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In Vitro Definition of the Yeast RNA Polymerase I Enhancer

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In vitro conditions are reported under which an *Eco*RI-*Hpa*I fragment of the *Saccharomyces cerevisiae* ribosomal gene spacer will enhance transcription from an adjacent RNA polymerase I promoter. Enhancement is largely independent of orientation and distance and is proportional to copy number. Mapping experiments reveal that two separate regions of the *Eco*RI-*Hpa*I fragment are independently capable of promoter stimulation. These regions appear to correspond to elements which have been shown by previous workers to cause enhancement in vivo. Using the detergent Sarkosyl to limit the number of rounds of transcription from each promoter, we found that the degree of enhancement is similar whether one or many rounds of transcription occur. This finding supports a model in which the enhancer increases the number of stable promoter complexes but does not alter the loading of polymerase on an active promoter. Once the stable promoter complex is formed, the enhancer can be physically severed from the promoter with no loss of enhancement. Likewise, the upstream activation region of the promoter can be severed from the core promoter domain once the stable complex has been formed. These results are interpreted to mean that the enhancer functions only to assist stable complex formation and, once that is accomplished, the enhancer is dispensable.

The genes coding for 35S rRNA precursor in the yeast *Saccharomyces cerevisiae* are known to contain two distinct DNA elements which influence transcription initiation by RNA polymerase I. One of these elements is the gene promoter situated at the 5′ end of the 35S coding region. Analysis of the gene promoter both in vivo (32) and in vitro (5, 23) shows that it consists of about 150 bp of sequence which slightly overlaps the site of transcription initiation and that it has a structure similar to that of vertebrate ribosomal gene promoters.

The other DNA element that influences initiation is the ribosomal gene enhancer. The enhancer was originally described as a 180-bp *Eco*RI-to-*Hin*dIII fragment that is located on the other side of the intergenic spacer from the gene promoter (10, 11), close to the 3′ end of the 35S precursor (Fig. 1A). When this fragment is placed in *cis* to a ribosomal gene promoter and assayed in vivo, it can cause a 20- to 50-fold increase in the activity of the promoter. In addition to its activity as an enhancer, the *Eco*RI-to-*Hin*dIII fragment has other activities. For example, in vivo sites of RNA 3′ end formation have been mapped to this fragment (20, 31, 41), and we have shown that a short region near the *Eco*RI site behaves in vitro as a terminator for RNA polymerase I (27). Some naturally occurring variants of this fragment can also support in vitro initiation by an α-amanitin-resistant polymerase, presumably RNA polymerase I (31, 39).

We have recently reported a simple procedure for obtaining whole cell extracts from yeast cells that are active for polymerase I transcription as well as for transcription by polymerases II and III (35). Under the proper conditions, the enhancer fragment will reproducibly stimulate ribosomal gene promoter activity in this extract. In this study, we took advantage of this finding to begin definition of the yeast ribosomal gene enhancer as it functions in vitro and to explore its mechanism of action.

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MATERIALS AND METHODS

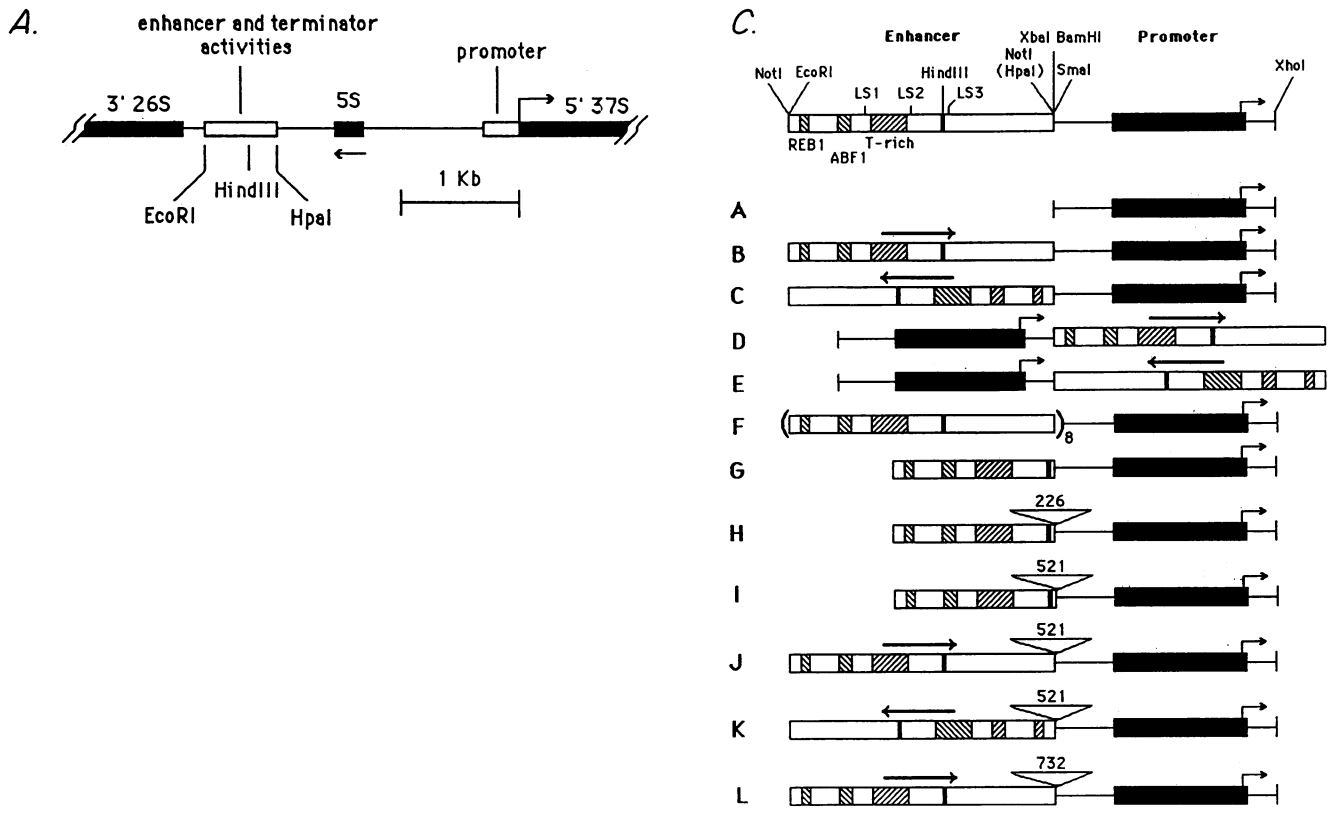
Constructs. Diagrams of the constructs used are shown in Fig. 1C (constructs A through L) and Fig. 6A (constructs M through Q). Details of their structure are as follows.

Construct A is a yeast RNA polymerase I promoter in which the *Sma*I-*Xho*I fragment of pYr11A (5), bearing the yeast ribosomal gene promoter, is cloned into the large *Sma*I-*Xho*I fragment of pBluescript SK$^-$ (Stratagene). This construct was previously referred to as pBYr11A (36).

Constructs B and C contain the polymerase I promoter with the enhancer immediately upstream in the forward and reverse orientations. The 306-bp *Eco*RI-*Hpa*I fragment of the yeast rDNA repeat, located approximately 2.1 kb upstream of the ribosomal gene promoter, was isolated from pBD4 (1) and then cloned into the *Not*I site of pBYr11A after addition of *Not*I linkers to the *Eco*RI and *Hpa*I sites. The enhancer was obtained in the forward (B) and reverse (C) orientations. Its sequence (Fig. 1B) on both strands was redetermined by the chain termination method, using Sequenase (U.S. Biochemical).

Constructs D and E contain the polymerase I promoter with the enhancer immediately downstream in the forward and reverse orientations. The promoter fragment of pBYr11A was subcloned to put a *Kpn*I site immediately upstream of its *Sma*I site (*Sma*I is at −216 with respect to +1) and a *Bam*HI site immediately downstream of its *Xho*I site. To place the enhancer downstream of the promoter, the *Kpn*I-*Bam*HI promoter fragment was inserted into the large *Bam*HI-*Kpn*I fragment of pBluescript SK$^-$ containing the enhancer fragment (with *Not*I adapters) in its *Not*I site in both orientations (see constructs B and C). The net result of these manipulations is that for constructs B and C, the polymerase I promoter transcribes in the same direction as the ampicillin gene on the vector; in constructs D and E, the polymerase I promoter and the ampicillin gene are divergent.

