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In vitro definition of the yeast RNA polymerase I promoter

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ABSTRACT

The structure of the ribosomal gene promoter from *Saccharomyces cerevisiae* has been analyzed in a whole cell in vitro extract. The promoter contains at least two essential domains, an upstream domain located at the 5′ boundary near position −150 and a core promoter domain around the site of transcription initiation at +1. The upstream domain augments transcription in vitro but is not absolutely required. Maintenance of correct spacing between the two domains is critical. The in vitro analysis agrees well with prior in vivo analysis and it appears that the yeast promoter has a structure very similar to that of vertebrate ribosomal gene promoters.

INTRODUCTION

The genes coding for 35S ribosomal RNA precursor in the yeast, *Saccharomyces cerevisiae*, are known to contain two distinct DNA elements which influence transcription initiation by RNA polymerase I. These elements are the gene promoter, situated at the 5′ end of the 35S coding region (11), and the 'enhancer', an element located at the 3′ end of the 35S coding region that strongly augments the activity of the gene promoter in vivo (3). The relative location of these two transcription control elements is diagrammed in Figure 1.

In this paper we use a newly developed in vitro transcription extract from yeast (15) to analyze the structure of the ribosomal gene promoter. Two general conclusions emerge from this work. First, the in vitro response of this promoter to mutagenesis corresponds closely to the in vivo analysis that was previously published by (11). Both the 5′ and 3′ boundaries as well as the internal domain structure of the promoter are very similar in both types of analysis. The second conclusion is that the yeast ribosomal gene promoter has a structure that is closely related to the structure of vertebrate ribosomal gene promoters. Yeast and vertebrate promoters are of similar size, both can be separated into core and upstream domains, and in both types of promoter there appears to be a critical need for transcription factors to be positioned on the correct face of the DNA helix.

These studies prepare the ground for eventual elucidation of the precise interaction between the yeast ribosomal gene promoter and its cognate transcription factors.

MATERIALS and METHODS

Construction of plasmids

pYr11A was the parent plasmid for all constructs. It contains the 35S ribosomal gene promoter isolated as a SmaI-TaqI fragment (−216 to +25) from pBD4 (1). The fragment was modified by ligating a 16 bp Xho I linker (underlined uppercase sequence in Fig. 1) to the TaqI site at +25 and then it was cloned into the large HincII-XhoI fragment of a pGEM3 derivative (pGEM3EX) in which the HindIII site was converted to an XhoI site by filling in and addition of a linker. Resequencing of this promoter fragment reveals an extra 10bp (positions −180 to −189 in Figure 1) plus two single base changes (A to C at −110, G to A at −130; Fig. 1) which differ from the original published sequence (1). We are confident, however, that the promoter fragment we are using is physiologically significant since this promoter, by itself, is capable of maintaining yeast at normal growth rates (Ed Morgan, personal communication).

Novel XbaI sites were introduced into the promoter by oligonucleotide-directed mutagenesis (8) to make the small linker scanner mutations shown in Figure 1. Large linker scanner mutations were made from the small linker scanners in three steps. First, internal 20bp deletions of the promoter were made by ligating together appropriate SmaI-XbaI fragments from the small linker scanners (the XbaI site in the polylinker was eliminated to facilitate this process). Then an appropriate double stranded oligonucleotide was ligated into the XbaI site in the deleted promoter to restore the correct spacing and create a 20bp linker scanner mutation (sequences shown in Figure 5A). 5′ deletions were made from the small linker scanners by digesting with XbaI and recircularizing the large fragment. This results in the joining of the XbaI site in the polylinker to the XbaI site of a particular small linker scanner mutation with the deletion of the intervening sequence. Mutations around the 3′ boundary of the promoter were also made by oligonucleotide-directed mutagenesis.

Spacing changes within the gene promoter were constructed at two different locations. One set of spacing mutants was based on the large linker scanner, LS −129/−102. A −20 space change mutant was made by digesting LS −129/−102 with XbaI and religating. A −16 space change was made by digesting LS −129/−102 with XbaI, filling the sticky ends, and religating. All other spacing change mutants were made by cutting LS −129/−102 with XbaI and ligating in the appropriate double

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stranded oligonucleotide (sequences of the final constructs are shown in Figure 8A). A similar set of spacing change mutants was constructed based on the large linker scanner, LS −49/−22 (sequences shown in Figure 8B).

