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The REB1 Site Is an Essential Component of a Terminator for RNA Polymerase I in *Saccharomyces cerevisiae*

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We have identified a terminator for transcription by RNA polymerase I in the genes coding for rRNA of the yeast *Saccharomyces cerevisiae*. The terminator is located 108 bp downstream of the 3′ end of the mature 25S rRNA and shares several characteristics with previously studied polymerase I terminators in the vertebrates. For example, the yeast terminator is orientation dependent, is inhibited by its own sequence, and forms RNA 3′ ends 17 ± 2 bp upstream of an essential protein binding site. The recognition sequence for binding of the previously cloned REB1 protein (Q. Ju, B. E. Morrow, and J. R. Warner, Mol. Cell. Biol. 10:5226–5234, 1990) is an essential component of the terminator. In addition, the efficiency of termination depends upon sequence context extending at least 12 bp upstream of the REB1 site.

Studies with both mouse and frog systems have shown that termination of transcription by RNA polymerase I requires the binding of a termination protein to a specific sequence in the DNA template. In mice, the protein has been called TTF1, and its specific binding site is an 18-bp-long element called the Sal box (10) (the element fortuitously contains an *Sal*I restriction site). In frogs, the binding protein has been christened Rib2 (21) and it binds to the site GACTTGCNC (19). In both systems, the terminator sequence will only cause termination when situated in the normal orientation and ceases to function when reversed. Partially purified TTF1 stimulates termination when added to a crude extract, and termination is abolished when an excess of an oligonucleotide containing the Sal box sequence is added to the reaction. Likewise, partially purified Rib2 causes specific DNase I footprints over the terminator element, and both footprinting and termination are abolished by competition with oligonucleotides containing the terminator sequence. Further progress in understanding the mechanism of termination has been hampered by the inability so far to completely purify either TTF1 or Rib2 or to clone the relevant cDNAs coding for these proteins.

In the hope that we could circumvent the problems of the vertebrate systems, we have begun to study termination by RNA polymerase I in the yeast *Saccharomyces cerevisiae*. The yeasts offer at least two potential advantages over the vertebrates: first, it is easier to obtain the large amounts of extract needed for biochemical purification, and second, the possibility of using the well-developed genetics of yeasts to supplement or replace biochemical approaches exists. The recent development of yeast extracts capable of supporting accurate initiation by RNA polymerase I in vitro (20, 27, 28) also offers the opportunity to develop in vitro assays for both DNA and protein elements involved in termination.

In this paper, we show that the yeast whole-cell extract developed by Schultz et al. (28) is capable of supporting RNA 3′-end formation of RNA polymerase I transcripts. One of these sites has features similar to the previously characterized vertebrate polymerase I terminators. Furthermore, fine-scale mutagenesis indicates that the terminator includes the binding site for REB1, an essential yeast protein that has previously been cloned (11, 23, 24).

MATERIALS AND METHODS

Plasmids. The yeast rRNA genes (rDNA) used in this study were derived originally from an *Sma*I repeat unit that was cloned as pBD4 (1).

A termination test vector was constructed by arranging in serial order a ribosomal gene promoter, a polylinker (for insertion of sequences to be tested for termination activity), and a series of unique restriction sites for linearizing the template. This construct is diagrammed in Fig. 1A and was made as follows. A 1,078-bp fragment of rDNA (extending from an *Sma*I site 220 bp upstream of transcription initiation to an *Xba*I site 858 bp downstream of initiation) was inserted into the polylinker of pBluescript SK$^+$ (Stratagene). The *Xba*I site, as well as an *Nde*I site in the rDNA portion, was then destroyed by cutting, filling in, and religation. The rDNA fragment was then excised by cutting with *Sma*I and cutting at an *Sst*I site in the pBluescript polylinker, and the fragment was inserted into pUC18. (The pUC18 recipient was cut with *Eco*RI, the end was filled in to blunt it, and then it was recut with *Sst*I; insertion of the rDNA fragment destroyed the *Sma*I site upstream of the rDNA promoter.) As shown in Fig. 1, the final construct has a polylinker located about 900 bp downstream of the transcription initiation site.

Fragments of the intergenic spacer were tested for 3′-end-forming ability by insertion into various restriction sites within the polylinker. The *Stu*I-*Hpa*I (positions −245 to +309), *Acc*I-*Hpa*I (positions −36 to +309), *Eco*RI-*Hpa*I (positions +2 to +309), *Eco*RI-*Pvu*II (positions +2 to +871), and *Eco*RV-*Sma*I (positions +1955 to +2162) fragments were all blunt ended and were inserted in either orientation into the *Sma*I site of the polylinker (Fig. 1A, position +897 of the termination test vector). Nucleotides are numbered according to the system used in Fig. 1B and 2A, which reflects the actual sequences inserted into the test vector. Position +1 designates the G residue in the *Eco*RI site adjacent to the REB1 protein binding site.

Transcription assays. Transcription extracts were prepared (either from the multiply protease-deficient strain BJ2168 or from a strain containing the *rpo41* mutation) as

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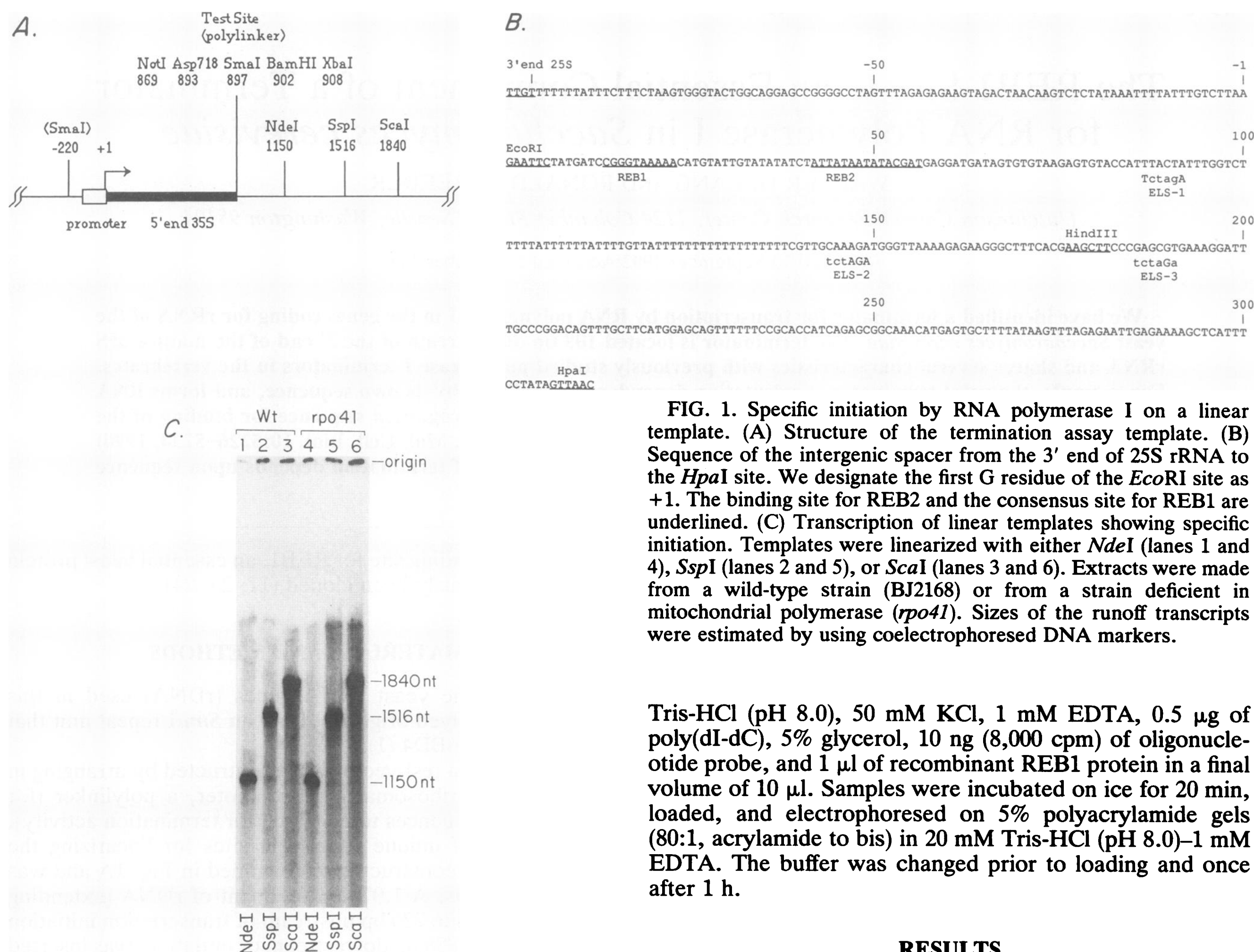