To make construct F (polymerase I promoter with eight head-to-tail copies of the enhancer cloned immediately upstream in the forward orientation), the *Eco*RI site of construct B was changed to a *Bgl*II site by fill-in with Klenow fragment and ligating on a *Bgl*II linker. Two fragments were



B.

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        +1                  +20                 +40                 +60                 +80
GCGGCCGCGAATTCTATGATCCGGGTAAAAACATGTATTGTATATATCTATTATAATATACGATGAGGATGATAGTGTGTAAGAGTGTACCATTTACTAT
NotI    EcoRI       REB 1                       ABF 1                                   TctagA
                                                                                        ELS 1

      +100                +120                +140                +160                +180
TTGGTCTTTTTATTTTTTATTTTGTTATTTTTTTTTTTTTTTTTTCGTTGCAAAGATGGGTTAAAAGAGAAGGGCTTTCACGAAGCTTCCCGAGCGTGAA
                                            tctAGA                              Hind III  tctaGa
                                            ELS 2                                         ELS 3

      +200                +220                +240                +260                +280
AGGATTTGCCCGGACAGTTTGCTTCATGGAGCAGTTTTTTCCGCACCATCAGAGCGGCAAACATGAGTGCTTTTATAAGTTTAGAGAATTGAGAAAAGCT

      +300
CATTTCCTATAGTTAGCTTGCGGCCGC
      (HpaI)  NotI
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FIG. 1. Location and sequence of the enhancer-containing fragment from yeast rDNA. (A) Diagram of an intergenic spacer region from the *S. cerevisiae* ribosomal genes showing the location of the *Eco*RI-*Hpa*I fragment which has been shown to contain enhancer elements by in vivo assays. (B) Nucleotide sequence of the *Eco*RI-*Hpa*I enhancer fragment. *Not*I linkers (double underlines) were added to this fragment to facilitate further manipulation. Single underlines indicate key restriction sites as well as the binding sites for two known proteins, REB1 (18) and ABF1 (reviewed in reference 8). The locations of three linker scanner mutations are indicated below the main sequence line (enhancer linker scanner [ELS] mutations 1, 2, and 3). (C) Structures of various enhancer-promoter combinations which have been assayed for enhancer activity. Details of these constructs are described in Materials and Methods.

then isolated: an *Xho*I-*Bam*HI fragment containing the enhancer and vector sequences, and an *Xho*I-*Bgl*II fragment including only the enhancer and promoter. These fragments were ligated to produce an enhancer head-to-tail dimer upstream of the promoter. This process was repeated twice to create construct F.

Construct G is an enhancer deletion mutant (sequences from 184 to 306 removed) cloned immediately upstream of the promoter. Construct B was used to make three linker scanner mutants (LS1 to LS3) of the enhancer. Unique *Xba*I sites were inserted by site-directed mutagenesis (25) at positions indicated on the map (Fig. 1B). Construct G was made by cutting LS3 with *Xba*I and religating the large fragment.

Construct H is the same as construct G except that the enhancer fragment is pushed 226 bp apart from the promoter by insertion of a 226-bp *Alu*I fragment from pBR322 into the *Sma*I site.

Construct I is the same as construct G except the enhancer fragment is pushed 521 bp apart from the promoter by insertion of a 521-bp *Alu*I fragment from pBR322 into the *Sma*I site.

Constructs J and K are the same as construct B except that the entire enhancer, in the forward (J) and reverse (K) orientations, is pushed 521 bp apart from the promoter as described for construct I.

In construct L, the entire enhancer was pushed 732 bp away from the promoter by insertion of the 732-bp *Bam*HI-*Bgl*II fragment of pCITExUBF LSA (30) into the *Bam*HI site between the enhancer and promoter in construct B.

Construct M is a promoter-only construct identical to construct A except that the 521-bp *Alu*I fragment of pBR322 is present in the *Sma*I site in the same orientation as in constructs I to K.

Constructs N to Q are deletion mutants of the enhancer pushed 521 bp apart from the promoter as in constructs I to K. The deletion mutants were made from the enhancer linker scanners described above. Construct N is a 5′ deletion of the *Eco*RI-LS2 region of the enhancer. This deletion removes the REB 1, ABF1, and T-rich sites. Construct O is a 5′ deletion of the *Eco*RI-LS3 region of the enhancer. This deletion removes all sequences to just 3′ of the *Hin*dIII site of the enhancer. Construct P is a 3′ deletion of the *Hpa*I-LS2 region of the enhancer. This deletion removes sequences up to the start of the T-rich region. Construct Q is a 3′ deletion of the *Hpa*I-LS3 region of the enhancer. This deletion removes sequences up to but not including the *Hin*dIII site and three nucleotides on its 3′ side.

The internal control used in some reactions was a promoter-only construct equivalent to construct A except that the RNA product was tagged with a different linker sequence (described as pYr12-5 [35]).

Preparation of transcription extracts. Extracts (100,000 × *g* supernatants) were prepared according to Schultz et al. (35) from cells grown in YEPD to an optical density at 600 nm of 1 to 4 and broken with a mortar and pestle under liquid nitrogen. Extracts that supported enhancer activity were routinely obtained from the protease-deficient strain BJ2168 (*MAT***a** *leu2 trp1 ura3-52 prb1-1122 pep4-3 prc1-407*) as well as strains with the normal complement of cellular proteases, e.g., rpo 41, described in reference 12 [*MAT***a** (*pet*) *ade2-101 lys2-801 his3Δ300 tyr1 RPO41*::Tn*10URA3*] and W303-1a, originally from R. Rothstein, Columbia University (*MAT***a** *ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3-1*). Strain W303-1a has been used by Warner and colleagues to characterize the yeast enhancer in vivo (10, 17).

Transcription reactions and assay of transcription products. Transcription reactions were performed at 22°C as described previously (35) except that they were in 20 μl, they contained 90 mM KCl, and the nucleotides were at 500 μM each. All reactions in a series received the same amount of extract, in the range of 40 to 80 μg of protein, and were run for 30 to 60 min. To detect stimulation by the enhancer in crude extracts, it was necessary to titrate out nonspecific DNA-binding activities by preincubation with vector DNA. This DNA was usually added in the linear form, since one inhibitory activity apparently has a high affinity for DNA ends. The preincubation with the nonspecific competitor was performed for 5 min, usually using 20 μg of pBluescript SK^- cut with *Pvu*II per ml. In some experiments, we used pBR322 cut with *Hpa*II instead. The incubation with the nonspecific competitor was followed by a 5-min incubation with a specific template. Unless otherwise indicated, templates transcribed as linear DNAs were cut at the *Sca*I site in the vector, which places the cut end 1.5 to 1.8 kb upstream of the rDNA sequences being tested, depending upon the construct. In most cases, single templates were added in the range of 5 to 25 ng/ml. When an internal control was included, it was added in the same concentration as the test construct, to give a total template concentration of 10 to 50 ng/ml. Subsequent steps in the reactions are described in the text and figure legends. RNA products from the test constructs (A to Q; see above) were detected by S1 nuclease protection analysis using a 50-base oligonucleotide probe as described previously (35). In Fig. 6, the internal control promoter was pYr12-5. A 60-base probe complementary to the region from −15 to +45 of pYr12-5 was used to specifically detect products from this construct.

Signals were quantitated by using a Molecular Dynamics PhosphorImager. This instrument has a large dynamic range and is capable of quantitating signals which appear overexposed on autoradiographs as well as signals which are not visible with normal exposure times (for example, Fig. 7).

RESULTS

The *Eco*RI-*Hpa*I spacer fragment will enhance RNA polymerase I transcription in vitro. The initial in vivo studies of Elion and Warner identified enhancer activity within a 180-bp *Eco*RI-*Hin*dIII fragment of the yeast rDNA intergenic spacer (11, 10). Later studies have suggested, however, that sequences immediately to the right of the *Hin*dIII site can also influence enhancer activity (31). In addition, Stewart and Roeder (38) also found evidence for transcriptional enhancement activity in a region even further to the right, just to the left of the *Hpa*I site. Considering all of these studies, we thought it prudent to begin work with the 310-bp *Eco*RI-*Hpa*I fragment whose sequence is shown in Fig. 1B. This fragment contains all regions that have ever been reported to enhance RNA polymerase I transcription in vivo. To facilitate working with this fragment, *Not*I linkers were added to each end, a process which destroyed the *Hpa*I site on the right-hand end. Nonetheless, we will continue to refer to this fragment as the *Eco*RI-*Hpa*I fragment for the remainder of this report.