All mutations were verified by direct sequencing of the final construct.

In vitro transcription assays

100,000×g supernatants and DEAE fractions were prepared according to (15) from BJ2168 cells grown to an OD600 of 2. Transcription reactions were performed as described (15), except that KCl was at 90 mM in each reaction and each reaction contained about 2 μg/ml of extract protein. For most experiments the extract was pre-incubated at 25° with pGEM3 DNA (5 μg/ml) for 5 minutes. Then the template was added (0.5 μg/ml) and incubated an additional 5 minutes before adding nucleotides and starting the reaction. Exceptions were the experiments in Figure 2 where template only was added at 30 μg/ml and Figure 3B where competitor was added at 5 μg/ml and template was added at 10 μg/ml. Reactions were stopped after 45 min and RNA products were analyzed by S1 nuclease protection using single-stranded DNA probes labeled at the 5′ end. Most reactions were probed with a 50 nt single-stranded oligonucleotide (probe A in reference 15) that yielded a 35 nt protected fragment when hybridized to correctly initiated polymerase I transcripts. A 275 nt probe was used to detect possible initiation events further upstream in the promoter. This was prepared from the EcoRI-XhoI small fragment of pYr11-316 (15) by strand-separation. The long probe included the region of the promoter from −220 to +40.

RESULTS

Assay conditions

All of the work in this paper was done with a promoter fragment which extends from an SmaI site at position −216 upstream of transcription initiation to a TaqI site at position +25 downstream of initiation. At the Taq I site a 16 bp linker has been inserted to facilitate distinguishing transcripts from this promoter from endogenous transcripts. The sequence of this fragment is shown in Figure 1.

Promoter mutants were assayed for transcription activity in a whole cell S-100 extract (15) using an S1 nuclease protection assay. Most S1 nuclease protection assays utilized a 50nt end labeled oligonucleotide probe which yields two protected bands, one band due to specific initiation by polymerase I at position +1 and a second band due to readthrough transcripts (representative examples can be seen in Figure 3). In some experiments additional protected bands are also seen in the vicinity of +5. These apparently arise from post-transcriptional processing of the primary transcript. We deduce this because the +5 signal rises and falls in proportion to the +1 signal and we never observe the +5 signal in the absence of the +1 signal.

In many experiments readthrough signal increases and decreases in concert with variations in polymerase I initiation activity at +1. From this correlation we have previously argued that most of the readthrough is due to RNA polymerase I transcription that has traveled clear around the plasmid circle (15). A second potential source of readthrough transcripts is initiation by mitochondrial RNA polymerase at an adventitious promoter that lies within the ribosomal gene promoter. These initiation events occur at position −75 (relative to the +1 site where polymerase I initiates) and can be detected by using a longer S1 nuclease probe. As shown in Figure 2, lane 1, an S1 probe that spans the region from −220 to +40 can detect a band at −75 in addition to the +1 and readthrough bands. We deduce that the initiation at −75 is due to mitochondrial polymerase since the responsible activity flows through a DEAE column in 100mM KCl (Figure 2, lane 2) whereas polymerase I activity is not eluted until 350mM KCl (Figure 2, lane 3; in this experiment a certain amount of polymerase I activity also came off in the 100mM eluate). Riggs and Nomura (14) have previously shown that mitochondrial RNA polymerase and RNA polymerase I can be separated in this manner. In addition, the activity initiating at −75 has a response to divalent cations that is characteristic of mitochondrial polymerase (data not shown). Initiation by mitochondrial RNA polymerase is not a complication in most of our experiments because it is not detectable at the low template concentration (0.5 μg/ml) usually employed. The exceptions are the experiment in Figure 2, where we purposely raised the template concentration to 30 μg/ml in order to detect initiation at −75 and the series of assays shown in Figure 3B which were done at 10 ug/ml. Even under the higher template conditions mitochrondrial RNA polymerase appears to compete poorly with the RNA polymerase I transcription machinery and the presence of an adventitious binding site is unlikely to affect the conclusions we have drawn.