FIG. 1. Specific initiation by RNA polymerase I on a linear template. (A) Structure of the termination assay template. (B) Sequence of the intergenic spacer from the 3′ end of 25S rRNA to the *Hpa*I site. We designate the first G residue of the *Eco*RI site as +1. The binding site for REB2 and the consensus site for REB1 are underlined. (C) Transcription of linear templates showing specific initiation. Templates were linearized with either *Nde*I (lanes 1 and 4), *Ssp*I (lanes 2 and 5), or *Sca*I (lanes 3 and 6). Extracts were made from a wild-type strain (BJ2168) or from a strain deficient in mitochondrial polymerase (*rpo41*). Sizes of the runoff transcripts were estimated by using coelectrophoresed DNA markers.

described previously (28), except that cells were ground the minimum amount to achieve 90% breakage. More extensive grinding resulted in higher background in the runoff assay. Protein concentrations in the extracts were in the range of 15 to 25 mg/ml as assayed by the Bradford reaction (2).

Templates for runoff transcription were linearized at the *Nde*I site unless stated otherwise. Typical reactions were run at room temperature in a volume of 40 μl containing 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid)-KOH (pH 7.9), 50 mM KCl, 5 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid], 0.05 mM EDTA, 2.5 mM dithiothreitol, 10 μg of α-amanitin per ml, 10% glycerol, 0.5 mM (each) ATP, GTP, and UTP, 0.1 mM CTP, 10 μCi of α-^{32}P-CTP (800 Ci/mmol), 10 μl of extract, and 25 ng of template. Reactions were stopped by adding 250 μl 7 M urea–100 mM LiCl–0.5% sodium dodecyl sulfate–10 mM EDTA–10 mM Tris-HCl (pH 8.0) and were extracted twice with phenol-chloroform. tRNA carrier (10 μg) was added to each reaction, and nucleic acids were precipitated with 2.5 volumes of ethanol. Pellets were resuspended in a mixture of 5 μl of 98% formamide, 20 mM EDTA, and 0.025% (wt/vol) (each) xylene cyanole and bromphenol blue, boiled for 3 min, and loaded onto 3% polyacrylamide gels containing 7 M urea.

Gel retardation assays. Each reaction contained 20 mM Tris-HCl (pH 8.0), 50 mM KCl, 1 mM EDTA, 0.5 μg of poly(dI-dC), 5% glycerol, 10 ng (8,000 cpm) of oligonucleotide probe, and 1 μl of recombinant REB1 protein in a final volume of 10 μl. Samples were incubated on ice for 20 min, loaded, and electrophoresed on 5% polyacrylamide gels (80:1, acrylamide to bis) in 20 mM Tris-HCl (pH 8.0)–1 mM EDTA. The buffer was changed prior to loading and once after 1 h.

RESULTS

Establishment of an in vitro termination assay for RNA polymerase I. A template containing a yeast ribosomal gene promoter with convenient unique restriction sites located downstream was constructed (Fig. 1A). Aliquots of this template were cut at either the *Nde*I, *Ssp*I, or *Sca*I site and transcribed in vitro, and labeled runoff RNA was subjected to gel electrophoresis. As shown in Fig. 1C, RNA was generated in the various reactions to the correct length to extend from the initiation site at the gene promoter to each unique restriction site. We have previously shown that this extract supports specific initiation by RNA polymerase I on circular templates (5, 28). The data in Fig. 1C support the conclusion that specific initiation at the ribosomal gene promoter is also occurring on linear templates.

All of the transcription reactions described in this paper contained enough α-amanitin to eliminate transcription by RNA polymerase II. Previous analysis of the DNA fragment used as a promoter cassette (5, 28) showed that it contains no promoters for RNA polymerase III. Furthermore, the signals are insensitive to Tagetin (Epicentre Technologies, Madison, Wis.), a specific inhibitor of RNA polymerase III (data not shown). The only other possible interfering RNA polymerase activity is mitochondrial polymerase. A cryptic promoter for mitochondrial RNA polymerase is located at about position −75 relative to the initiation site for RNA polymerase I (5, 27), and at the level of resolution of the gel shown in Fig. 1C, RNA initiated at position −75 might