To test for enhancer activity of the *Eco*RI-*Hpa*I fragment in vitro, the fragment was linked to a yeast ribosomal gene promoter by inserting it at an *Sma*I site located 216 bp upstream of the site of transcription initiation. The structure of this enhancer-promoter construct is shown schematically in Fig. 1C (construct B). The promoter used in all of our enhancer assays is the same yeast ribosomal gene promoter

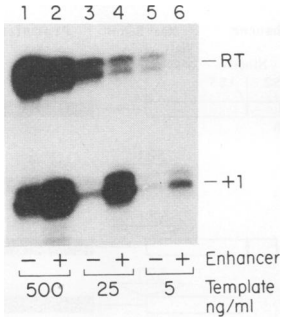

FIG. 2. Evidence that the *Eco*RI-*Hpa*I fragment enhances RNA polymerase I transcription in vitro. Construct A (without the *Eco*RI-*Hpa*I fragment; Fig. 1C) or construct B (with the *Eco*RI-*Hpa*I fragment) was transcribed in whole cell extract at the indicated concentrations. Constructs were all linearized at the *Sca*I site in the vector. Signals indicated by +1 correspond to specific initiation at the ribosomal gene promoter. Transcription due to read-through from upstream of the promoter is indicated by RT.

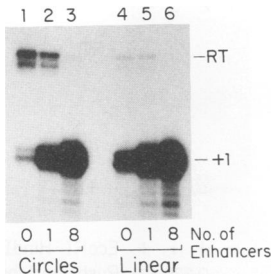

FIG. 4. Evidence that enhancement increases with enhancer copy number. Linear and supercoiled templates were transcribed in whole cell extract at 25 ng/ml (final concentration). Lanes: 1 and 4, construct A (no enhancer); 2 and 5, construct B (one enhancer); 3 and 6, construct F (eight enhancers). Constructs are diagrammed in Fig. 1C. RT, read-through transcript.

that we previously characterized by deletion and linker scanner mutagenesis (5).

The ability of construct B, containing the *Eco*RI-*Hpa*I fragment, to direct RNA polymerase I-specific transcription in vitro was then compared with that of construct A, an identical construct except that it lacks the *Eco*RI-*Hpa*I fragment. As shown in Fig. 2, the *Eco*RI-*Hpa*I fragment stimulates transcription over at least a 100-fold range of template concentrations. From doing many experiments of this type, we have learned that relative enhancement is generally higher at lower template concentrations and the optimum concentration varies somewhat for different preparations of extract. At template concentrations above 1 μg/ml, enhancement is nearly absent. At the optimum template concentration (usually between 5 and 25 ng/ml), the degree of enhancement is in the range of 5- to 10-fold.

Enhancement is relatively independent of distance, orientation, and template topology. The distance and orientation dependence of enhancement by the *Eco*RI-*Hpa*I fragment is explored in Fig. 3 (structures of templates tested are shown diagrammatically in Fig. 1C). To begin with, comparison of lanes 1 and 2 reinforces the observation that construct B, with the *Eco*RI-*Hpa*I fragment situated 216 bp upstream of initiation, shows enhanced transcription compared with the promoter alone (construct A). The result for construct C (lane 3) shows that the degree of enhancement is the same when the *Eco*RI-*Hpa*I fragment is reversed in orientation at the −216 site. In constructs D and E (lanes 4 and 5), the *Eco*RI-*Hpa*I fragment was inserted in both orientations at a position 42 bp downstream of initiation. Enhancement occurs with both of these constructs as well. In constructs J and K (lanes 6 and 7), the *Eco*RI-*Hpa*I fragment is present in both orientations upstream of the promoter, but a 521-bp piece of pBR322 DNA has been inserted to increase the distance between the enhancer and the promoter. Increasing the distance has no visible effect on the degree of enhancement observed in either orientation. Finally, in construct L (lane 8), the enhancer has been pushed even further away from the promoter by insertion of a 732-bp piece of nonspecific DNA. Again, no decrease in the fold of enhancement is observed.

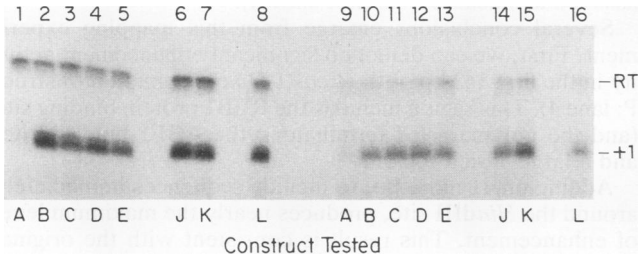

FIG. 3. Distance and orientation independence of enhancement. Constructs diagrammed in Fig. 1C were transcribed in whole cell extract at 5 ng/ml (final concentration). Lanes 1 through 8 contained closed circular templates; lanes 9 through 16 contained linear templates cut at the *Sca*I site. RT, read-through transcript.

All of the experiments shown in lanes 1 through 8 used templates that were added to the transcription reactions as closed circular molecules. In lanes 9 through 16, the same series of templates was assayed except that in these lanes the templates were all linearized. The overall level of transcription is about twofold lower on the linear templates than on the circular templates. However, linearization has little effect on the relative fold of enhancement.

From the experiments shown in Fig. 3, we can conclude that enhancement of RNA polymerase I transcription by the *Eco*RI-*Hpa*I fragment is insensitive both to distance from the ribosomal gene promoter and to orientation. In addition, enhancement works essentially as well on linear templates as it does on templates that are closed circular.

It is important when using linear templates to cut the DNA at some distance from the promoter. Free ends can have a profound negative effect on transcription when they are too close to the promoter, possibly because they are attractive sites for nonspecific protein binding. We believe that this is the explanation for the results of Lue and Kornberg (29), who showed that moving a free DNA end closer and closer to the promoter caused progressive inactivation of transcription. In the experiments shown in Fig. 3, lanes 9 through 16, the DNA was cut at a unique *Sca*I site in the vector, at least 1.5 kb away from the promoter.

Enhancement is proportional to the number of enhancers. It has been shown for many transcriptional activator elements, including the vertebrate ribosomal gene enhancers (26), that multiple copies yield more activation than do single copies. Figure 4 shows that this result is also observed with the *Eco*RI-*Hpa*I fragment. In this experiment, eight tandem head-to-tail copies of the *Eco*RI-*Hpa*I fragment were inserted at the *Sma*I site 216 bp upstream of initiation (con-

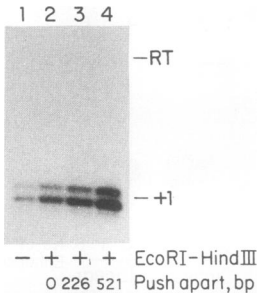

FIG. 5. Evidence that the *Eco*RI-*Hin*dIII fragment will not enhance when located too close to the promoter. All templates were cut with *Sca*I and assayed at 12.5 ng/ml. Lanes: 1, construct A (no enhancer); 2, construct G (*Eco*RI-*Hin*dIII fragment inserted at position −216); 3, construct H (*Eco*RI-*Hin*dIII fragment moved 226 bp further upstream); 4, construct I (*Eco*RI-*Hin*dIII fragment moved 521 bp further upstream). Constructs are diagrammed in Fig. 1C. RT, read-through transcript.