Analysis of 5′ deletions

Oligo-directed mutagenesis was used to insert a novel XbaI site within the promoter at 20bp intervals as shown in Figure 1. Note

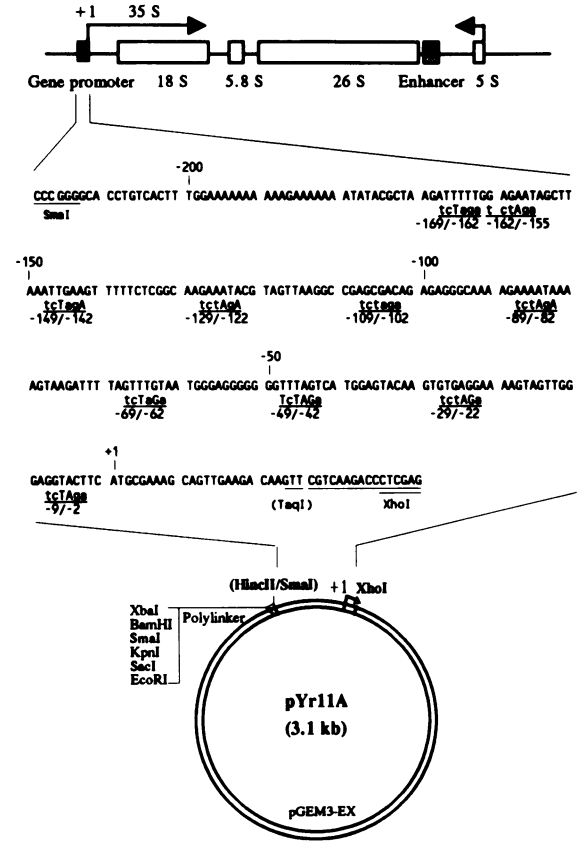


Figure 1. Diagram of the yeast ribosomal gene spacer showing the location of enhancer, promoter, and key restriction sites.

that in every case the insertion was done without changing any spatial relationships within the promoter. The polylinker into which the gene promoter fragment was inserted contained an XbaI site on the 5′ side of the promoter insert which facilitated turning each one of the LS mutants into a corresponding 5′ deletion. This was accomplished by digestion of the plasmid with XbaI and religation of the XbaI site in the polylinker to the XbaI site within the promoter with concomitant deletion of the intervening DNA.

The set of 5′ deletions was assayed for the ability to direct accurate initiation at +1 in the whole cell extract at two different template concentrations. The results of typical S1 nuclease protection assays are shown in Figure 3 and are summarized graphically in Figure 6A. Deletions extending down to 5′ −158 have initiation activity equal to the wild-type construct. Further deletion to 5′ −145 severely reduces activity. This suggests that a major 5′ boundary is located at approximately −150. When the deletions are assayed at relatively low template concentration (0.5 μg/ml) deletion to 5′ −145 completely inactivates the promoter (Figure 3A, lane 4). However, reassay at a higher template concentration (10 μg/ml) shows some residual initiation activity remaining after deletion to 5′ −145 (Figure 3B). This lower level of activity remains as more and more of the promoter is deleted until the deletions reach 5′ −25 at which point all activity is lost (Figure 3B, lane 10). This type of response to sequential 5′ deletion has been previously observed on other polymerase I promoters (10, 5, 16) and indicates that the promoter contains at least two essential domains, one near the 5′ boundary and a second domain close to +1. For the remainder of this paper we will refer to these two domains as the 'upstream' domain and the 'core' domain.

Figure 2. Identification of a promoter for mitochondrial RNA polymerase within the ribosomal gene promoter. Transcription from an intact ribosomal gene promoter (pYR11A) was assayed by S1 nuclease protection using an end labeled probe that spans the region from −220 to +40. Lane 1, transcription in an S-100 whole cell extract. Lane 2, transcription in the 100mM KCl flowthrough from a DEAE Sepharose column fractionation. Lane 3, transcription in the 350mM KCl eluate from DEAE Sepharose. RT indicates full length protection of the probe, −75 indicates 5′end formation that is apparently due to mitochondrial RNA polymerase, and +1 indicates 5′end formation due to RNA polymerase I (justification for assigning the +1 signal to polymerase I is given in (15).

