antibody, but not by preimmune sera (data not shown; Ref. 33). These data confirm that REB1 binds to Site 1 sequences.

A second DNA binding activity was detected with the wild-type modulator region and the Ribo2 oligonucleotide probe with a major activity peak in fraction 31 (Fig. 2). Surprisingly, this binding activity co-chromatographed with ABF1 protein detected by gel mobility shift assays with the HMRB oligonucleotide probe (Fig. 2). Cleavage of the EcoRI/FokI probe with MnlI at position –120 abolished binding, consistent with the published observations for REB2 (data not shown; Ref. 31). Although these data suggested that the REB2 binding activity corresponds to ABF1, no strong ABF1 consensus binding site was evident at Site 2. Utilizing a degenerate ABF1 consensus binding site (RTCRYKHDDACG at 75% certainty) developed from strong ABF1 binding sites (28) and a table of nucleotide frequencies at all positions within known ABF1 binding sites, putative ABF1 binding sites in the vicinity of Site 2 were statistically ranked. This analysis revealed two poor overlapping matches to the degenerate consensus (one on each DNA strand), which varied from the consensus at 75% certainty in one of the five “invariant” positions, but preserved biases at degenerate positions (Fig. 1D). A 4-base substitution mutation that abolished both putative ABF1 binding sites without introducing a significant novel ABF1 binding site was generated in the enhancer element. An EcoRI/FokI probe containing these base substitution mutations, designated site2m, failed to bind REB2 in vitro (Fig. 2). To confirm that the binding activity that interacts with Site 2 is ABF1, gel mobility supershift assays were performed using the EcoRI/FokI probe, fraction 31 after Mono S chromatography, and a monoclonal antibody directed against ABF1 (23). As illustrated in Fig. 3, the gel mobility shift complex was supershifted in the presence of the ABF1 antibody. The specificity of the ABF1 monoclonal antibody was verified by showing that REB1 polyclonal antibody and a monoclonal antibody directed against the Glu-Glu epitope tag from SV40 T antigen failed to supershift the ABF1:EcoRI/FokI complex (data not shown). These data confirm that ABF1 binds to Site 2 within the rDNA enhancer element and that REB2 is ABF1.

No specific DNA binding events were detected by gel mobility shift assay with the Ribo3 oligonucleotide (data not shown).
rDNA enhancer. Enhancer elements containing base substitution mutations in Sites 1 and 2, which abolish REB1 and ABF1 binding, respectively, and Site 3 were cloned upstream from the RNA polymerase I gene promoter in prib1 (Fig. 1B). The activities of these mutant enhancer elements were monitored in vivo and compared to prib1 containing a wild-type enhancer element (Fig. 1B). The activities of these mutant enhancer elements were monitored in vivo and compared to prib1 containing a wild-type enhancer element (Fig. 1B). The activities of these mutant enhancer elements were monitored in vivo and compared to prib1 containing a wild-type enhancer element (Fig. 1B). The activities of these mutant enhancer elements were monitored in vivo and compared to prib1 containing a wild-type enhancer element (Fig. 1B). The activities of these mutant enhancer elements were monitored in vivo and compared to prib1 containing a wild-type enhancer element (Fig. 1B). 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RNA polymerase I minigene transcription unit in prib1 (19). This abundant transcript apparently initiates at BAT1, a previously identified fortuitous RNA polymerase II promoter present in pBR322-derived sequences (34). Thus, the site3m mutation disrupted fortuitous termination of RNA polymerase II transcripts by the rDNA enhancer modulator region in prib1.
The REB1 Binding Site Is Essential for an RNA Polymerase I-Dependent Termination Event in Vivo—