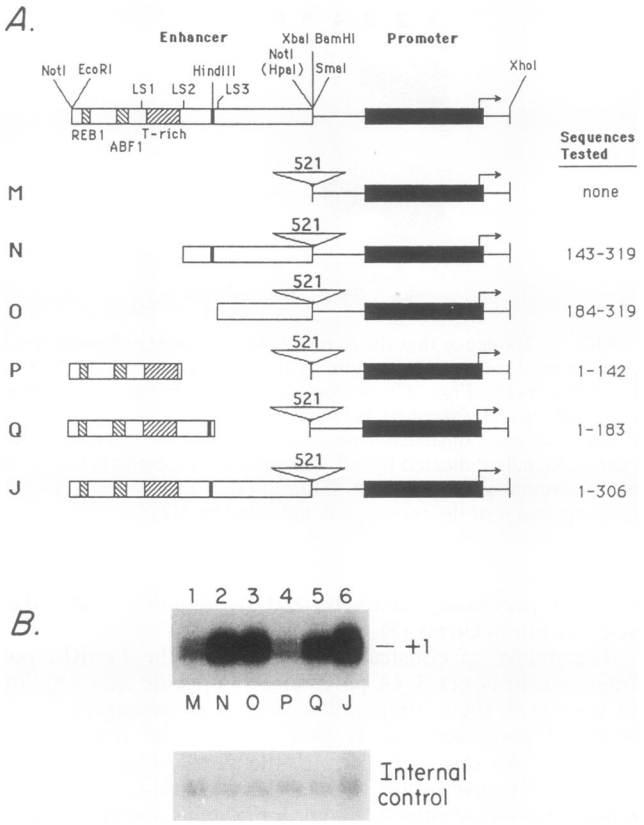

FIG. 6. Mapping sequences required for enhancer activity within the *Eco*RI-*Hpa*I fragment. (A) Structures of the constructs tested. 5′ and 3′ deletions of the *Eco*RI-*Hpa*I fragment were made by using LS1, LS2, and LS3 as endpoints. To avoid proximity effects, all fragments to be tested were inserted at a point 737 bp upstream of transcription initiation. (B) Assay of enhancer deletion constructs. All reactions contained a second polymerase I promoter as an internal control (similar to construct A; Fig. 1C). Both the internal control and the test constructs (indicated below the lanes) were circular and were assayed at 25 ng/ml.

struct F; Fig. 1C). Eight copies of the *Eco*RI-*Hpa*I fragment gave a considerably stronger stimulation (lanes 3 and 6) than was obtained with only one copy (lanes 2 and 5). As was observed in Fig. 3, the level of enhancement was only marginally affected by whether the template was circular (lanes 1 through 3) or linear (lanes 4 through 6).

We have shown elsewhere (27) that a terminator for RNA polymerase I is located within the *Eco*RI-*Hpa*I fragment. Sequences required for termination in vitro consist of the REB1 protein binding site and a few nucleotides between the *Eco*RI site and the REB1 site. In other work, we have also shown that most of the read-through transcription observed on circular templates is probably due to polymerase I initiating at the ribosomal gene promoter and then transcribing completely around the template circle (mutations that eliminate specific promoter initiation by polymerase I generally eliminate the read-through as well [5]). In agreement with these two earlier observations, Figure 4 shows that the read-through transcription detected on a circular template is progressively decreased by increasing the number of *Eco*RI-*Hpa*I fragments located upstream of the promoter (compare lanes 1, 2, and 3). Termination in vitro is often inefficient, and it has been observed in other systems as well that increasing the number of terminator elements causes a progressive decrease the the overall amount of read-through (13).

Enhancement is inhibited when the element is located too close to the promoter. It has been demonstrated that the 180-bp *Eco*RI-*Hin*dIII fragment is capable of yielding significant enhancement by itself (without the additional *Hin*dIII-*Hpa*I sequences) when assayed in vivo (11, 10). Initial attempts to detect activity in the *Eco*RI-*Hin*dIII fragment in vitro, by placing it 216 bp upstream of initiation at the *Sma*I site, yielded little or no activity (construct G in Fig. 1C; assayed in Fig. 5, lane 2). The results shown in Fig. 5 suggest that the reason for this initial failure is that in construct G, the enhancing element is located too close to the ribosomal gene promoter. Pushing the *Eco*RI-*Hin*dIII fragment and the promoter apart by 226 bp (construct H) causes a detectable increase in enhancer activity (Fig. 5, lane 3). Pushing the two elements apart by 521 bp (construct I) brings the enhancer activity of the *Eco*RI-*Hin*dIII fragment nearly up to the level of the entire *Eco*RI-*Hpa*I fragment (lane 4).

Mapping of regions sufficient for enhancement within the *Eco*RI-*Hpa*I fragment. We have made a series of deletions mutants to approximately map the regions within the *Eco*RI-*Hpa*I fragment that are required for enhancement. After considering the results of Fig. 5, we assayed all of these deletion mutants by inserting them into a test vector at a point 521 bp upstream of the promoter-proximal *Sma*I site. The various deletion mutants are shown diagrammatically in Fig. 6A, and their ability to enhance transcription when assayed on circular templates is shown in Fig. 6B. Essentially identical results are obtained when the templates are assayed as linear DNAs (not shown).

Several conclusions emerge from this mapping experiment. First, we can detect no significant enhancement activity in the first 142 bp of the *Eco*RI-*Hpa*I fragment (construct P; lane 4). This region includes the REB1 protein binding site (and the polymerase I terminator), the ABF1 binding site, and a large T-rich region.

Adding on 41 more bp, to include sequences immediately around the *Hin*dIII site, produces nearly the maximum level of enhancement. This result is consistent with the original observation that the *Eco*RI-*Hin*dIII fragment is an active enhancer in vivo (11). It further suggests that, in vitro at least, all of the detectable enhancer activity in the *Eco*RI-*Hin*dIII fragment is due to the 41 bp between positions 142 and 183.

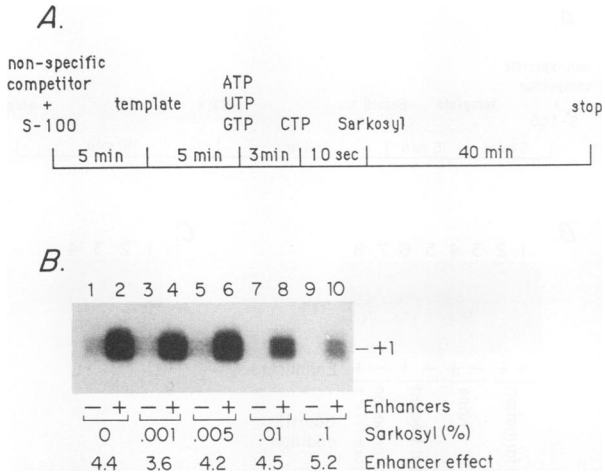

FIG. 7. Evidence that the level of enhancement is not dependent upon the number of rounds of transcription occurring at a promoter. (A) Protocol of the experiment. (B) Transcription result. Sarkosyl limits the number of rounds of transcription but does not decrease the magnitude of enhancement. Lanes: 1, 3, 5, 7, and 9, construct A (no enhancer); 2, 4, 6, 8, and 10, construct F (eight enhancers). Sarkosyl was added 10 s after the last triphosphate, following the protocol shown in panel A. Dried gels were exposed in a PhosphorImager for quantitation. Longer exposures reveal signals in the lightest lanes which are not visible at this exposure.

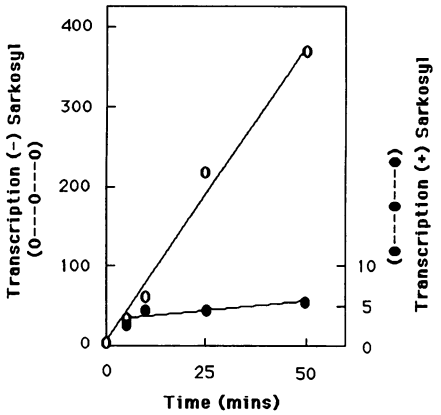

FIG. 8. Time course of transcription from a promoter-only construct (Fig. 1C, construct A) in the presence and absence of 0.1% Sarkosyl. Detergent was added 10 s after the last triphosphate, using the protocol outlined in Fig. 7A. Note that the scale on the right is enlarged 10-fold relative to the scale on the left.

Somewhat to our surprise, we also find strong enhancement activity in the region between the *Hin*dIII and *Hpa*I sites (construct O; lane 3). The activity of the sequence from 184 to 319 is not additive with the activity of the sequence between 1 and 183 (compare constructs O and Q with construct J; lanes 3, 5, and 6). This finding suggests that we have mapped at least two independent but nonadditive enhancing elements within the *Eco*RI-*Hpa*I fragment. In Discussion, we will consider the extent to which our mapping data agree with prior in vivo mapping of enhancer elements by other laboratories.

Enhancement increases the number of active promoters rather than the activity of a constant number of promoters. Initiation by mammalian nuclear RNA polymerases occurs in a series of discrete steps which can be distinguished by their sensitivity to the detergent Sarkosyl (polymerase II [15], polymerase III [22], and polymerase I [19]). A critical step in initiation is the assembly of polymerase into a tightly bound complex that is resistant to Sarkosyl concentrations in the range of 0.05 to 0.1%, a step which can occur in the presence or absence of nucleoside triphosphates. At this concentration of detergent, free polymerase is unable to initiate and transcription is limited to chain elongation by polymerases that have already entered the tightly bound complex. By adding Sarkosyl immediately after adding the triphosphates, transcription can thus be limited to that due to a single polymerase initiation at each promoter. We have used this technique to examine the degree of enhancement obtained under conditions in which either single or multiple rounds of transcription are allowed to occur on each promoter.