Analysis of the small linker scanner mutants

Introduction of novel XbaI sites at various locations within the promoter caused clustered base substitutions ranging from 2 to 6 bases depending upon the precise location (see Figure 1). We will refer to this set of linker scanner mutants as the 'small' linker scanners in contrast to larger clustered mutations which will be discussed later. Transcription analysis of the small LS mutants is shown in Figure 4 with a quantitative summary shown in Figure 6B. One of the small LS mutants, LS −9/−2, completely inactivates the promoter (Figure 4, lane 11). We interpret this

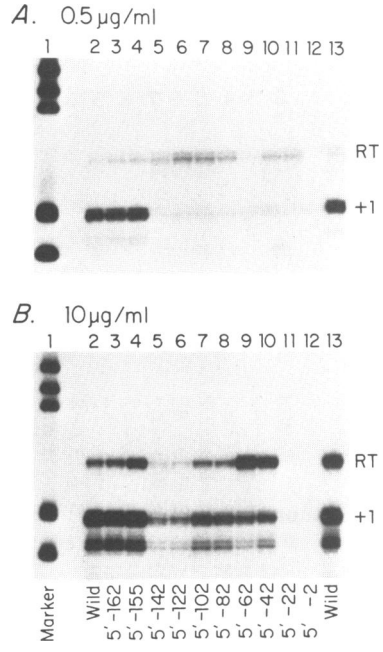


Figure 3. Assay of 5′ deletion mutants at low and high template concentrations. Promoter constructs were transcribed in an S-100 extract and assayed for specific initiation by S1 nuclease protection using a 50nt probe as described in Materials and Methods. RT indicates full length probe protection while +1 indicates initiation due to RNA polymerase I. A. Assay at low (0.5 μg/ml) template concentration. B. Assay at high (10 μg/ml) template concentration. Endpoints of the 5′ deletions are indicated at the bottom of the Figure.

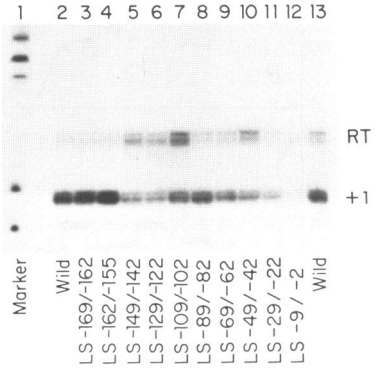


Figure 4. Assay of the small linker scanner mutants. Constructs were transcribed in an S-100 extract at a template concentration of 0.5 μg/ml.

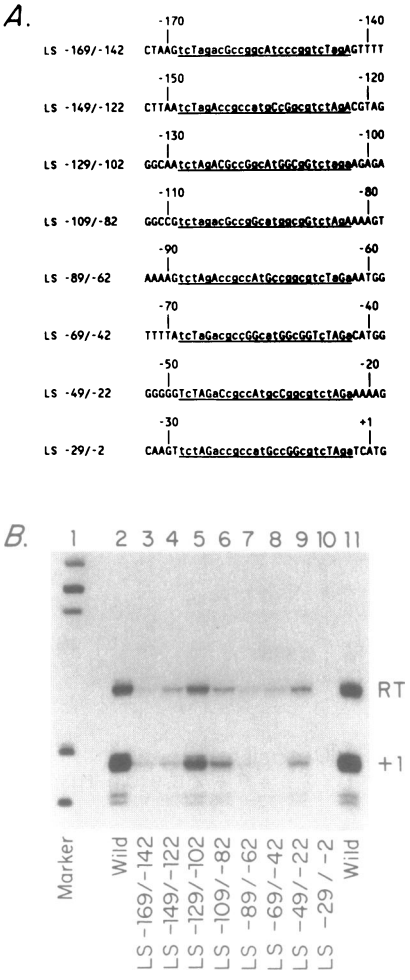


Figure 5. A. Sequence and location of 20bp LS mutants. B. Typical assays of 20bp LS mutants.