Table I

| Name        | Length | Sequence                        |
|-------------|--------|---------------------------------|
| Double-stranded oligos |        |                                 |
| Ribo1       | 23bp   | CTA T G A T C C G G T A A A A A ACATGT G A T A C T A G G C C C A T T T T T G T A C A | |
| Ribo2       | 38bp   | ATT G T A T A T A T C T A T T A T A A T A A T A C G A T G A G G A T G A T A T A A C AT A T A G A T A A T A T T G C A T T C C T A C T A T | |
| Ribo3       | 32bp   | T A A A A A A C AT T G A T T T G T A T A T C T A T T A T A A T A T A T T T T G T A C A | |
| HMRB        | 24bp   | CTT G C A A A A A C C A T C A A C C T T G G A A A A A A G T A G A C G A A C G T T T T T G G T A G T T G G A A C T T T T T T T C A T C T G | |
| HMRE        | 36bp   | C A A T A C A T C A T A A A A A A C A G G A A C G A G T A T G A T A G T G T G C | |
| Ribo11      | 23bp   | C T A T G A T C A T T G T A A A A A A C A T G T G A T A C T A G A T A A A A C A T G | |
| Ribo21      | 31bp   | T A T T A T A A T A T T A T T T G A G G A T G A T A G T G T G | |
| Single-stranded oligos |        |                                 |
| muta1       | 35 b   | C A A G A A T T C T A T G A T C A A T T G A A A A A C A T G A T T G | |
| muta3       | 60 b   | C A A G A A T T C T A T G A T C C G G G T A A A A A A C A T G T | |
| pBR322H3ccw  | 23 b   | T A G G C C T A C A C G G G C C C T T C | |
| pBR322H3ccw  | 24 b   | G A C T G C G T T A G C A A T T T A A A C T G T G | |

**Fig. 3.** Gel mobility supershift analysis with antibodies directed against REB1 and ABF1. Gel mobility shift assays contained a $^{32}$P-labeled DNA probe corresponding to the rDNA enhancer modulator region (EcoRI/ FokI probe) and Mono S fractionated REB1 (fraction 42) or ABF1 (fraction 31). Gel mobility shift assays were performed as described under “Experimental Procedures” using the indicated dilutions of a REB1 polyclonal antibody or an ABF1 monoclonal antibody. Probe Only indicates binding reactions containing only the $^{32}$P-labeled DNA probe. No Antibody indicates binding reactions lacking REB1 or ABF1 antibody, respectively.

mut3.

Most surprisingly, expression of a minigene, designated prib1 mut2, containing the 4-base substitution at Site 2 that disrupted ABF1 binding in vitro was reduced 7-8 fold relative to wild-type prib1 in vivo (Fig. 4). Once again, the effect of the Site 2 base substitution mutations on enhancer function was comparable to a 5' deletion mutation that removed all three sites. However, unlike such a deletion mutation, no high molecular weight read-through transcript was observed for prib1 mut2.

The REB1 Binding Site Is Essential for an RNA Polymerase I-Dependent Termination Event in Vivo—Both genetic and biochemical analyses have shown that the 3' terminus of yeast $^{35}$S-rRNA is generated by processing of a primary transcript (35–37). Therefore, we set out to determine the requirements for RNA polymerase I-dependent 3' end formation utilizing an rDNA minigene on plasmid prib2 (Fig. 1B). We previously showed that sequences near the 5' end of the rDNA enhancer element directed RNA polymerase I-dependent termination or processing in vivo (19). The prib2 minigene contains a second copy of the rDNA enhancer, designated the test enhancer, at a site downstream of pBR322-derived reporter sequences. Transcripts from prib2 initiate at the $^{35}$S-rRNA gene promoter and end near the downstream test enhancer (Fig. 1B).

The prib2 minigene containing a wild-type test enhancer in the native orientation directs RNA polymerase I-dependent termination in vivo, reflected by the production of a 480-base fusion transcript (Ref. 19, Fig. 5). The size of this transcript was determined by comparison to RNA standards using both log(molecular weight) versus relative mobility and square root(molecular weight) versus log(relative mobility) plots (38). The 480-base fusion transcript did not hybridize with vector sequences upstream or downstream of the rDNA minigene present in prib2 (data not shown). Based on electrophoretic mobility, the 3' terminus of the 480-base fusion transcript is located near the 5' end of the test enhancer (Fig. 1C). The steady state level of the 480-base fusion transcript was lower than the steady state level of the $^{35}$S-fusion transcript synthesized from plasmid prib1, despite the presence of a second enhancer in prib2 transcripts. As shown below for prib3 and prib2 containing a mutation in Site 2, both enhancers in prib2 probably function. Therefore, the 480-base fusion transcript probably has a significantly shorter half-life than prib1 transcripts, perhaps related to the different 3' termini of the two transcripts.