Experiments with Sarkosyl followed the protocol shown in Fig. 7A. Template was preincubated in the extract in the absence of triphosphates for 5 min to allow stable complex formation to occur. Three of the nucleoside triphosphates (A, U, and G) were then added for a further 3 min of incubation. This presumably allowed a single polymerase to elongate a few nucleotides on each active template. Finally, the fourth triphosphate (C) was added, followed 10 s later by the addition of Sarkosyl. Transcription was then allowed to proceed for 40 min before termination of the reaction.

In preliminary experiments (data not shown), we tested the inhibitory effect of Sarkosyl in concentrations ranging from 0 to 0.1%. We noted that in the range of 0.05 to 0.1%, a plateau occurred, with no further decrease in the amount of transcription. This result agrees with work on mammalian RNA polymerases and indicates that in this range, already initiated polymerase is stable but no new initiation is possible. We also observed (data not shown) that addition of this amount of Sarkosyl to a reaction that had been running for 10 min caused an immediate cessation of transcription (further evidence that this concentration prevents any new initiation).

Armed with this information, we performed the experiment shown in Fig. 7. Transcription from a template without an enhancer (construct A; Fig. 1C) or with eight enhancers (construct F) was assayed in the presence of Sarkosyl concentrations ranging from 0 to 0.1%. As expected, the overall amount of transcription decreases as the Sarkosyl concentration increases (compare lanes 1 and 2 with lanes 9 and 10). The striking result, however, is that the relative enhancer effect remains nearly the same over the entire range of detergent concentrations.

Since Sarkosyl was added 10 s following the last triphosphate, we presume that the amount of transcription observed in 0.1% Sarkosyl represents a single round of transcription per promoter. Thus, we should be able to calculate the number of rounds of reinitiation occurring in the absence of detergent by dividing the transcription signal in 0.1% Sarkosyl into that obtained in the absence of detergent. To examine this possibility more closely, we monitored the time course of transcription in the presence and absence of 0.1% Sarkosyl (Fig. 8). In the absence of detergent, transcription continues linearly for at least 50 min. In 0.1% detergent, transcription is much less and has essentially stopped by 5 min. Since we are measuring the 5′ end of the transcript in this assay, one would expect that in 0.1% Sarkosyl, the reaction would plateau well before the earliest 5-min time point. In fact, the apparent signal doubles between 5 and 50

min. Inspection of the original autoradiogram, however, suggests that this doubling is largely due to an increase of background in the later lanes which is difficult to correct for. For these reasons, we suspect that the amount of signal in the 5-min time point is nearer to that produced by single round transcription rather than that measured at 50 min. Regardless, dividing either the 5-min or the 50-min signal (in the presence of Sarkosyl) into the 50-min signal (in the absence of Sarkosyl) leads to the conclusion that between 70 and 150 rounds of initiation occurred per promoter in the absence of detergent. This number is similar to the estimate of about 100 rounds of initiation per hour on an active polymerase III promoter (22).

Without much more experimentation, we cannot be certain that we are measuring precisely one round of transcription per promoter in the presence of 0.1% Sarkosyl. However, it does appear likely that we have altered the number of rounds of initiation per promoter over a very wide range without causing a significant change in the relative level of enhancement (Fig. 7). This result argues strongly that the enhancement that we are studying acts by increasing the number of active, stable promoter complexes rather than by altering the rate of polymerase loading on a constant number of active promoters.

The enhancer functions only during the setup of the stable promoter complex and is dispensable thereafter. The Sarkosyl experiment shown in Fig. 7 argues that enhancers function to increase the number of stable promoter complexes that are formed. The experiment shown in Fig. 9 suggests that, in fact, enhancers act only at the formation of the stable complex and are dispensable once that point is past. In this experiment, an enhancer-bearing construct (construct F; Fig. 1C) was incubated in extract for 5 min in the absence of added triphosphates. No transcription signal is detected following such an incubation, but presumably the stable initiation complex is formed during this time. Then, a restriction enzyme was added for a further 15 min to cleave the enhancer from the template (Fig. 9C shows that the restriction enzyme will cut to completion under these conditions). Finally, triphosphates were added and transcription was allowed to proceed for 40 min. Under this protocol, the enhancer effect is still detected (Fig. 9; compare lanes 7 and 8). Comparison of lane 8 with lane 4 shows that the magnitude of the enhancer effect under the cutoff conditions is just as great as is obtained when no restriction enzyme is added. Lane 6 shows that the same enhancer effect is obtained when heat-inactivated restriction enzyme is used. However, if the restriction enzyme is added prior to stable complex formation (i.e., before addition of the DNA to the S-100 extract), the enhancer effect is completely abolished (lanes 1 and 2). Not only does the enhancer act at the time of stable complex formation, but once the complex is formed, the enhancer appears to be dispensable.

The ribosomal gene promoter can also be bisected after the stable complex is formed. The yeast ribosomal gene promoter is approximately 150 bp in length and contains at least two essential domains (5, 23, 32). One domain, called the core promoter, overlaps the site of transcription initiation. The second, or upstream, domain is located at the 5′ boundary of the promoter. Precise spacing between these two domains is essential for promoter activity, but much of the sequence between the two domains can be replaced with little effect as long as the original spacing is maintained (5). Covalent linkage between the two domains appears to be essential to allow stable promoter complex formation. We wondered whether covalent linkage was still essential after the stable complex had been formed.

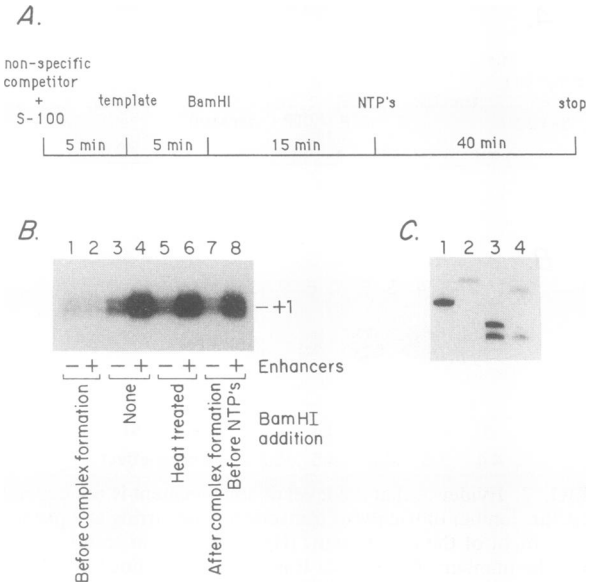

FIG. 9. Evidence that the enhancer can be severed from the promoter, following stable complex formation, with no loss of enhancement. (A) Protocol of the enhancer cutoff experiment. (B) Transcription result. All templates were linearized at the *Sca*I site and were assayed at 25 ng/ml. Lanes: 1, 3, 5, and 7, construct A (no enhancer); 2, 4, 6, and 8, construct F (eight enhancers); 1 and 2, *Bam*HI added to the template before stable complex formation; 3 and 4, no *Bam*HI addition; 5 and 6, heat-treated *Bam*HI added after stable complex formation, before nucleoside triphosphates (NTP's); 7 and 8, active *Bam*HI added after stable complex formation, before nucleoside triphosphates. (C) Demonstration that *Bam*HI digests templates in the presence of whole cell extract. Constructs were cut with *Sca*I, end labeled, and then used in the reaction protocol to the point of addition of nucleotides. The reactions were stopped, and the nucleic acids were isolated and resolved on a 1% agarose gel for visualization by autoradiography. Lanes: 1, construct A, intact *Sca*I fragment; 2, construct F, intact *Sca*I fragment; 3, digestion of construct A by *Bam*HI added after stable complex formation; 4, digestion of construct F by *Bam*HI added after stable complex formation. Comparison of lane 1 with lane 3 (and lane 2 with lane 4) shows that stable complex formation does not inhibit restriction enzyme cutting.

To answer this question, we incubated a promoter in extract, in the absence of triphosphates, to allow stable complex formation, and then added a restriction enzyme to cut the promoter between the core and upstream domains, as was done in the enhancer cutoff experiment. Finally, triphosphates were added and transcription capacity was measured. Figure 10B shows the results of this type of experiment as performed on three different variants of the promoter. The first promoter tested was a wild-type promoter (pYr11A) which does not contain an *Xba*I site. Addition of the enzyme *Xba*I, either before stable complex formation (lane 2) or after complex formation (lane 4), had no effect on transcription.