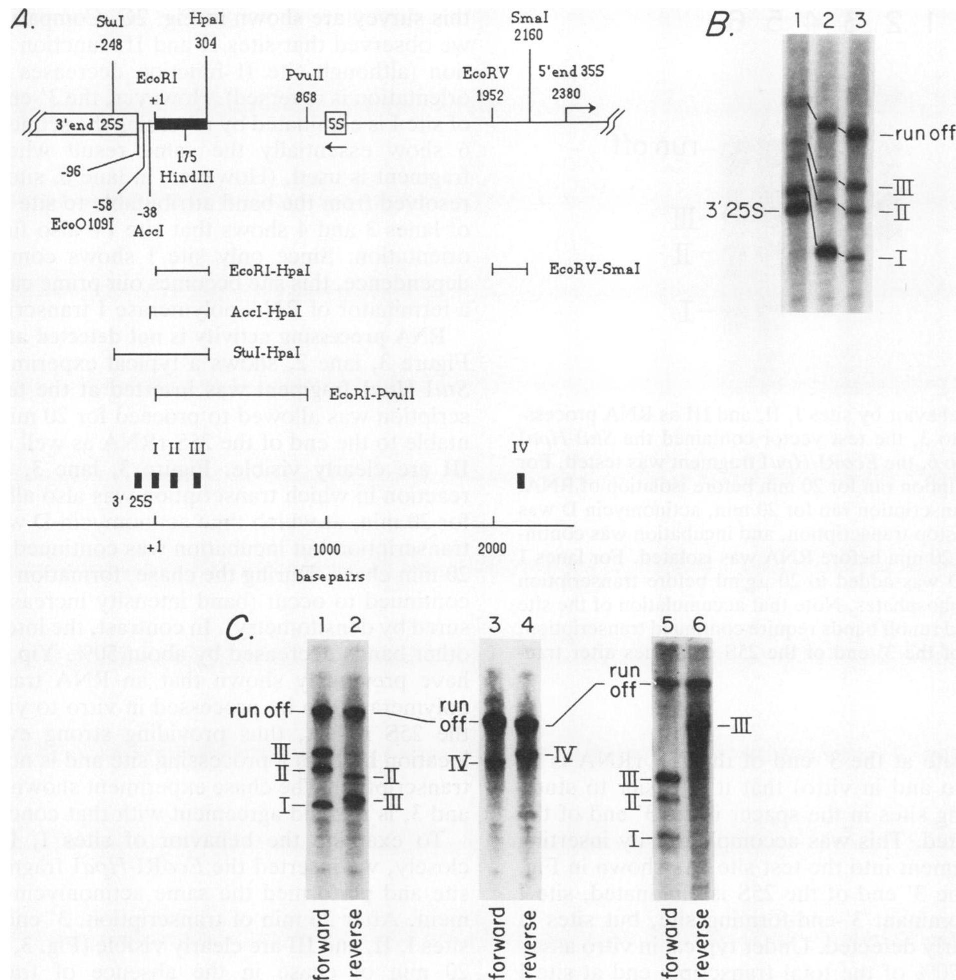

FIG. 2. Survey of the intergenic spacer for RNA 3'-end-forming sites. (A) Map of the intergenic spacer showing location of fragments that were tested. (B) Survey of 3'-end-forming sites between the *Stu*I site (position −248) and the *Hpa*I site (position +307). For lane 1, the *Stu*I-*Hpa*I fragment was inserted into the *Sma*I site of the test vector and transcribed after linearization further downstream at the *Nde*I site. For lane 2, the *Acc*I-*Hpa*I fragment was inserted into the test site. For lane 3, the *Eco*RI-*Hpa*I fragment was tested. The locations of runoff RNA bands attributable to the 3' end of the 25S rRNA, 3'-end formation at sites I, II, and III, and full-length runoff are indicated. (C) Orientation dependence of 3'-end-forming sites. The *Eco*RI-*Hpa*I fragment was inserted into the test site in either the forward (lane 1) or reverse (lane 2) orientation and tested in runoff transcription. Likewise, the *Eco*RV-*Sma*I fragment was tested in either the forward (lane 3) or reverse (lane 4) orientation, and the *Eco*RI-*Pvu*II fragment was tested in either the forward (lane 5) or reverse (lane 6) orientation. The locations of runoff RNA bands attributable to 3'-end formation at sites I, II, III, and IV are indicated.

possibly be confused with RNA initiated at position +1. However, mitochondrial polymerase makes little, if any, contribution to the runoff signal seen in this assay system. Figure 1C, lanes 1 through 3, shows the runoff signal generated in a whole-cell extract made from wild-type yeast cells. Lanes 4 through 6 show transcripts generated from the same truncated templates but utilizing an extract from an *rpo41* strain which lacks mitochondrial RNA polymerase activity (9). The relative size and intensity of the runoff RNAs are essentially identical in both cases. We conclude, therefore, that the RNA signals generated in this assay system are attributable to specific initiation by RNA polymerase I.

To assay for RNA 3'-end-forming elements, a polylinker was inserted into the template, thus locating a unique *Sma*I site 897 bp downstream from the position +1 initiation site. Various DNA fragments were then inserted into this *Sma*I site to see whether they were capable of generating additional, shorter runoff products indicative of RNA 3'-end formation.

Survey of the ribosomal gene spacer for RNA 3'-end-forming sites. We began by inserting into the test site a 550-bp *Stu*I-*Hpa*I fragment that extends from within the 25S rRNA coding region to a point downstream of the ribosomal gene enhancer. (The sequence of the relevant portion of this fragment is shown in Fig. 1B; the location of this fragment within the spacer is shown in Fig. 2A.) As shown in Fig. 2B, lane 1, the *Stu*I-*Hpa*I fragment caused the appearance of four shorter RNAs in the runoff assay. The shortest RNA has a length suggesting that its 3' end corresponds to the 3' end of the 25S rRNA itself. Other workers have presented evidence that this 3' end is formed by processing (26, 32), and our own experiments agree with that conclusion (Fig. 3). The 3' ends of the other three RNAs are formed at sites I, II, and III, which we have numbered in order of increasing distance from the ribosomal gene promoter.

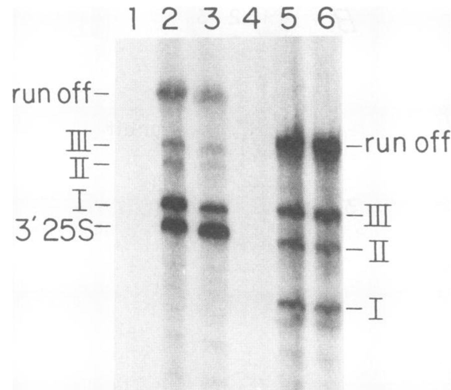

FIG. 3. Lack of behavior by sites I, II, and III as RNA processing sites. In lanes 1 to 3, the test vector contained the *Stu*I-*Hpa*I fragment. In lanes 4 to 6, the *Eco*RI-*Hpa*I fragment was tested. For lanes 2 and 5, transcription ran for 20 min before isolation of RNA. For lanes 3 and 6, transcription ran for 20 min, actinomycin D was added to 20 μg/ml to stop transcription, and incubation was continued for an additional 20 min before RNA was isolated. For lanes 1 and 4, actinomycin D was added to 20 μg/ml before transcription was initiated with triphosphates. Note that accumulation of the site I, II, and III bands and runoff bands require continued transcription, while accumulation of the 3′ end of the 25S continues after transcription is stopped.

The processing site at the 3′ end of the 25S rRNA is so active (both in vivo and in vitro) that it is easier to study other 3′-end-forming sites in the spacer if the 3′ end of the 25S is first eliminated. This was accomplished by inserting the *Acc*I-*Hpa*I fragment into the test site. As shown in Fig. 2B, lane 2, once the 3′ end of the 25S is eliminated, site I becomes the predominant 3′-end-forming site, but sites II and III are still clearly detected. Under typical in vitro assay conditions, about 70% of the total transcripts end at site I when the *Acc*I-*Hpa*I fragment is assayed. Figure 2B, lane 3, shows the result obtained when the *Eco*RI-*Hpa*I fragment is inserted into the test site. Shorter RNAs attributable to sites I, II, and III appear as seen previously with the *Acc*I-*Hpa*I fragment (compare lane 2 with lane 3), but in the case of the shorter fragment, only about 25% of the total transcripts are attributable to site I.

In Fig. 2C, the survey is extended to other parts of the intergenic spacer. Lane 1 shows a repeated experiment in which the *Eco*RI-*Hpa*I fragment was inserted into the test site. Lane 5 shows the results of inserting a larger *Eco*RI-*Pvu*II fragment. Again, only RNAs attributable to sites I, II, and III appear. Finally, lane 3 shows the result of inserting an *Eco*RV-*Sma*I fragment into the test site. With this fragment, a new truncated RNA appears, indicating another RNA 3′-end-forming location, which we have numbered site IV.