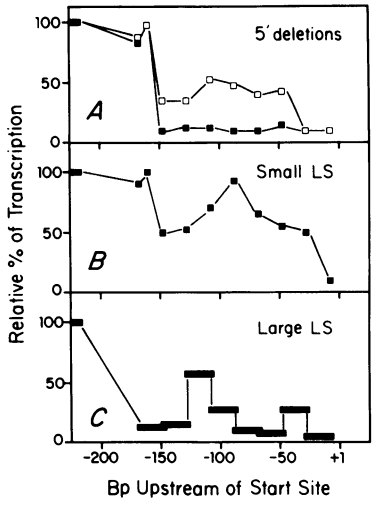
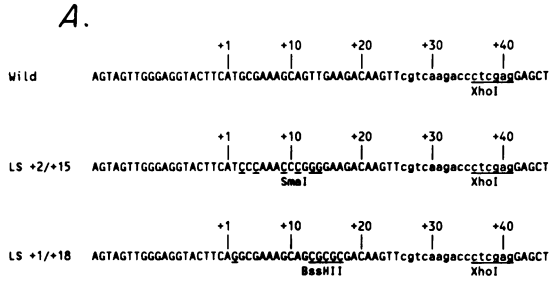


Figure 6. Graphic summary of assays of 5′ deletions, small LS mutants, and large LS mutants. A. 5′ deletions, summary of data from Figure 3. B. Small linker scanner mutants, summary of data from Figure 4. C. Large linker scanner mutants, summary of data from Figure 5.

result as further evidence that an essential core promoter domain is located in the vicinity of +1. The rest of the small LS mutants have relatively less effect on initiation, with some of them being nearly neutral (ie, LS −89/−82, lane 7). We note, however, that LS −162/−155 (lane 3) shows wild-type activity while the next two mutants, LS −149/−142 and LS −129/−122 are decreased in activity by about 50% (lanes 4 and 5). These results agree with the assays of the 5′ deletion mutants which indicated that the 5′ boundary of the promoter is located at approximately −150.

Analysis of large linker scanner mutants

Since the small linker scanner mutants had relatively little effect on the promoter (except for LS −9/−2) we examined the effect of making an ordered set of 20bp mutations across the promoter (the sequences of these large LS mutants are shown in Figure 5A, S1 analysis of their transcription activity is in Figure 5B, and a quantitative summary of their activity is shown in Figure 6C). As expected, many of these large LS mutations seriously decrease promoter activity. In particular, two large LS mutants (LS −169/−142 and LS −149/−122) appear to damage the essential



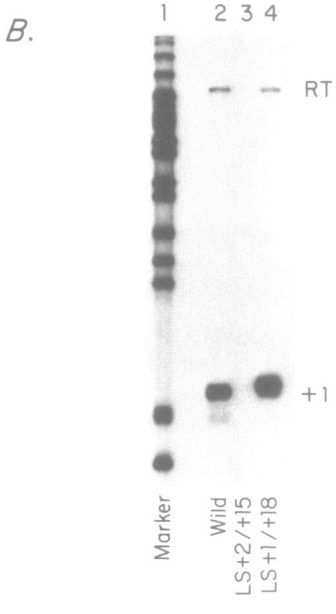


Figure 7. Locating the approximate 3′ boundary of the promoter. A. Sequence of wild type and mutant promoters. B. Transcription activity of promoters shown in Part A. A separate end labeled probe was made for each promoter, extending from the XhoI site at +40 to a KpnI site in the polylinker at −240. All probes were labeled to the same specific activity. Note that mitochondrial polymerase activity (initiation at −75) is absent in this particular extract.

domain at the 5′ boundary and have the same negative effect as deletion past the 5′ boundary. Likewise, LS −29/−2 damages the essential core promoter domain and completely inactivates the promoter. The surprising result is that several of the large linker scanner mutants are still relatively neutral. For example, LS −129/−102 causes less than a two-fold decrease in promoter activity while LS −109/−82 and −49/−22 cause about three-fold decreases. We interpret this to mean that relatively large regions of the interior of the promoter are neutral and perhaps serve only to maintain the correct spacing between essential domains.