The same experiment was then performed with use of a promoter containing a large substitution of foreign sequence between positions −129 and −102 (LS−129/−102). An *Xba*I restriction site is present at either end of this substitution. As we have shown previously, this substitution mutation is relatively neutral when the intact promoter is assayed (5). If

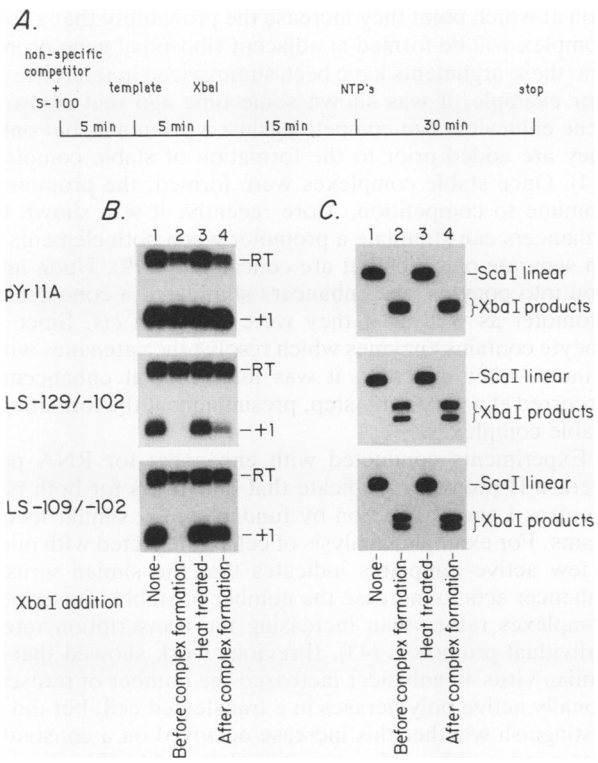

FIG. 10. Evidence that the upstream activation element of the promoter can be severed from the core region, following stable complex formation, with minimal loss of activity. (A) Protocol of promoter cut-apart experiment. The promoter constructs used were pYr11A (construct A in pGEM3 rather than pBluescript) and two linker scanner mutants described previously (5), LS−129/−102 and LS−109/−102. Extract was preincubated with nonspecific linear competitor (20 μg/ml) for 5 min and then with the template (500 ng/ml) for 5 min. The enzyme *Xba*I (25 U) or buffer only was added, and after 15 min, nucleoside triphosphates (NTP's) were added. The reactions were stopped after 40 min. (B) Transcription result. As we reported previously, these mutants have relatively little effect on initiation and define a neutral domain in the promoter (compare pYr11A with LS−129/−102 and LS−109/−102; lane 1). Lane 2, addition of *Xba*I prior to stable complex formation. Note that pYr11A does not contain an *Xba*I site. Lane 3, addition of heat-inactivated *Xba*I after stable complex formation. Lane 4, addition of active *Xba*I after stable complex formation, prior to addition of nucleoside triphosphates. RT, read-through transcript. (C) Demonstration that *Xba*I completely digests templates in the presence of whole cell extract. Constructs were cut with *Sca*I, end labeled with ^{32}P, and subjected to the reaction protocol up to the point of adding nucleotides, at which point nucleic acids were isolated for electrophoresis. pYr11A contains a single *Xba*I site upstream of the promoter which cleaves the *Sca*I fragment into two nearly equal fragments. In the linker scanner mutants, this *Xba*I site has been removed to allow use of the *Xba*I site as a linker scanner within the promoter.

LS−129/−102 is digested with *Xba*I prior to stable complex formation, transcription is nearly eliminated as expected (7% of control; Fig. 10B, lane 2). However, if *Xba*I digestion is performed after stable complex formation, about 31% of the control activity remains (Fig. 10B, lane 4). Figure 10C, lane 4, shows that digestion was complete in this particular reaction, even in the presence of the preformed stable complex. We repeated the experiment with another mutated promoter, LS−109/−102, which contains a single *Xba*I site substituted at the indicated location between the upstream and core domains. Cutting at this site prior to stable complex formation also destroys promoter activity (4% of control). However, complete cutting after stable complex formation still allows about 58% of the control activity (Fig. 10B, lane 4).

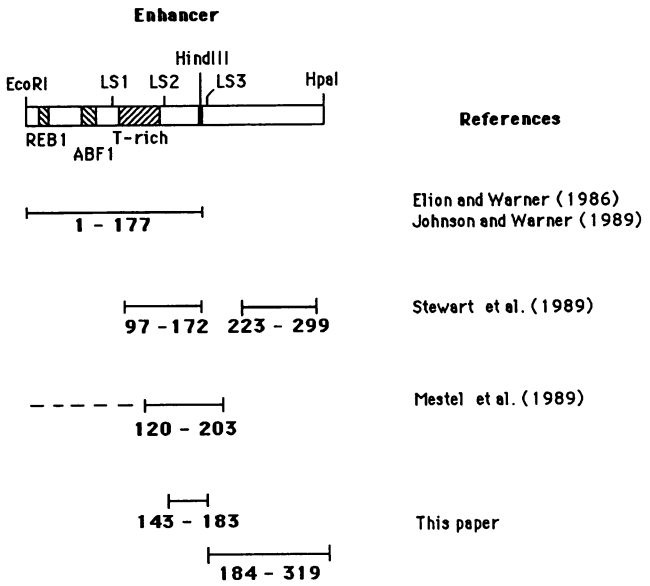

FIG. 11. Summary of regions within the *Eco*RI-*Hpa*I fragment which have been reported to show enhancement activity in vivo. At the bottom, we summarize the regions which have been found to give enhancement in vitro (this report).

These experiments suggest that the upstream domain of the promoter, like the enhancer, is essential for assembly of the stable promoter complex but physical linkage is not needed after that assembly has occurred.

DISCUSSION

Are the same sequences responsible for enhancement in vitro as well as in vivo? Figure 11 summarizes our attempt to bring together all published data concerning which sequences of the *Eco*RI-*Hpa*I enhancer fragment are required for enhancement in the living cell. In Fig. 11, the published data have been related to the numbering system used in this report.

Studies from the Warner laboratory (10, 11, 17) have shown that sequences from positions 1 to 177 are sufficient for enhancement in vivo. In particular, Elion and Warner (10) report that removing a few nucleotides from the *Hin*dIII site severely damages enhancement, emphasizing that the *Hin*dIII site is located in a critically important region. Stewart and Roeder (38), using an assay which measured the frequency of recombination events stimulated by polymerase I transcription, found that mutations in the region between positions 97 and 172 decreased recombination (presumably by eliminating enhancement). A third study, by Mestel et al. (31), identified a 3′ boundary for enhancement just to the right of the *Hin*dIII site and a 5′ boundary for full enhancement around position 120. Finally, a recent report by Kulkens et al. (24) verifies that removing the entire *Eco*RI-*Hpa*I region eliminates enhancement. All of these reports agree with the conclusion that sequences to the left of the *Hin*dIII site, and the *Hin*dIII site itself, are important for enhancer action even though the precise boundaries are

not clearly established. Our in vitro deletion analysis identifies sequences between 143 and 183 as critical. To this extent, our data and the available in vivo data are in excellent agreement.

What is less clear is the possible involvement of sequences outside of this core region. Both Mestel et al. (31) and Kulkens et al. (24) report that eliminating the REB1 site causes a modest decrease in transcription. As we have recently shown, however, the REB1 site is an essential element of the polymerase I terminator (27). Eliminating termination would allow transcription to proceed over the core enhancer and could conceivably decrease its activity. Therefore, it remains unclear whether the REB1 site is a component of the enhancer or simply protects it. Kulkens et al. (24) also observed a twofold effect of mutating a second REB1 site upstream of the ribosomal gene promoter. That site is not included in any of our current constructs, and we have no information on it at present. Both in vivo and in vitro evidence has been reported that the AT-rich stretch (approximately nucleotides 80 to 140) has polymerase I enhancer activity (29). We have found, however, that moving a cut end closer to the promoter causes a progressive inactivation of transcription. We suspect that this artifact is responsible for the in vitro effect that they report. In our own in vitro work, we have not been able to detect any enhancer activity in the entire region from 1 to 143.

Unexpectedly, we do observe enhancer activity in the region from 184 to 319. The only in vivo report of activity in this region is that of Stewart and Roeder (38), who found that mutations in the region from 223 to 299 decreased transcription-dependent recombination. No other workers have reported enhancer activity in this region. However, it has recently been shown that a terminator of replication maps to this vicinity (21). All we can say at present is that this region merits further investigation.