The relative locations of all of the 3′-end-forming sites we have identified in this survey are summarized at the bottom of Fig. 2A. This survey omits 1,100 bp of the intergenic spacer (from the *Pvu*II site to the *Eco*RV site). Apparently some element in this region is unstable in the vectors we are using, because we only recovered rearranged inserts (if any) in repeated attempts to subclone this region.

Only site I is orientation dependent. RNA polymerase I terminators in both frogs and mice function only when positioned in the forward orientation (10, 18). We therefore surveyed the yeast sites I, II, III, and IV to see whether any of them had similar orientation dependence. The results of this survey are shown in Fig. 2C. Comparing lanes 1 and 2, we observed that sites II and III function in either orientation (although site II function decreases somewhat when orientation is reversed). However, the 3′-end-forming ability of site I is eliminated by reversing the sequence. Lanes 5 and 6 show essentially the same result when a longer test fragment is used. (However, in lane 6, site II is not clearly resolved from the band attributable to site III.) Comparison of lanes 3 and 4 shows that site IV also functions in either orientation. Since only site I shows complete orientation dependence, this site becomes our prime candidate for being a terminator of RNA polymerase I transcription.

RNA processing activity is not detected at site I, II, or III. Figure 3, lane 2, shows a typical experiment in which the *Stu*I-*Hpa*I fragment was inserted at the test site and transcription was allowed to proceed for 20 min. 3′ ends attributable to the end of the 25S rRNA as well as sites I, II, and III are clearly visible. Figure 3, lane 3, shows a parallel reaction in which transcription was also allowed to proceed for 20 min, at which time actinomycin D was added to stop transcription but incubation was continued for an additional 20-min chase. During the chase, formation of the 25S 3′ end continued to occur (band intensity increased 60%, as measured by densitometry). In contrast, the intensity of all of the other bands decreased by about 50%. Yip and Holland (32) have previously shown that an RNA transcribed by SP6 polymerase can be processed in vitro to yield the 3′ end of the 25S rRNA, thus providing strong evidence that this location harbors a processing site and is not a terminator of transcription. The chase experiment shown in Fig. 3, lanes 2 and 3, is in good agreement with that conclusion.

To examine the behavior of sites I, II, and III more closely, we inserted the *Eco*RI-*Hpa*I fragment into the test site and performed the same actinomycin D chase experiment. After 20 min of transcription, 3′ ends attributable to sites I, II, and III are clearly visible (Fig. 3, lane 5), and after 20 min of chase in the absence of transcription, their intensities have decreased by about 20% (compare lanes 5 and 6). We conclude that sites I, II, and III require continued RNA transcription in order to form 3′ ends. In this respect, they all have the behavior expected of termination sites, in contrast to the 3′ end of the 25S rRNA, which has the behavior expected of a processing site.

Site I is sensitive to competition with its own DNA sequence. Another distinguishing characteristic of the vertebrate polymerase I terminators is that they require the binding of a sequence-specific protein in order to cause termination (10, 21). Because of this, termination in both mouse and frog systems is highly sensitive to competition caused by adding an excess of a DNA fragment containing the terminator sequence. We have tested yeast sites I, II, and III to see whether they have a similar sensitivity to competition. As shown in Fig. 4A, addition of increasing amounts of the *Eco*RI-*Hpa*I fragment (which contains sites I, II, and III) causes a rapid loss of 3′-end formation at site I. Site II is also sensitive to competition, but site III is completely resistant within this range of competitor.

The sensitivity of site I to competition is examined more closely in Fig. 4B. In this experiment, the competitor was a polymerized 30-bp double-stranded oligonucleotide (from positions 7 to 30 within the *Eco*RI-*Hpa*I fragment shown in Fig. 1B). 3′-end formation at site I is specifically and rapidly inhibited by this oligonucleotide, while sites II and III are unaffected.

We conclude that, among the RNA 3′-end-forming sites we have identified within the intergenic spacer, site I is the

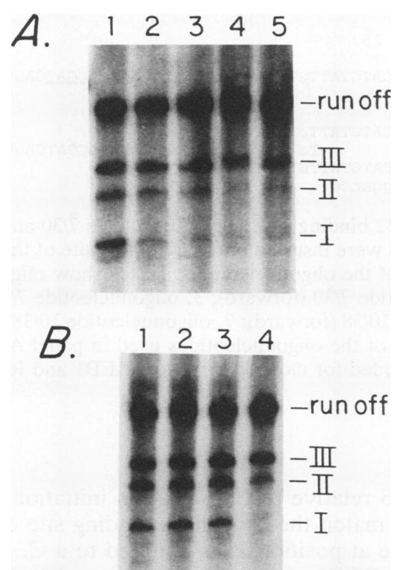

FIG. 4. Sensitivity of sites I, II, and III to competition with their own sequence. Test vector (25 ng) containing the *Eco*RI-*Hpa*I fragment was added to each transcription reaction. (A) Competition with the *Eco*RI-*Hpa*I fragment. The following amounts of gel-purified restriction fragment were added to each reaction before transcription began: none (lane 1), 50 ng (lane 2), 100 ng (lane 3), 200 ng (lane 4), and 400 ng (lane 5). (B) Competition with an oligonucleotide containing the REB1 site. Oligonucleotide 7/30 (sequence shown in Fig. 6B) was polymerized, and an aliquot was added to each reaction before transcription began, as follows: no competitor (lane 1), 0.5 ng (lane 2), 2 ng (lane 3), and 10 ng (lane 4).

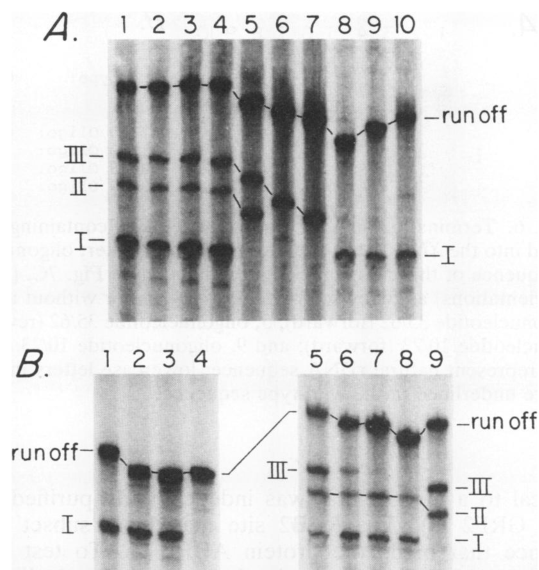

FIG. 5. 5′ and 3′ deletion mapping of sites I, II, and III. (A) Assay of deletions of the *Eco*RI-*Hpa*I fragment. For lane 1, *Not*I adaptors were added to the *Eco*RI-*Hpa*I fragment, and it was inserted into the *Not*I site at position 869 within the test vector polylinker. The procedure for lanes 2, 3, and 4 was the same as that for lane 1 but with a novel *Xba*I site introduced at positions 87, 144, and 185, respectively. For lanes 5 to 10, the *Xba*I sites were used to create and test various deletions of the *Eco*RI-*Hpa*I fragment. Coordinates of the sequences tested are as follows: 92 to 309 (lane 5), 147 to 309 (lane 6), 192 to 309 (lane 7), 1 to 87 (lane 8), 1 to 143 (lane 9), and 1 to 185 (lane 10). (B) Assay of finer deletions made by *Exo*III digestion. Lanes 1 to 4 show tests of 5′ deletions of the *Eco*RI-*Hpa*I fragment. Coordinates of the sequences tested are as follows: 1 to 87 (lane 1), 1 to 137 (lane 2), 1 to 23 (lane 3), 1 to 16 (lane 4), 2 to 309 (lane 5), 2 to 288 (lane 6), 2 to 266 (lane 7), 2 to 194 (lane 8), and 15 to 309 (lane 9).

only one which passes all the tests we have applied to be a terminator. It requires continued transcription for 3′-end formation, it is orientation dependent, and it is sensitive to competition. Although the precise molecular events occurring at site I remain to be determined, we will refer to it as a terminator in the rest of this paper.