Mutations in the 3′ boundary of the promoter

In order to mark a ribosomal gene and to distinguish its transcripts from the endogenous transcripts, we originally inserted a 16bp linker at position +25. This construct is actively transcribed in vitro (it is the 'wild-type' or starting point for most of the mutants examined in this paper) and Dr. Ed Morgan has shown that ribosomal genes carrying this insert are able to support normal growth rates in intact yeast (personal communication). Therefore, we think that the linker insertion at +25 is probably outside of the 3′ boundary of the yeast ribosomal gene promoter.

We have also altered the sequence from +12 to +17 to form a BssHII restriction site, changing 5 out of 6 bp in that region. At the same time nucleotide +2 was changed from a T to a G (our aim was to eliminate all T residues from the immediate 5′ end of the transcript so that we could make a T-free cassette for this promoter). These combined alterations cause essentially no change in promoter activity (see LS +1/+18, Figure 7A), leading us to conclude that the 3′ boundary is probably upstream of +12. However, these alterations do change the site of transcription initiation so that the transcript is longer by one nucleotide (Figure 7B, lane 4). We presume this change in initiation site is due to the T to G change at +2 but we have not tested this directly.

In the process of making a G-free cassette for the ribosomal gene promoter we mutated several nucleotides between +3 and +14 (LS +2/+15, Figure 7A). These alterations completely kill the promoter (Figure 7B, lane 3). Thus, we conclude that the 3′ boundary of the yeast ribosomal gene promoter overlaps the site of transcription initiation and probably lies between +3 and +12.

Spacing between domains of the promoter is critical

To examine the possible role of domain spacing in the yeast ribosomal gene promoter we made various push apart and pull together spacing mutants. For one set of spacing mutants we took as our starting point the large linker scanner introduced at position −129/−102 which decreases promoter activity about 25% (see summary, Figure 6C). As shown in Figure 8A we first removed the large linker scanner mutation in increments to create pull together mutants. Secondly, we inserted additional nucleotides in the linker scanner to create push apart mutants. The transcription activity of both the pull together and push apart