Enhancement occurs at the level of stable complex formation. Experiments reported in this paper provide direct support for a model in which ribosomal gene enhancers aid formation of the stable promoter complex and are dispensable once that complex is formed. The Sarkosyl experiments, for example, show that the level of enhancement is essentially the same regardless of the number of rounds of transcription occurring at each promoter (Fig. 7). This result argues against any model in which enhancement is due to increased loading of RNA polymerase per promoter. It is consistent, however, with the enhancer increasing the number of stable complexes that are formed.

Figure 9 shows that once the stable complex has formed, the enhancer can be severed from the promoter with no loss of enhancement. The same is largely true for the upstream activation domain of the promoter itself (Fig. 10). This finding argues that the role of both of these elements is to aid stable complex formation at the core promoter and they are both dispensable once the complex is formed. One caveat to this conclusion is that we do not know the composition of the stable complex. It is possible that either the enhancer, the upstream activation domain, or both remain bound to the core promoter via protein-protein interactions even though their DNA connection has been severed.

Vertebrate ribosomal genes have multiple copies of relatively weak enhancers, while yeast genes have a single copy per repeat of a strong enhancer. Despite these difference of detail, it appears that both vertebrate and yeast enhancers function by the same basic mechanism. A variety of indirect arguments have also led to the conclusion that in vertebrates, the enhancers act only at the beginning of transcription at which point they increase the probability that a stable complex will be formed at adjacent ribosomal gene promoters (these arguments have been summarized in reference 33). For example, it was shown some time ago that ribosomal gene enhancers can compete against a promoter, but only if they are added prior to the formation of stable complexes (34). Once stable complexes were formed, the promoter is immune to competition. More recently, it was shown that enhancers can stimulate a promoter when both elements are on separate plasmids but are concatenated (9). Upon injection into oocytes, the enhancers stimulated a concatenated promoter as well as if they were linked in *cis*. Since the oocyte contains enzymes which resolve the catenanes within minutes after injection, it was inferred that enhancement occurred at a very early step, presumably at the formation of stable complexes.

Experiments conducted with enhancers for RNA polymerase II promoters indicate that enhancers for both polymerases I and II function by fundamentally similar mechanisms. For example, analysis of cells transfected with one or a few active templates indicates that the simian virus 40 enhancer acts to increase the number of stable transcription complexes rather than increasing the transcription rate of individual promoters (43). (Previous work showed that the simian virus 40 enhancer increased the number of transcriptionally active polymerases in a transfected cell, but did not distinguish whether this increase occurred on a constant or increased number of active templates [40, 42].) In vitro analysis of the upstream activator, ATF, showed that the activator stimulated the formation of a stable promoter complex involving TFIID (16). Once the stable complex was formed, ATF could be removed by specific competition with an oligonucleotide without decreasing the level of transcription (14). This latter experiment suggests that the ATF-binding element, like the yeast ribosomal gene enhancer, is dispensable once the stable complex has been formed. Considering the fact that polymerases I and II both utilize the TATA-binding protein as a basal transcription factor (6, 7, 36), it seems entirely reasonable that their enhancers would operate via similar mechanisms.

DNA looping and enhancer action. The favored model for how a distant enhancer can interact with the promoter is via formation of a DNA loop (evidence for DNA looping was recently reviewed in reference 2). Our data are consistent with looping, especially the result showing that an enhancer cannot function if it is located too close to the promoter (construct G; assayed in Fig. 5, lane 2). The mapping experiments shown in Fig. 6 indicate that the critical enhancer element in construct G is within the 30 bp just to the left of the *Hin*dIII site. In construct G, this element is located about 70 bp upstream of the 5′ boundary of the promoter. Measurements of the probability of DNA ring closure as a function of chain length have shown that very short molecules resist ring closure due to the natural stiffness of double-stranded DNA, while very long molecules resist closure as a result of the low probability of one terminus finding the other. For naked DNA in vitro, the optimum length for ring closure turns out to be about 500 bp (37). It is interesting to note that for the enhancer element surrounding the *Hin*dIII site, removing it a total of about 590 bp from the upstream boundary of the promoter affords the maximum enhancement that we have observed with this element (construct I; Fig. 5, lane 4).

For a yeast ribosomal protein gene, it has previously been shown in vivo that an upstream activating element is active

when located 390 bp from the start site of transcription but is inactive when located at a closer distance of 120 bp (44).

Models have been proposed (17, 20) in which looping between the enhancer and the promoter facilitates recycling of RNA polymerase from the terminator-enhancer region back to the promoter. Although such loops may exist, our data argue against the enhancer having any direct role in recycling RNA polymerase. In the first place, the enhancer functions well either upstream or downstream of the promoter and in either orientation (Fig. 3). When located downstream in the reverse orientation, the terminator within the *Eco*RI-*Hpa*I fragment probably does not function and the enhancer is subject to being run over by elongating RNA polymerase. This view argues against formation of a continuously required complex between the enhancer and promoter. A more direct experiment is the enhancer cutoff experiment shown in Fig. 9. Once the stable complex is formed, the enhancer can be physically severed from the promoter with no detectable effect. This result would seem to eliminate any possible role for the enhancer in recycling RNA polymerase. Other workers have arrived at this same conclusion on the basis of less direct in vivo experiments (3).

Why does the enhancer function at a distance in this particular extract? It is commonly observed that upstream activators can be made to function in vitro when placed close to the promoter that they influence. However, successful activation from far upstream is much more difficult to achieve. The question arises, therefore, as to why the ribosomal gene enhancer displays such position independence in this particular extract. We can think of two possible answers to this question. First, the yeast rDNA enhancer is probably a relatively strong upstream activator. In contrast to vertebrate cells, in which enhancement occurs via multiple copies of individually weak elements, the yeast ribosomal genes contain a single element per repeat which by itself is sufficient for the task. Previous workers have suggested that strong activators are better able to function from a distance in vitro (4).

A second point to consider is the possible influence of chromatin assembly on long range activation in vitro. It has been reported that assembling the template into chromatin permits the detection of long-range activation in an RNA polymerase II transcription system (28). We have found (34a) that the yeast whole cell extract is quite efficient at assembling added DNA into chromatin. Thus, the combination of a strong activator (the yeast rDNA enhancer) coupled with a transcription system which actively assembles chromatin (the whole cell extract) may explain our success in detecting long-range activation.

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ADDENDUM IN PROOF

Morrow et al. (B. E. Morrow, S. P. Johnson, and J. R. Warner, Mol. Cell. Biol. **13**:1283–1289, 1993) have recently published an in vivo analysis of the yeast rDNA enhancer as it functions during growth on different carbon sources. They found that full enhancer activity is obtained with only the 45 bp situated between the T-rich stretch and the *Hin*dIII site. This agrees well with our in vitro deletion mapping (summarized in Fig. 11), which indicates that full enhancer activity is obtained with sequences between positions 143 and 183. The discrepancies between our two approaches are that Morrow et al. detected a small amount of enhancer activity from the REB1 and ABF1 sites or the T-rich stretch alone while we did not and that, conversely, they did not detect any activity in the region between positions 184 and 319.