As all RNA polymerase I terminators examined in vitro function only in the native orientation (39–41), we tested the orientation dependence of the putative yeast terminator in
abolished RNA polymerase I-dependent termination in \textit{vivo}. 1 base substitution mutations that disrupted REB1 binding downstream of the test enhancer. As illustrated in Fig. 5, acting factors required for RNA polymerase I termination at Sites 1, 2, and 3 were cloned at the downstream test enhancer site and tested for their effects on enhancer function.

Two rDNA Enhancer Elements Positioned Upstream and Downstream from the 35S-rRNA Gene Promoter Function Together to Activate Transcription of an rDNA Minigene. Formally, the absence of the 480-base fusion transcript from prib2 minigenes containing mutations that eliminate the REB1 binding site in the downstream test enhancer could have resulted from loss of either 3' end formation or enhancer function. To evaluate the enhancer activity of the downstream test enhancer element in the prib2 minigene, a new minigene was constructed in plasmid prib3. The minigene in prib3 differs from the prib2 minigene by insertion of 35S-rRNA 3' terminal processing sequences between the pBR322 reporter sequences and the downstream test enhancer (Fig. 1B). Since the minigenes in prib1 and prib3 direct synthesis of the same 35S-fusion transcript, steady state transcript levels should reflect the relative rate of transcriptional initiation from the 35S-labeled gene promoter in each minigene without interference from differential RNA turnover. Transcription of the prib3 minigene with two wild-type enhancers was stimulated almost 20-fold over that observed for the prib1 minigene with a single wild-type enhancer (Fig. 6). Since the single enhancer in the prib1 minigene stimulated transcription 17.5-fold (19), this result indicated that the two enhancer elements in the prib3 minigene stimulate transcription approximately 300-350-fold overall.

To test whether the downstream enhancer in prib3 functions by the same mechanism as the upstream enhancer, enhancer elements containing deletion mutations were cloned at the downstream test enhancer site and tested for their effects on 35S-fusion transcript synthesis. A series of 5' deletion mutations extending from position −161 to −92 caused progressive loss of enhancer activity, whereas 3' deletion mutations extending from position +131 to positions −35 or −71 caused complete loss of test enhancer activity (Fig. 6).

Overall, deletion mutations in the downstream test enhancer of prib3 affected transcription in a quantitatively similar fashion to that observed previously when the identical deletion mutations were tested for their effects on the activity of the enhancer element in prib1 (19). Notably, deletion of sequences corresponding to Site 3 in prib3 (Fig. 6, −161/−122) did not decrease transcription as was observed in prib1. Interestingly, a 3' deletion mutation adjacent to the spacer promoter (prib3 +3/+131) caused either loss of enhancer activity or no effect on enhancer activity in approximately equal numbers of the multiple transformants analyzed. The bipolar behavior of this deletion mutation could reflect the importance for enhancer function of sequences within the HindIII recognition site (+1), demonstrated in previous studies (18). With the exception of these mutations, the similarity in requirement for cis-acting sequences suggests that the two enhancers in prib3 act to stimulate 35S-labeled gene promoter initiation by the same mechanism. Taken together, these results suggested that the failure of prib2 mut1 to direct synthesis of 480-base fusion transcript (Fig. 5) was due to loss of termination rather than enhancer function.

![Diagram](Image)

**Fig. 4.** The effects of base substitution mutations within the modulator region of the rDNA enhancer on enhancer activity in \textit{vivo}. Expression of wild-type (WT) prib1 and derivatives containing base substitution mutations in Site 1 (prib1 mut1), Site 3 (prib1 mut3), and Site 2 (prib1 mut2), was monitored by Northern blotting using a probe complementary to pBR322 reporter sequences within the minigene transcript. The positions of the prib1 encoded 35S-fusion transcript and a high molecular weight (HMW) read-through transcript are indicated. Site 1 and Site 2 correspond to REB1 and ABF1 binding sites, respectively. Lanes contained 5 μg of total cellular RNA from the yeast host strain S173-6B carrying the plasmid minigene indicated. The GCR1 control panel contained identical aliquots of total cellular RNA but was hybridized with a probe corresponding to GCR1 coding sequences and served as a control.

vivo. The terminator in the prib2 minigene functioned in an orientation-dependent manner, evident by its inability to direct 480-base fusion transcript synthesis when the test enhancer was in the reverse orientation (Fig. 5A, prib2 WT reverse). Loss of 480-base fusion transcript synthesis was accompanied by the appearance of a 2200-base read-through transcript that hybridized with vector sequences downstream of the test enhancer (Fig. 5A and data not shown).