5′ and 3′ deletion mapping of 3′-end-forming sites within the *Eco*RI-*Hpa*I fragment. The RNA 3′ ends formed by sites I, II, and III map to the approximate positions shown at the bottom of Fig. 2A. We also wished to know the location of the DNA sequences responsible for forming these 3′ ends. Accordingly, a series of mutants were made in which three novel *Xba*I sites were introduced at intervals across the *Eco*RI-*Hpa*I fragment (the novel *Xba*I sites are designated ELS-1, -2, and -3, and their sequence locations are shown in Fig. 1B). As shown in Fig. 5A, lanes 2 to 4, introduction of these novel *Xba*I sites did not cause any alteration in RNA 3′-end formation. The *Xba*I sites were then used as end points to produce a small series of 5′- and 3′-terminal deletions of the *Eco*RI-*Hpa*I fragment.

Results from assay of the 5′ deletion mutants are shown in Fig. 5A, lanes 5 through 7. Removing sequences from positions 1 to 91 eliminates 3′-end formation by site 1 (lane 5). Further deletion to position 146 eliminates 3′-end formation at site II (lane 6). Site III remains even after 5′ deletion to position 191 (lane 7).

The corresponding set of 3′ deletions is shown in Fig. 5, lanes 8 through 10. The deletion from positions 186 to 309 eliminates site III and greatly reduces site II. Site I is unaffected by any of the 3′ deletions of sequences between positions 88 and 309 (lanes 8 and 9).

To map the boundaries of sites I, II, and III further, we assayed a finer set of deletions within the *Eco*RI-*Hpa*I fragment, as shown in Fig. 5B. Deleting positions 24 to 309 still allows 3′-end formation at site I (lane 3), whereas removal of positions 17 to 309 (lane 4) and positions 1 to 14 (lane 9) abolishes it. Deleting positions 289 to 309 still allows 3′-end formation at site III (lane 6), but further removal of sequence to position 267 abolishes it (lane 7). Site II is not affected by deletion of positions 195 to 309 (lane 8).

The conclusions from these experiments are that sequences essential for site I map between positions 1 and 23, sequences for site II map between positions 106 and 194, and sequences for site III map between positions 192 and 288.

At this level of resolution, each of the RNA 3′ ends formed by sites I, II, and III maps to the same general location, as do the DNA sequences that cause the 3′ ends. Site II and its 3′ end map near a region of sequence that is almost pure AT. Site III maps between the *Hin*dIII site and the *Hpa*I site. Site I, the putative terminator, maps close to the *Eco*RI site at the 5′ end of the *Eco*RI-*Hpa*I fragment. We will now examine this latter region in more detail.

The terminator (site I) contains the binding site for REB1. DNase I footprinting and gel shift analyses have identified binding sites for two proteins, the so-called REB1 and REB2 sites, just downstream of the *Eco*RI restriction site (23, 24) (Fig. 1B). The REB1 site consists of 8 bp from positions 13 to 20, and the REB2 site is located at positions 42 to 56. The REB1 protein has been cloned (11) and is likely to be

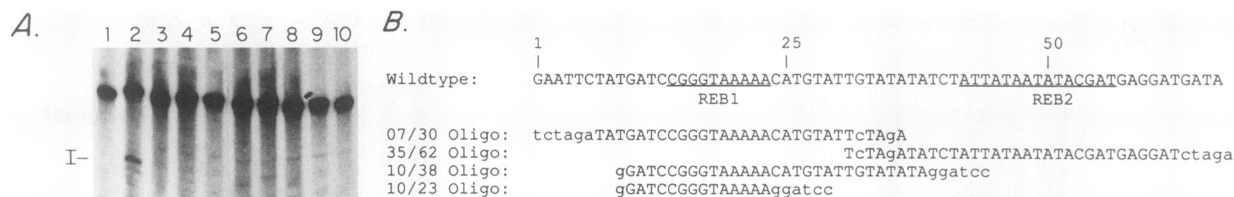

FIG. 6. Termination activity of oligonucleotides containing either the REB1 or REB2 binding site. Oligonucleotides 7/30 and 35/62 were inserted into the *Xba*I site of the test vector polylinker; oligonucleotides 10/38 and 10/23 were inserted into the *Bam*HI site of the polylinker. The sequence of these oligonucleotides is shown in Fig. 7C. (A) Termination activity of the oligonucleotides. Lanes show oligonucleotides (and orientations) as follows: 1 and 10, assay vector without any insert; 2, oligonucleotide 7/30 (forward); 3, oligonucleotide 7/30 (reverse); 4, oligonucleotide 35/62 (forward); 5, oligonucleotide 35/62 (reverse); 6, oligonucleotide 10/38 (forward); 7, oligonucleotide 10/38 (reverse); 8, oligonucleotide 10/23 (forward); and 9, oligonucleotide 10/23 (reverse). (B) Sequences of the oligonucleotides used in panel A. Uppercase letters represent natural rDNA sequence; lowercase letters indicate linker sequence added for cloning purposes. REB1 and REB2 binding sites are underlined in the wild-type sequence.

identical to a protein that was independently purified and called GRF2 (4). The REB2 site may be a subset of a sequence that binds the protein ABF1 (3). To test their possible involvement in termination, we prepared oligonucleotides which contained either the REB1 or REB2 site and cloned them into the test site. Results of the termination assay are shown in Fig. 6A.

The oligonucleotide containing the REB2 site showed no detectable termination (oligonucleotide 35/62) (Fig. 6A, lane 4). However, an oligonucleotide containing the REB1 site did exhibit termination activity (oligonucleotide 7/30, lane 2). This oligonucleotide contains 6 bp of sequence upstream of the CCGGGTAA core sequence which was identified by DNase I footprinting (23, 24). Other oligonucleotides containing the core sequence but which had only 3 bp of upstream sequence failed to cause termination (oligonucleotides 10/38 and 10/23, lanes 6 and 8). Lane 3 shows that the REB1 oligonucleotide which did cause termination still shows the same orientation dependence that is characteristic of RNA polymerase I terminators.