REFERENCES

1. **Bell, G. I., L. J. DeGennaro, D. H. Gelfand, R. J. Bishop, P. Valenzuela, and W. J. Rutter.** 1977. Ribosomal RNA genes of Saccharomyces cerevisiae. I. Physical map of the repeating unit and location of the regions coding for 5S, 5.8S, 18S, and 25S ribosomal RNAs. J. Biol. Chem. **252**:8118–8125.
2. **Bellomy, G. R., and M. T. Record, Jr.** 1990. Stable DNA loops in vivo and in vitro: roles in gene regulation at a distance and in biophysical characterization of DNA. Prog. Nucleic Acid Res. Mol. Biol. **39**:81–128.
3. **Butlin, M., and R. Quincey.** 1991. The yeast rRNA gene enhancer does not function by recycling RNA polymerase I and cannot act as a UAS. Curr. Genet. **20**:9–16.
4. **Carey, M., J. Leatherwood, and M. Ptashne.** 1990. A potent GAL4 derivative activates transcription at a distance in vitro. Science **247**:710–712.
5. **Choe, S. Y., M. C. Schultz, and R. H. Reeder.** 1992. In vitro definition of the yeast RNA polymerase I promoter. Nucleic Acids Res. **20**:279–285.
6. **Comai, L., N. Tanese, and R. Tjian.** 1992. The TATA-binding protein and associated factors are integral components of the RNA polymerase I transcription factor, SL1. Cell **68**:965–976.
7. **Cormack, B. P., and K. Struhl.** 1992. The TATA-binding protein is required for transcription by all three nuclear RNA polymerases in yeast cells. Cell **69**:685–696.
8. **Doorenbosch, T., W. Mager, and R. Planta.** 1992. Multifunctional DNA-binding proteins in yeast. Gene Expression **2**:193–201.
9. **Dunaway, M., and P. Droge.** 1989. Transactivation of the Xenopus rRNA gene promoter by its enhancer. Nature (London) **341**:657–659.
10. **Elion, E., and J. R. Warner.** 1986. An RNA polymerase I enhancer in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **6**:2089–2097.
11. **Elion, E. A., and J. R. Warner.** 1984. The major promoter element of rRNA transcription in yeast lies 2 kb upstream. Cell **39**:663–673.
12. **Greenleaf, A., J. Kelly, and I. R. Lehman.** 1986. Yeast RPO41 gene product is required for transcription and maintenance of the mitochondrial genome. Proc. Natl. Acad. Sci. USA **83**: 3391–3394.
13. **Grummt, I., H. Rosenbauer, I. Neidermeyer, U. Maier, and A. Oehrlein.** 1986. A repeated 18bp sequence motif in the mouse rDNA spacer mediates binding of a nuclear factor and transcription termination. Cell **45**:837–846.
14. **Hai, T., M. Horikoshi, R. G. Roeder, and M. R. Green.** 1988. Analysis of the role of the transcription factor ATF in the assembly of a functional preinitiation complex. Cell **54**:1043–1051.
15. **Hawley, D. K., and R. G. Roeder.** 1987. Functional steps in initiation and reinitiation from the major late promoter in a HeLa nuclear extract. J. Biol. Chem. **262**:3452–3461.
16. **Horikoshi, M., T. Hai, Y.-S. Lin, M. R. Green, and R. G. Roeder.** 1988. Transcription factor ATF interacts with the TATA factor to facilitate establishment of a preinitiation complex. Cell **54**:1033–1042.
17. **Johnson, S. P., and J. R. Warner.** 1989. Unusual enhancer function in yeast rRNA transcription. Mol. Cell. Biol. **9**:4986–4993.
18. **Ju, Q., B. E. Morrow, and J. R. Warner.** 1990. REB1, a yeast DNA-binding protein with many targets, is essential for cell growth and bears some resemblance to the oncogene *myb*. Mol. Cell. Biol. **10**:5226–5234.
19. **Kato, H., M. Nagamine, R. Kominami, and M. Muramatsu.** 1986. Formation of the transcription initiation complex on mammalian rDNA. Mol. Cell. Biol. **6**:3418–3427.
20. **Kempers-Veenstra, A. E., J. Oliemans, H. Offenberg, A. F. Dekker, P. W. Piper, R. J. Planta, and J. Klootwijk.** 1986. 3′ end

formation of transcripts from the yeast rRNA operon. EMBO J. **5**:2703–2710.

21. **Kobayashi, T., M. Hidaka, M. Nishizawa, and T. Horiuchi.** 1992. Identification of a site required for DNA replication fork blocking activity in the rRNA gene cluster in Saccharomyces cerevisiae. Mol. Gen. Genet. **233**:355–362.
22. **Kovelman, R., and R. G. Roeder.** 1989. Sarkosyl defines three intermediate steps in transcription initiation by RNA polymerase III: application to stimulation of transcription by E1A. Genes Dev. **4**:646–658.
23. **Kulkens, T., D. L. Riggs, J. D. Heck, R. J. Planta, and M. Nomura.** 1991. The yeast RNA polymerase I promoter: ribosomal DNA sequences involved in transcription initiation and complex formation in vitro. Nucleic Acids Res. **19**:5363–5370.
24. **Kulkens, T., C. A. F. M. van der Sande, A. F. Dekker, H. van Heerikhuizen, and R. J. Planta.** 1992. A system to study transcription by yeast RNA polymerase I within the chromosomal context: functional analysis of the ribosomal DNA enhancer and the RBP1/REB1 binding sites. EMBO J. **11**:4665–4674.
25. **Kunkel, T. A.** 1985. Rapid and efficient site-specific mutagenesis without phenotypic selection. Proc. Natl. Acad. Sci. USA **82**:488–492.
26. **Labhart, P., and R. H. Reeder.** 1984. Enhancer-like properties of the 60/81 bp elements in the ribosomal gene spacer of Xenopus laevis. Cell **37**:285–289.
27. **Lang, W., and R. H. Reeder.** 1993. The REB1 site is an essential component of a terminator for RNA polymerase I in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **13**:649–658.
28. **Laybourn, P. J., and J. T. Kadonoga.** 1992. Threshold phenomena and long-distance activation of transcription by RNA polymerase II. Science **257**:1682–1685.
29. **Lue, N. F., and R. D. Kornberg.** 1990. Accurately initiated, enhancer-dependent transcription by RNA polymerase I in yeast extracts. J. Biol. Chem. **265**:18091–18094.
30. **McStay, B., C.-H. Hu, C. S. Pikaard, and R. H. Reeder.** 1991. xUBF and Rib1 are both required for formation of a stable polymerase I promoter complex in X. laevis. EMBO J. **10**:2297–2903.
31. **Mestel, R., M. Yip, J. P. Holland, E. Wang, J. Kang, and M. J. Holland.** 1989. Sequences within the spacer region of yeast rRNA cistrons that stimulate 35S rRNA synthesis in vivo mediate RNA polymerase I-dependent promoter and terminator activities. Mol. Cell. Biol. **9**:1243–1254.
32. **Musters, W., J. Knol, P. Maas, A. F. Dekker, H. van Heerikhuizen, and R. J. Planta.** 1989. Linker scanning of the yeast RNA polymerase I promoter. Nucleic Acids Res. **17**:9661–9678.
33. **Reeder, R. H.** 1992. The regulation of transcription by RNA polymerase I, p. 315–347. *In* K. Yamamoto and S. L. McKnight (ed.), Transcriptional regulation. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
34. **Reeder, R. H., J. G. Roan, and M. Dunaway.** 1983. Spacer regulation of Xenopus ribosomal gene transcription: competition in oocytes. Cell **35**:449–456.

34a. **Schultz, M. C.** Unpublished data.

35. **Schultz, M. C., S. Y. Choe, and R. H. Reeder.** 1991. Specific initiation by RNA polymerase I in a whole-cell extract from yeast. Proc. Natl. Acad. Sci. USA **88**:1004–1008.
36. **Schultz, M. C., R. H. Reeder, and S. Hahn.** 1992. Variants of the TATA binding protein can distinguish subsets of RNA polymerase I, II, and III promoters. Cell **69**:697–702.
37. **Shore, D., and R. L. Baldwin.** 1983. Energetics of DNA twisting. I. Relation between twist and cyclization probability. J. Mol. Biol. **170**:957–981.
38. **Stewart, S. E., and G. S. Roeder.** 1989. Transcription by RNA polymerase I stimulates mitotic recombination in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **9**:3463–3472.
39. **Swanson, M. E., and M. J. Holland.** 1983. RNA polymerase I-dependent selective transcription of yeast ribosomal DNA. Identification of a new cellular ribosomal RNA precursor. J. Biol. Chem. **258**:3242–3250.
40. **Treisman, R., and T. Maniatis.** 1985. The simian virus 40 enhancer increases number of RNA polymerase II molecules on linked DNA. Nature (London) **315**:73–75.
41. **van der Sande, C. A. F., T. Kulkens, A. B. Kramer, I. J. de Wijs, H. van Heerikhuizen, J. Klootwijk, and R. J. Planta.** 1989. Termination of transcription by RNA polymerase I. Nucleic Acids Res. **17**:9127–9146.
42. **Weber, R., and W. Schaffner.** 1985. Simian virus 40 enhancer increases RNA polymerase density within the linked gene. Nature (London) **315**:75–77.
43. **Weintraub, H.** 1988. Formation of stable transcription complexes as assayed by analysis of individual templates. Proc. Natl. Acad. Sci. USA **85**:5819–5823.
44. **Woudt, L. P., W. H. Mager, R. T. M. Nieuwint, G. M. Wassenaar, A. C. van der Kuyl, J. J. Murre, M. F. M. Hockman, P. G. M. Brockhoff, and R. J. Planta.** 1987. Analysis of upstream activation sites of yeast ribosomal protein genes. Nucleic Acids Res. **15**:6037–6048.