To further define the cis-acting sequences and the trans-acting factors required for RNA polymerase I termination in \textit{vivo}, enhancer fragments containing base substitution mutations at Sites 1, 2, and 3 were cloned at the downstream test enhancer position in prib2. In addition to the 480-base fusion transcript, a series of larger transcripts was present that, presumably, reflect read-throughs of the termination site (Fig. 5B). The major read-through species detected have molecular weights consistent with 3' termini in the vector sequences downstream of the test enhancer. As illustrated in Fig. 5B, Site 1 base substitution mutations that disrupted REB1 binding in vitro abolished RNA polymerase I-dependent termination in \textit{vivo}. No new read-through transcripts appeared, nor did the level of read-through transcripts rise commensurate with loss of the 480-base fusion transcript, suggesting either that the resulting transcripts are unstable or that only a small portion of the total transcripts share the 480-base end point (see "Discussion"). Base substitution mutations at Site 3 and Site 2 did not affect 3' end formation. Thus, RNA polymerase I-dependent termination in \textit{vivo} appears to require REB1 binding to Site 1. Base substitution mutations at Site 2 caused a decrease in enhancer activity, suggesting that the downstream enhancer in prib2 activated synthesis of the 480-base fusion transcript. In contrast to the observations made in prib1, however, mutations in Site 3 did not decrease the level of prib2 expression.
Fig. 5. Enhancer sequences required for RNA polymerase I-dependent termination or processing. Expression of prib1 and prib2 was monitored by Northern blotting utilizing a hybridization probe complementary to pBR322 reporter sequences within the rDNA minigene. Lanes contain 5 μg of total cellular RNA from the yeast host strain S173-6B carrying the plasmid minigene indicated. All minigenes contained rDNA elements in their native orientation except prib2 WT reverse in which the downstream test enhancer element was in the reverse orientation. The wild-type enhancer includes sequences from -161 to +29 relative to the HindIII site. The GCR1 control panels contained identical aliquots of total cellular RNA but was hybridized with a probe corresponding to GCR1 coding sequences and served as a control. A, orientation dependence of termination. The positions of the 35S-fusion transcript (35S FT) synthesized from prib1, the 480-base fusion transcript (480 FT) synthesized from prib2, and a 2.2-kilobase pair read-through transcript (2200 RT) synthesized from prib2 WT reverse are indicated. B, identification of cis-acting sites required for RNA polymerase I-dependent termination in vivo. Expression of wild-type prib2 (prib2 WT) and derivatives containing base substitution mutations in Site 1 (prib2 mut1), Site 3 (prib2 mut3), and Site 2 (prib2 mut2) was monitored by Northern blotting using a probe complementary to pBR322 reporter sequences within the minigene transcript. Site 1 and Site 2 correspond to REB1 and ABF1 binding sites, respectively. The position of the prib2 encoded 480 base fusion transcript is indicated. RNA standard lanes were transferred to the same filter and visualized by methyl blue staining. Standard sizes are indicated in kilobases to the right of the panel.

Discussion

Deletion mutations extending from the 5' end of the enhancer element cause a progressive loss of enhancer activity consistent with the removal of two or more cis-acting regulatory sites (19). Loss of REB1 binding caused by a base substitution mutation (prib1 mut1) in Site 1 had only a modest effect (2-fold) on transcriptional stimulation by the rDNA enhancer. This result agrees with our previous observations for a deletion mutation (prib1 -161/-148) that eliminates REB1 binding (19). Others have also reported small effects of REB1 site deletion mutations on the activity of plasmid borne rDNA minigenes (42) as well as a tagged rDNA cistron integrated in the chromosomal rDNA tandem array (43).