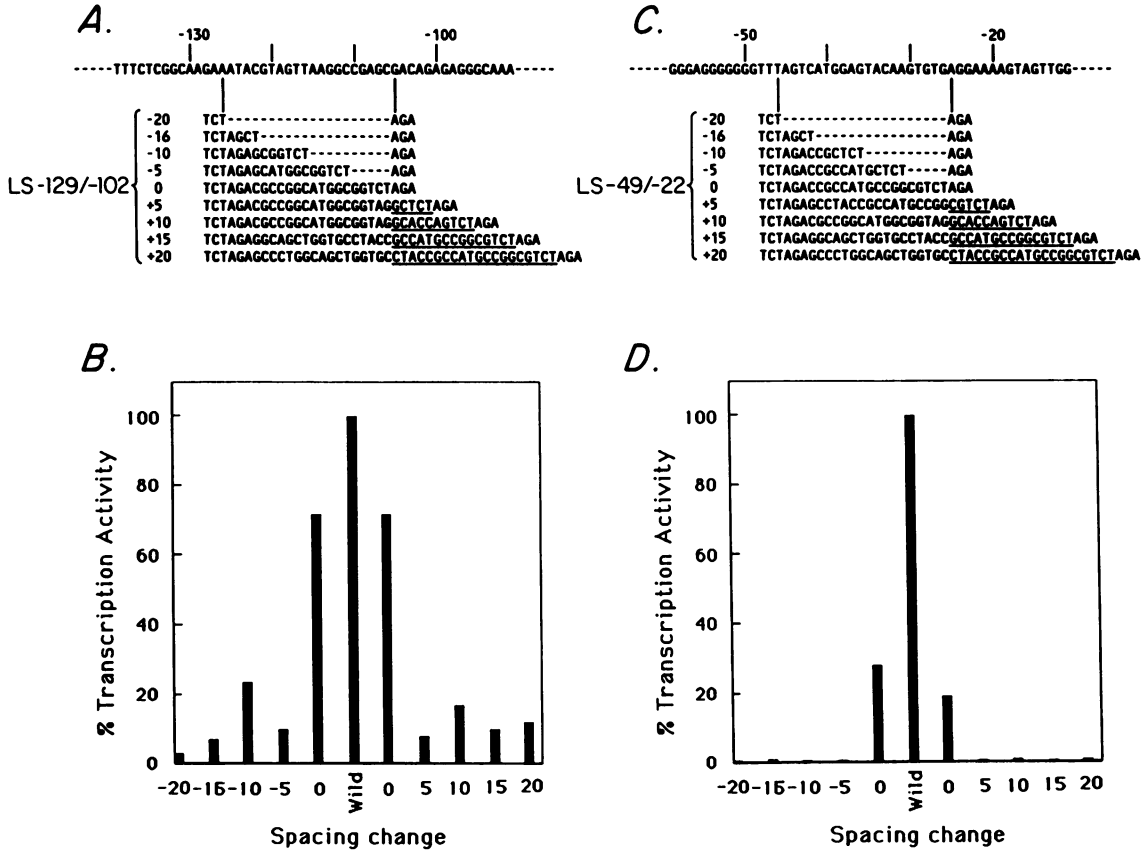


Figure 8. Effect of changing the spacing between upstream and core promoter domains. A. Sequence of spacing change mutants based on LS −129/−102. B. Transcription activity of the LS −129/−102 spacing mutants. C. Sequence of spacing change mutants based on LS −49/−22. D. Transcription activity of the LS −49/−22 spacing mutants. Transcription activity was quantited by measuring the intensity of the +1 signal with a densitometer and comparing it to the activity of a wild type promoter.

spacing mutants is shown in Figure 8B. In this particular experiment the −129/−102 linker scanner mutation exhibited typical transcription activity that was about 75% of wild type. Changing the spacing at this location, in contrast, is very deleterious to promoter activity. For example, either increasing or decreasing the spacing by 5bp causes activity to drop to about 10% of wild type (Figure 8B). The most interesting result, however, is that a partial rescue of promoter activity is observed when the spacing is further increased or decreased to10bp. This type of periodic dependence on spacing has been previously described for domains of the X. laevis ribosomal gene promoter (12, 9). In the present case we interpret these results to mean that spacing between the core and upstream domains of the yeast promoter is critical. Furthermore, proteins bound to the core and upstream domains of the yeast promoter must be positioned on the correct face of the DNA helix for maximal activity.

We also made a similar set of spacing mutants starting with the large linker scanner located at position −49/−22 (Figure 8C). In this case increasing or decreasing the spacing by 5bp completely inactivates the promoter (Figure 8D). Further increases or decreases do not cause detectable rescue of promoter activity. This result reinforces the conclusion that correct spacing is of critical importance for the activity of this promoter.

DISCUSSION

In vitro assay of the yeast ribosomal gene promoter agrees well with previous mutagenesis assayed in vivo

Our in vitro results agree remarkably well with the previous in vivo analysis of the yeast ribosomal gene promoter published by Musters, et al. (11). They concluded that the 5′ boundary of the promoter was located between −155 and −134 while our mutagenesis locates the boundary at between −158 and −145. Both sets of data agree that deleting past this boundary severely damages the promoter. Likewise, the in vivo study concluded that the 3′ boundary of the promoter lies between +27 and −2 while our in vitro data indicate that the boundary is between +3 and +12.

Musters, et al, also assayed a set of linker scanner mutants and identified three domains of the promoter that were sensitive to mutagenesis. One domain was located at the 5′ boundary (defined by their mutant LS −146/−134) and this agrees with what we are calling the upstream promoter domain (defined by our LS −169/−102 and LS −149/−122). They also showed that two mutations near the initiation site (LS −28/−17 and LS −4/+8) severely affected transcription. This would correspond to what we are calling the core domain (defined by our LS −9/−2 and LS −29/−2). Finally, they observed a third region of mutation sensitivity as defined by mutations in the region from −70 to −51. Once again, our own mutations in that region have a similar negative effect on the promoter (LS −89/−62 and LS −69/−42). If there is any significant difference between the two types of analyses, it is that small mutations appear to have a more severe effect when assayed in vivo. In vitro we had to make considerably larger mutations in order to seriously affect transcription in many instances. This minor difference aside, it appears that our in vitro data agree very well with the in vivo analyses of Musters, et al.