The experiments shown in Fig. 5 and 6 suggest that nucleotides between positions 7 and 23 are important for termination activity, a region which includes the REB1 consensus binding site. To examine this region in more detail, we made a series of mutants in which just two nucleotides at a time were mutated in stepwise fashion starting at one side of the consensus REB1 binding site and proceeding across to the other side. The sequences of these mutants are shown in Fig. 7C, and their ability to cause termination when inserted into the test site is shown in Fig. 7A. Beginning upstream of the REB1 site, the pairwise mutants at positions 7 to 8, 9 to 10, and 11 to 12 (designated 7/8, 9/10, and 11/12—lanes 2, 3, and 4, respectively) have no effect on termination. All of these mutations are also outside of the REB1 binding site. Mutation 13/14, which changes the first nucleotide of the binding site, eliminates termination (lane 5). The same is true for other mutations within the site (mutations 15/16, 17/18, and 19/20—lanes 6, 7, and 8, respectively). So far, the correlation between mutations which affect the REB1 site and mutations which eliminate termination is perfect. The apparent anomaly is the next cluster of mutations (21/22/23), in which three A residues were all changed to C's. These changes are within the REB1 consensus binding site (4), but they do not eliminate termination (lane 9). After making this cluster of mutations, however, we realized that the three changes inadvertently altered the sequence to that of a second REB1 binding site that is known to exist upstream of the ribosomal gene promoter (positions −216 to −225 relative to transcription initiation [24]). This site does not match the consensus binding site exactly; the purine residue at position 23 is mutated to a C.

Each of the REB1 mutants that was tested for termination activity in Fig. 7A was also tested for its ability to bind the REB1 protein in a gel shift assay (Fig. 7B). The correlation between termination activity and the ability to bind REB1 protein is complete. The behavior of mutant 21/22/23 is

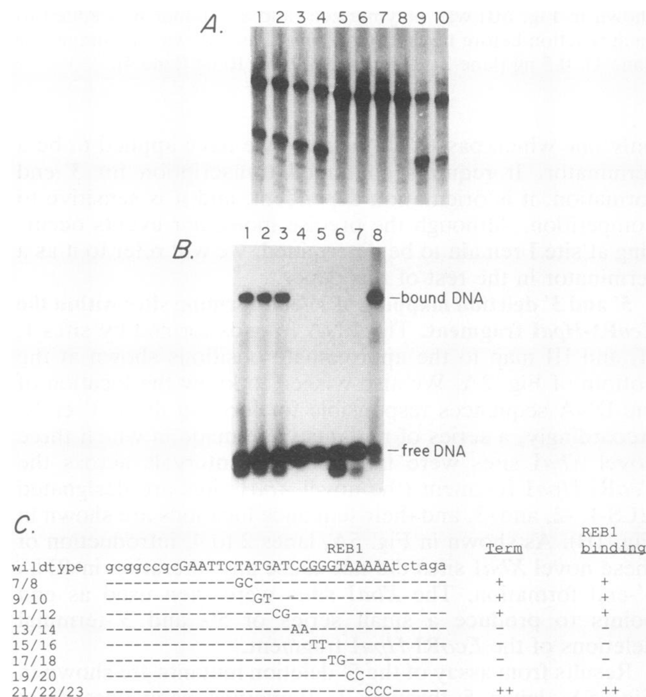

FIG. 7. REB1 site as an essential part of the polymerase I terminator. (A) Assay of REB1 binding site mutants for termination activity. Lanes: 1 and 10, termination by wild-type sequences 1 to 23; 2, mutant 7/8; 3, mutant 9/10; 4, mutant 11/12; 5, mutant 13/14; 6, mutant 15/16; 7, mutant 17/18; 8, mutant 19/20; and 9, mutant 21/22/23. (B) Assay of REB1 binding site mutants for binding to the REB1 protein. Lanes: 1, mutant 7/8; 2, mutant 9/10; 3, mutant 11/12; 4, mutant 13/14; 5, mutant 15/16; 6, mutant 17/18; 7, mutant 19/20; and 8, mutant 21/22/23. (C) Sequences of REB1 site mutants. The sequence of the consensus REB1 site is underlined. Term, termination activity. −, absent; +, present; ++, strongly present.

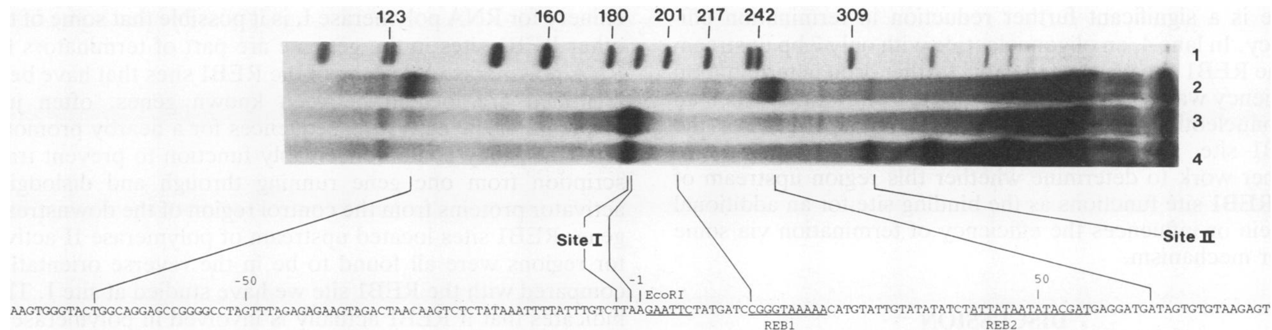

FIG. 8. Map of the 3′ end formed at site I 17 ± 2 bp upstream of the REB1 binding site. The length of the transcript specified by site I was compared with the length of runoff RNA products generated by transcription of vectors truncated at known sites. Lane 1 shows 5′-end-labeled DNA size standards (*Hpa*I digest of pBR322). DNA size standards do not run precisely the same as RNA standards of the same sequence. These DNA standards are included only to illustrate the degree of resolution obtained in this region of the gel. Lane 2 shows RNA size standards generated when the assay vector (with no insert) is transcribed after linearization at either the *Bgl*II or *Hin*dIII site. These two RNA standards have lengths of 123 and 256 nucleotides, corresponding to map positions −69 and +64, respectively. Lane 3, a third RNA size standard, was generated by deleting part of the assay vector (to shorten it) and linearizing at the Asp-718 site. This produces an RNA 191 nucleotides in length whose 3′ end corresponds to map position −2. Lane 4, an assay template containing the *Eco*RI-*Hpa*I fragment at the test site, was modified by removing 706 bp of vector sequence between the polymerase I promoter and the test site. Upon transcription of this template, RNA generated by site I migrates slightly faster than the position −2 marker RNA. This places the 3′ end formed by site I 17 ± 2 bp upstream of the REB1 consensus binding site.

especially interesting. It has been shown that this particular REB1 binding sequence has a higher level of affinity for the REB1 protein (4) than wild-type sequence does. Our data agree with this observation in that the 21/22/23 mutant reproducibly shifts a larger fraction of the probe than does the wild-type sequence in gel shift assays (Fig. 7B, compare lane 3 with lane 8). In many assays, mutant 21/22/23 also is a more efficient terminator than the wild-type sequence is, but this effect is not clearly seen in Fig. 7B. Thus, within this set of mutants, the correlation between sequences required for REB1 binding and sequences required for termination by RNA polymerase I is perfect.