Morrow et al. (31) identified REB2 as an activity that binds weakly to the modulator region at Site 2. The weak binding hindered previous attempts to biochemically characterize REB2. Lorch et al. (44) hypothesized that REB2 might be ABF1, based on limited sequence similarity between their binding sites. We now have demonstrated directly that REB2 and ABF1 behave identically with respect to binding site selection, co-chromatography, and gel mobility supershift assays using a monoclonal antibody directed against ABF1. While we cannot unequivocally exclude all other possibilities, it appears highly probable that ABF1 is identical to REB2.

Introduction of a mutation (site2m) that abolished ABF1 binding into the rDNA enhancer elements in prib1 and prib2 caused a 7-8-fold and a 4-5-fold loss of enhancer activity, respectively. For prib1, this mutation decreased enhancer activity to a level previously observed for a 5' deletion mutation extending from position -161 to -91 that removed the REB1 and ABF1 binding sites (19). These results are in contrast with those obtained utilizing two rDNA minigenes separated by a complete yeast rDNA spacer (45). In this latter study, a deletion mutation that removed the ABF1 (REB2) binding site did not cause a loss of expression of either rDNA minigene, whereas a deletion mutation that removed critical sequences near the 3' end of the enhancer element caused a 3-5-fold loss of expression of both rDNA minigenes (45). While we cannot reconcile the apparent lack of a requirement for the ABF1 (REB2) site observed in these latter experiments with those reported here, we speculate that the complete rDNA spacer used in this latter study may contain sequence elements that can compensate for loss of the ABF1 (REB2) site located within the enhancer element.

A 4-base transversion mutation at a third site (site3m) shown to be important for enhancer activity (19) had no measurable effect on REB1 binding to Site 1 or ABF1 binding to Site 2 in vitro. The site3m mutation in prib1 caused a 4-5-fold reduction in enhancer activity consistent with the results obtained for a deletion mutation in prib1, which removed the REB1 binding site and Site 3 (19). Interestingly, this latter deletion mutation did not affect the activity of the downstream enhancer in prib3, nor did the site3m mutation affect expression of the prib2 minigene. Taken together these results show
that the requirement for Site 3 is only observed in the context of the prib1 rDNA minigene. A high molecular weight read-through transcript, previously observed for prib1 deletion mutations that removed Site 3 (19), was also observed for prib1 carrying the site3m base substitution mutations. This read-through transcript probably initiates from a fortuitous RNA polymerase II promoter (designated BAT1) within plasmid pBR322 vector sequences upstream from the rDNA minigene (34). For prib1 derivatives lacking a functional Site 3, it is possible that synthesis of the read-through transcript interferes with enhancer element activity.

We showed previously that sequences near the 5' terminus of the rDNA enhancer element direct termination or processing of transcripts initiated from the 35S-rRNA promoter in prib2 (19). Here we show that REB1 binding to Site 1 is required for this termination/processing event in vitro. Unfortunately, one cannot distinguish transcription termination from RNA processing in vivo since template release of nascent RNA within cells cannot be readily assessed. Nevertheless, REB1-dependent release of RNA polymerase I initiated nascent RNA chains has been observed in vitro (46). REB1-dependent 3' end formation occurs in vitro at a site immediately upstream of the rDNA enhancer (41). The 3' terminus predicted from the size of the 480-base fusion transcript synthesized from prib2 is in close agreement with the termination site determined in vitro (41), suggesting that REB1 participates in transcription termination rather than RNA processing.

S1 nuclease mapping analysis of endogenous yeast rRNA transcripts revealed a 3' terminus (designated T2) (36) located approximately 60 bp downstream from the 3' terminus corresponding to the REB1-dependent termination site synthesized in vivo from the prib2 minigene. None of the read-through transcripts from prib2 or its mutant derivatives corresponded in size to that expected for termination at the T2 site. Unfortunately, van der Sande et al. (36) did not report an S1 nuclease mapping analysis that would have detected a 3' terminus corresponding to the 480-base fusion transcript synthesized in vivo from the prib2 minigene.