Kulkens, et al., (18) have recently published an in vitro analysis of the yeast ribosomal gene promoter using an extract that had been partially fractionated by elution from DEAE at 0.35M KCl (recall that this fractionation would remove mitochondrial RNA polymerase from the extract [14]). In the instances where we have done similar experiments their results and ours are in close agreement. They concluded that the promoter is located between positions −155 and +27 and identified a domain near the 5′ boundary (−146 to −91) plus a core promoter (−28 to +8). They also observed an effect of mutation of the center of the promoter similar to our own results and chose to interpret this as a separate domain.

Comparison of the yeast ribosomal gene promoter with its vertebrate counterparts

Four vertebrate ribosomal gene promoters have been analysed in sufficient detail to compare with the the yeast ribosomal gene promoter. These are the ribosomal gene promoters from human, mouse, rat, and frog. Little, if any, sequence conservation has been detected among these promoters, yet they share several common structural similarities.

To begin with, all of the vertebrate promoters, as well as the yeast promoter, are of approximately the same length and they all overlap the site of transcription initiation. The human promoter extends from about −156 to +18 (5), the mouse promoter from −140 to +9 (10), rat from about −167 to +10 (2), frog from −142 to +4 (16, 13), and the yeast promoter from about −150 to between +3 and +12 (11, 18, and this paper).

Second, all of these promoters are composed of an essential core domain which is more or less dependent on a second upstream domain. In some organisms, such as mouse and human, the core domain is the dominant element under most in vitro conditions (5, 10, 4, 17). However, even in these organisms the upstream domain appears to be absolutely essential in vivo (7, 6). In other organisms, such as the frog, the upstream domain is dominant even in vitro (9) and it requires special effort to demonstrate the existence of a core promoter domain (16). Yeast appears to be somewhat intermediate between these two extremes. Like the frog, in vitro transcription is heavily dependent upon the upstream domain. However, at higher template concentrations one can readily observe significant transcription until the core domain is damaged (reference 18 and Figure 3).

It is a matter of argument whether these promoters contain essential internal domains in addition to the core and upstream domains. For example, depending upon the precise assay conditions, large segments of both the frog and yeast promoters appear relatively neutral. It is possible to damage both of these promoter by internal mutations, but it remains to be shown whether this is due to alteration of a critical protein binding site or to a more general change in bendability or some other structural feature of the promoter.

The third, and perhaps most striking similarity between the yeast and vertebrate promoters is the requirement for precise spacing between the core and upstream domains. In the frog it has been shown that changing the spacing between upstream and core domains will change a frog promoter into a highly active mouse promoter (12). In addition, such changes will cause the initiation site to move from an aberrant −4 site to the correct +1 site (when assayed in heterologous mouse extract). Utilization of the +1 site was observed to increase and decrease as the spacing changes went through a 10 bp periodicity.

With the yeast promoter, neither we nor Musters, et al,(11) observed any changes in the site of initiation upon introducing spacing changes. However, the activity of the promoter was highly sensitive to spacing changes, both in vivo and in vitro. Furthermore, our in vitro analysis shows a partial rescue of

activity when the spacing change was either increased or decreased to 10 bp. It appears, therefore, that both the frog and yeast promoters have a critical requirement for proteins binding to the core and upstream domains to be located at precise positions on the face of the DNA helix.

The fact that a frog promoter can be changed into a mouse promoter strongly argues that the apparent differences in domain sensitivity between the two species is obscuring the fact that they both have nearly identical architecture and protein binding sites. In our view, the yeast promoter also fits this general pattern and it is a reasonable working model that rDNA promoters from yeast to mammals have the same general architecture.

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