We conclude that the REB1 site is an essential component of the yeast polymerase I terminator. This implies that either REB1 itself or some unknown protein with identical DNA binding specificity is essential for termination.

3′ ends formed at site I map about 17 bp upstream of the REB1 site. An additional characteristic of both the mouse and frog polymerase I terminators is that the 3′ ends map about 11 to 15 bp upstream of the essential terminator protein binding site (15, 17). Finer mapping of the 3′ end specified by site I shows that the same is also true for the yeast terminator (Fig. 8). In this experiment, marker RNA molecules of precisely known length were generated by RNA polymerase I runoff transcription from the assay template truncated at appropriate sites. The three marker RNAs we used had lengths that would correspond to 3′-end formation at positions −69 and +64 (lane 2) and −2 (lane 3). In lane 4, we show that RNA produced by 3′-end formation at site I migrates slightly faster than the position −2 marker. This places the 3′ end specified by site I about 17 ± 2 bp upstream of the boundary of the REB1 site that is essential for its formation. Once again, the yeast terminator has characteristics similar to its vertebrate counterparts. Similar experiments with less resolution located the 3′ ends formed by site II and site III around positions 155 and 250, respectively (data not shown).

Efficiency of termination at site I is influenced by sequences upstream of the REB1 site. The mutagenesis shown in Fig. 7 demonstrates that the REB1 site is an essential component of the terminator. However, the efficiency of termination is dependent upon sequences upstream of the REB1 site. This is illustrated in Fig. 9. Figure 9, lane 1, shows the result of inserting the *Acc*I-*Hpa*I fragment into the test site. This fragment contains 49 bp of normal sequence upstream of the REB1 consensus binding site and is the most efficient terminator fragment we have assayed (about 70% of the transcripts terminate at site I). In lane 2, a shorter *Eco*RI-*Hpa*I fragment was blunt ended, *Not*I linkers were attached, and the result was inserted into the test site. These manipulations allowed retention of the first G of the *Eco*RI recognition site (13 bp upstream of the REB1 site). Termination occurs with this fragment, but it is clearly less efficient than with the *Acc*I-*Hpa*I fragment shown in lane 1. In lane 3, the *Eco*RI-*Hpa*I fragment is assayed again, but this time it was blunt ended and was inserted directly into the *Sma*I site of the test site. These manipulations result in the loss of the first G of the *Eco*RI recognition sequence, and

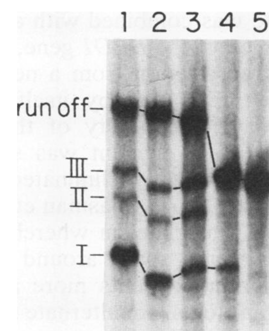

FIG. 9. Dependence of the efficiency of termination at site I upon sequences upstream of the REB1 site. DNA fragments with various amounts of natural sequence upstream of the REB1 consensus binding site were assayed for the relative efficiency of termination. Lanes are as follows: 1, assay of the *Acc*I-*Hpa*I fragment assayed (49 bp of upstream sequence); 2, *Eco*RI-*Hpa*I fragment with *Not*I adaptors (13 bp upstream); 3, *Eco*RI-*Hpa*I fragment blunt ended into the *Sma*I site (12 bp upstream); 4, oligonucleotide 7/30 (7 bp upstream); and 5, oligonucleotide 10/23 (4 bp upstream).

there is a significant further reduction in termination efficiency. In lane 4, an oligonucleotide with only 7 bp upstream of the REB1 site was tested and a further drop in termination efficiency was observed. Finally, lane 5 shows that when an oligonucleotide is tested with only 4 bp upstream of the REB1 site, termination is not detectable. It will require further work to determine whether this region upstream of the REB1 site functions as the binding site for an additional protein or influences the efficiency of termination via some other mechanism.

DISCUSSION

What is the function of the other REB1 binding sites in the yeast genome? The experiments reported in this paper show that a protein with DNA binding characteristics indistinguishable from those of REB1 is involved in termination of RNA polymerase I transcription in yeast. It will require further experiments to definitively determine whether or not REB1 is the terminator protein. But, on the reasonable assumption that the terminator protein actually is REB1, what is already known about this protein is worth reviewing.

REB1 is probably identical to GRF2, a protein that was independently identified and purified by the Kornberg group (4, 8). A computer search has revealed REB1 consensus binding sites in a number of locations throughout the yeast genome, including near UAS elements for many polymerase II promoters, at telomeres, and near a centromere (4). Within the rDNA, there are two REB1 sites, one which we have studied for this paper at site I, downstream of the 3′ end of the 25S rRNA, and a second located about 220 bp upstream of the gene promoter but in the opposite orientation (23). Considering the orientation dependence of site I, it is unlikely that the REB1 site near the gene promoter is involved in polymerase I termination.

REB1 is an essential gene in yeasts (11, 24). Since it also has numerous binding sites throughout the genome (4, 31), the possibility arises that it is involved in more than just termination of transcription for RNA polymerase I. One possibility is that REB1 sites function as part of complex activation regions to stimulate transcription from certain promoters. For example, Fedor et al. (8) have shown that REB1 binding to a site between the promoters for the *GAL1* and *GAL10* genes excludes nucleosomes from about 230 bp of DNA and phases nucleosome positioning to either side. When the REB1 site was combined with a T-rich activator from the control region of the *DED1* gene, a striking synergism in activating transcription from a nearby TATA box was observed. The REB1 site by itself was essentially inactive in this assay. The ability of the REB1 site to synergize with the *DED1* element was strongly distance dependent and was completely eliminated by spacing the two elements 121 bp apart. As Chasman et al. (4) point out, these results suggest a mechanism whereby REB1 binding creates a nucleosome free space around its site, thereby making nearby activator elements more accessible to the binding of activator proteins. An alternate suggestion is that REB1 is a repressor of RNA polymerase II transcription. Wang et al. (31) placed an REB1 site between the CYC1 upstream activator and its cognate TATA element and noted that this caused repression of the CYC1 promoter. However, it seems possible that both the activating and repressive effects of REB1 binding could be incidental to the fact that REB1 is a strong DNA binder and is likely to displace other nearby DNA binding proteins.

If the REB1 site actually is part of the termination machinery for RNA polymerase I, is it possible that some of the other REB1 sites in the genome are part of terminators for RNA polymerase II? Many of the REB1 sites that have been identified are located between known genes, often just upstream of the activating sequences for a nearby promoter (4). Thus, they could conceivably function to prevent transcription from one gene running through and dislodging activator proteins from the control region of the downstream gene. REB1 sites located upstream of polymerase II activator regions were all found to be in the reverse orientation compared with the REB1 site we have studied at site I. This indicates that if REB1 actually is involved in polymerase II termination, the orientation of the element is polymerase specific.

In vivo searches for the terminator of RNA polymerase I transcription. The 3′ end of the yeast rRNA coding region has previously been studied in vivo in attempts to locate possible terminators of polymerase I transcription. For example, Planta's group has studied transcription of ribosomal minigenes introduced on multicopy plasmids and reports identification of five sites of 3′-end formation (12, 30). The first site, T0, corresponds to the 3′ end of the 25S rRNA and was concluded to be an RNA processing site on the basis that this 3′ end largely disappears in yeast cells carrying the *rna 82.1* mutation, a mutation that inactivates an endonuclease involved in 3′-end processing of 5S RNA (26). Yip and Holland (32) have further shown that an artificial transcript can be processed in vitro at this site to yield the 3′ end of the 25S. In our in vitro transcription assays, we also observed a 3′ end that maps to the terminus of the 25S rRNA (Fig. 2B, lane 1; this corresponds to position −96 of the sequence shown in Fig. 1B). The results of actinomycin D chase experiment shown in Fig. 3 lend further support to the conclusion that this is an RNA processing site and not a termination site.