Base substitution mutations that abolished REB1 binding to the downstream enhancer element in prib2 (prib2 mut1) resulted in the loss of the 480-base fusion transcript, but did not cause the appearance of novel read-through transcripts or an increase in the levels of read-through transcripts observed for the wild-type rDNA minigene in prib2. Novel read-through transcripts with significantly shorter half-lives than the 480-base fusion transcript would not have been detected. Alternatively, the REB1-dependent 480-base fusion transcript might represent only a small fraction of the total transcripts synthesized from the prib2 rDNA minigene. In this case, loss of the 480-base fusion transcript caused by the site1m mutation would result in an unmeasurable redistribution of a small percentage of total transcripts over a set of preexisting read-through transcripts. This latter model is consistent with the observation that less than 20% of total transcripts terminate at the REB1-dependent site in vitro (46) and that the steady-state levels of endogenous cellular rRNA complementary to chromosomal rDNA sequences located immediately upstream and downstream from the REB1 binding site (Site 1) are similar (36).

The REB1-dependent terminator displayed strong orientation and factor dependence in vivo consistent with in vitro assays of mouse, Xenopus, and yeast RNA polymerase II terminators (39–41, 46–48). Experiments with purified factors suggest that REB1 is sufficient for termination in vitro (46). In light of REB1's role in RNA polymerase II transcription, however, it seems unlikely that REB1 functions primarily as an RNA polymerase I terminator factor. One possibility is that REB1's RNA polymerase I specific activities result from specific interactions with RNA polymerase I itself.

Much attention has been focused on the tandem arrangement of rRNA genes and the proposal that enhancers function by delivering RNA polymerase I and/or specific transcription factors to the gene promoter (11–17, 49–51). The rDNA minigene in prib3 provided an opportunity to compare the properties of one (prib1) versus two enhancer elements (prib3). Deletion mapping of the downstream enhancer in prib3 indicated that similar sequences are required for downstream enhancer function as observed for the upstream enhancer (19). In addition, the two enhancers in prib3 simulate transcription “multiplicatively.” As the product is greater than the sum of activation by each enhancer, such a multiplicative interaction represents a subset of synergistic effects (52). Synergy between enhancer elements further supports the model that the two enhancers act in the same pathway. Furthermore, one interpretation of multiplicative enhancer effects is that the energetic effects of the enhancers are independent, rather than acting through cooperative complex formation (53). It is not clear how such a model could be reconciled with models involving higher order template structures that co-localize enhancer elements (43, 54).

Our observation of multiplicative enhancer-dependent activation of transcription differs from that reported by Johnson and Warner (54), who observed an additive interaction of two enhancer elements and position-dependent enhancer strength when rDNA minigenes are integrated at the chromosomal URA3 locus. The topological difference between the circular plasmids used here and the linear chromosomal array used by Johnson and Warner might explain these quantitative differences.

The factors that bind the modulator region of the rDNA enhancer were previously shown to be involved in regulation of transcription by RNA polymerase II. REB1 binding sites have been identified in UAS of class II genes, centromeres, and telomeres (55, 56). ABF1 and RAP1 are structural homologs...
(57) and have been implicated in silencing of the silent mating type loci, telomere function, ARS function, and coordinate control of ribosomal protein gene transcription (23, 58–61). ABF1 and RAP1 appear functionally interchangeable for ARS function (61). REB1, ABF1, and RAP1 are abundant site-specific DNA-binding proteins, and their binding sites are ubiquitous. Given this array of seemingly unrelated biological processes, it is attractive to speculate that these are not multifunctional proteins, but rather serve an architectural function that is phenomenologically elaborated in the various activities observed.

Recent evidence suggests that transcription of vertebrate U6 genes by RNA polymerase III is regulated by OCT1, a factor that is involved in activation of RNA polymerase II-dependent genes by RNA polymerase III is regulated by OCT1, a factor phenomenologically elaborated in the various activities proteins, but rather serve an architectural function that is attractive to speculate that these are not multifunctional proteins, but rather serve an architectural function that is phenomenologically elaborated in the various activities observed.

Given this array of seemingly unrelated biological processes, it is attractive to speculate that these are not multifunctional proteins, but rather serve an architectural function that is phenomenologically elaborated in the various activities observed.

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