Other in vivo sites of 3′-end formation identified by Planta's group include T1, T2, T3, and Tp. T1 3′ ends are located downstream of the 3′ end of the 25S rRNA (Fig. 1B, between positions −81 and −50) and also appear to be attributable to processing (30). We have not observed any termini that would correspond to T1 in our in vitro assays.

T2 maps to the beginning of a T-rich stretch between the *Eco*RI and *Hin*dIII sites (about position 100 in Fig. 1B) and was concluded to be the most likely candidate for an authentic terminator (30). We do not observe a 3′ end at precisely this location in vitro. However, it is possible that T2 is the same as what we are calling site II. Site II maps to the other side of the T-rich stretch as compared with T2 (about position 155 in Fig. 1B). In S1 nuclease protection assays (as employed by the Planta group), the T-rich hybrid could be lost, yielding an apparent map position coinciding with T2. As we have shown in Fig. 2C, 3′-end formation at site II occurs when the sequence is in either orientation. Therefore, we have concluded that site II (and presumably T2 as well) is likely to be a simple roadblock caused by the tight binding of one or more proteins. It is unlikely, however, to be an authentic terminator of polymerase I transcription.

T3 refers to two locations, T3A and T3B, which would be at about map positions 550 and 890 in Fig. 2A. This would put them within the *Eco*RI-*Pvu*II fragment that was surveyed in Fig. 2C, lane 5. We found nothing in vitro that corresponds to these 3′ ends. Tp was reported to be about −300 bp upstream of the site of transcription initiation for the ribosomal gene promoter. Our approximate mapping suggests that site IV is near the *Sma*I site upstream of the

promoter at position −216; however, in the absence of finer mapping, it is possible that Tp and site IV are the same.

Previous in vivo searches have not found 3′ ends that would correspond to the site I we observed in vitro (12, 30). We can think of two possible reasons why this discrepancy exists. First, multicopy rDNA plasmids in yeast may be subject to transcription by more than one type of polymerase. Such transcription could have the dual effect of obscuring termini attributable to RNA polymerase I as well as creating additional termini having nothing to do with polymerase I transcription. A second problem is that the 3′ end formed at site I is located within a few nucleotides of the *Eco*RI site. Many of the S1 protection probes used in the in vivo studies (12, 30) were made from the *Eco*RI-*Hpa*I fragment and could not have detected that particular 3′ end.

Direct evidence that the REB1 site (and site I) is involved in terminator function in vivo as well as in vitro comes from the work of Holland's group (22). They assayed RNA 3′-end formation on ribosomal minigenes utilizing a low-copy-number *CEN* plasmid. When the *Eco*RI-*Hpa*I fragment was placed downstream of a ribosomal gene promoter, it generated a single 3′ terminus. They further showed that upon deletion of sequences corresponding to positions 8 to 46 (Fig. 1B), including the REB1 site, 3′-end formation was eliminated and longer read-through products were observed. It is interesting that in vivo on a low-copy-number plasmid, only the 3′ terminus specified by the REB1 binding element (site I) is observed and there are no 3′ termini corresponding to sites II and III. This leads us to think that termination at site I is very efficient in vivo and that transcription ordinarily never reaches sites II and III.

What causes 3′-end formation at sites II and III? At both of these locations, 3′-end formation is independent of orientation. This suggests that at both locations, tightly bound proteins may act as roadblocks for RNA polymerase I approaching from either direction. Lac repressor protein has been reported to block elongating RNA polymerase I and II (6, 14), and it is likely that other tightly bound proteins would have similar effects. The question then becomes, what proteins are likely to be bound at sites II and III? The 3′ ends formed at site II map closely to what appears to the the upstream boundary of the rDNA enhancer element. In vivo work (7, 22, 29) indicates that the enhancer element extends from about position 150 to slightly beyond the *Hin*dIII site. We have shown elsewhere that the enhancer element is highly active in the yeast whole-cell extract (27a), and thus we would expect proteins to be bound to the enhancer element in our termination assays as well. We conclude it is possible that the 3′ ends observed at site II are attributable to a roadblock imposed by one or more enhancer binding proteins.

The explanation for site III is more speculative. Some reports fail to detect any transcription enhancer activity in this region (7, 22). However, the work of Stewart and Roeder (29) clearly showed that there was something in this region that strongly affected recombination activity. We also note a recent paper showing that a terminator of replication maps in this same general region (13). We might be detecting a transcription roadblock caused by proteins binding to one or more of these elements in vitro.

Comparison of site I with other known terminators of polymerase I transcription. The yeast polymerase I terminator has characteristics very similar to those of terminators that have been previously studied in both mice and frogs. To begin with, the yeast terminator is located some distance (108 bp) downstream of the 3′ end of the mature 25S, and the 3′ end of the 25S is formed by processing (the only known exception to this rule is *Ascaris lumbricoides*, in which the terminator and the 3′ end of the largest mature rRNA coincide [25]). The yeast terminator requires a specific sequence in the DNA and is the binding site for a specific protein (REB1). Termination is not caused by a simple roadblock, however, as evidenced by the fact that the terminator only functions in one orientation. Finally, the 3′ ends formed at site I map about 17 bp upstream of the protein binding site.

An interesting characteristic of the yeast polymerase I terminator is the influence of sequences upstream of the REB1 protein binding site on the efficiency of 3′-end formation (Fig. 9). The related effects of context sequences have also been noted in the vertebrate systems. For example, in mice, sequences both upstream and downstream of the *Sal*I box influence termination efficiency and the precise location of the 3′ ends (16). In the frog system, we saw no context effect in vitro (21), but the efficiency of termination appeared to be context dependent in oocyte injection experiments (17).

Is REB1 the terminator protein? We believe we have shown in this paper that the REB1 binding site is an essential element of an RNA polymerase I terminator in yeast. The obvious question is whether or not it is the REB1 protein whose binding to that site causes termination. To summarize the results from a number of frustrating experiments, we still do not know the answer to that question. J. Warner and coworkers have generously supplied us with recombinant REB1 protein and with strains of yeast carrying temperature-sensitive mutations in the REB1 protein. To date, we have been unsuccessful in obtaining any significant stimulation of termination by adding back excess REB1 protein. We have added back recombinant protein made in bacteria as well as native protein partially purified from yeast extracts. The adding back has been made to extracts that were partially depleted in REB1 binding activity either by competition with specific oligonucleotides or by heat shock of temperature-sensitive extracts. Among the several possibilities, it may be that either (i) REB1 really is the relevant protein and we have not found the correct protocol for reconstitution; (ii) the real terminator protein is something other than REB1, which happens to bind to essentially the identical REB1 binding site; or (iii) an additional factor is involved in termination, and it is limiting in our extracts. It will require further work, including some fractionation of the extract, to decide between these possibilities.

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