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Two Distant and Precisely Positioned Domains Promote Transcription of *Xenopus laevis* rRNA Genes: Analysis with Linker-Scanning Mutants

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To examine the internal organization of the promoter of the *Xeonpus laevis* rRNA gene, we constructed a series of linker-scanning mutants that traverse the rDNA initiation region. The mutant genes, which have 3 to 11 clustered base substitutions set within an otherwise unaltered rDNA promoter sequence, were injected into *Xenopus* oocyte nuclei, and their transcriptional capacity was assessed by S1 nuclease analysis of the resultant RNA. The data demonstrate that there are two essential promoter domains, the distal boundaries of which coincide with the promoter boundaries established previously by analysis of 5′ and 3′ deletion mutants. The upstream promoter domain is relatively small and extends from residues ca. −140 to −128. The downstream domain is considerably larger, encompassing residues ca. −36 to +10, and exactly corresponds in both size and position to the mammalian minimal promoter region. The *Xenopus* rDNA sequence between these two essential domains has a much smaller effect on the level of transcriptional initiation. In light of the fact that a large portion of this intervening region consists of a segment (residues −114 to −72) that is duplicated many times in the upstream spacer to form an rDNA enhancer sequence, it is noteworthy that a "−115/−77 linker scanner," in which virtually this entire segment is replaced by a polylinker sequence, has full promoter activity in the injected *Xenopus borealis* oocytes. Analysis of a parallel series of spacing change linker-scanning mutants revealed the unexpected result that the relative positions of the upstream and downstream promoter domains are very critical: all spacing alterations of more than 2 base pairs within this 100-base-pair region virtually abolish promoter activity. We conclude that the factors that bind to these two distant promoter domains must interact in a very precise stereospecific manner.

An essential aspect of understanding the mechanisms that control eucaryotic gene expression is the identification of the *cis*-acting DNA sequences required for accurate and efficient initiation of transcription. Comparison of the DNA sequences immediately upstream of the initiation site of a number of genes transcribed by RNA polymerase II has revealed motifs that are conserved across a wide range of genes, for instance, the TATA box located ~30 base pairs (bp) upstream of the mRNA cap site of most polymerase II-transcribed genes (5, 6) and a CCAAT box and GGCGGG sequence located ~50 to 100 bp upstream of the cap site of many genes (3, 8). Similarly, A and B box sequence motifs are conserved within the ~80 bp following the site of transcriptional initiation in various genes transcribed by RNA polymerase III. The functional significance of these and other conserved elements has been demonstrated by assaying the transcriptional efficiency of deletion, point, or linker-scanning mutants in a number of systems.

For the rRNA gene transcribed by RNA polymerase I, it has not been possible to identify distinct conserved sequence elements within the promoter region of various species, a result consistent with the observation that the rRNA gene promoter appears to act in a fairly species-specific manner (10). Nonetheless, by assay of mutant rDNA templates, the promoter boundaries of the rRNA gene have been identified in a number of species, including *Xenopus laevis* (22, 29), mice (9, 21, 36), humans (16, 26), *Drosophila melanogaster* (14), and *Acanthamoeba castellanii* (13). These data indicate that rDNA promoters are contained within an ~170-bp region and consist of multiple distinct domains, the presence of which are revealed under different transcription conditions. With the *Xenopus* rRNA gene, a small core promoter region from residues −7 to +6 is sufficient to direct efficient transcription when deletion mutants are injected into *Xenopus* oocytes at a high rDNA concentration (29), and under these conditions the presence of upstream sequences does not augment the transcriptional level above that obtained from 5′Δ−7. However, by merely lowering the amount of injected rDNA promoter from 2 to 1 fmol per oocyte, the presence of an additional promoter domain that extends upstream to ca. residue −142 becomes essential for directing transcription initiation (35). Analogous results have also been obtained with the mouse rDNA promoter: when in vitro transcription reaction conditions are made increasingly stringent by any of a number of alterations, the apparent 5′ promoter boundary shifts from ca. residue −27 to ca. −35, −45, −100, and −140. Each border evidently demarks a promoter domain (21). Similarly, for human rDNA, sequences from ca. residues −52 to +7 are necessary and sufficient to direct transcription in vitro, but under other in vitro and in vivo conditions, sequences extending upstream to ca. position −150 are also found to be involved (16, 26).

These data have suggested a general model for the structure of the eucaryotic rDNA promoter (28). First, there is a core promoter domain which consists of sequences immediately flanking the initiation site and is required under all reaction conditions. This region is capable of binding the necessary transcription factors in the absence of the upstream sequences, since it is sufficient for transcription under appropriate conditions. In addition, there are upstream domains whose effect is only observed under more stringent reaction conditions. Template commitment studies

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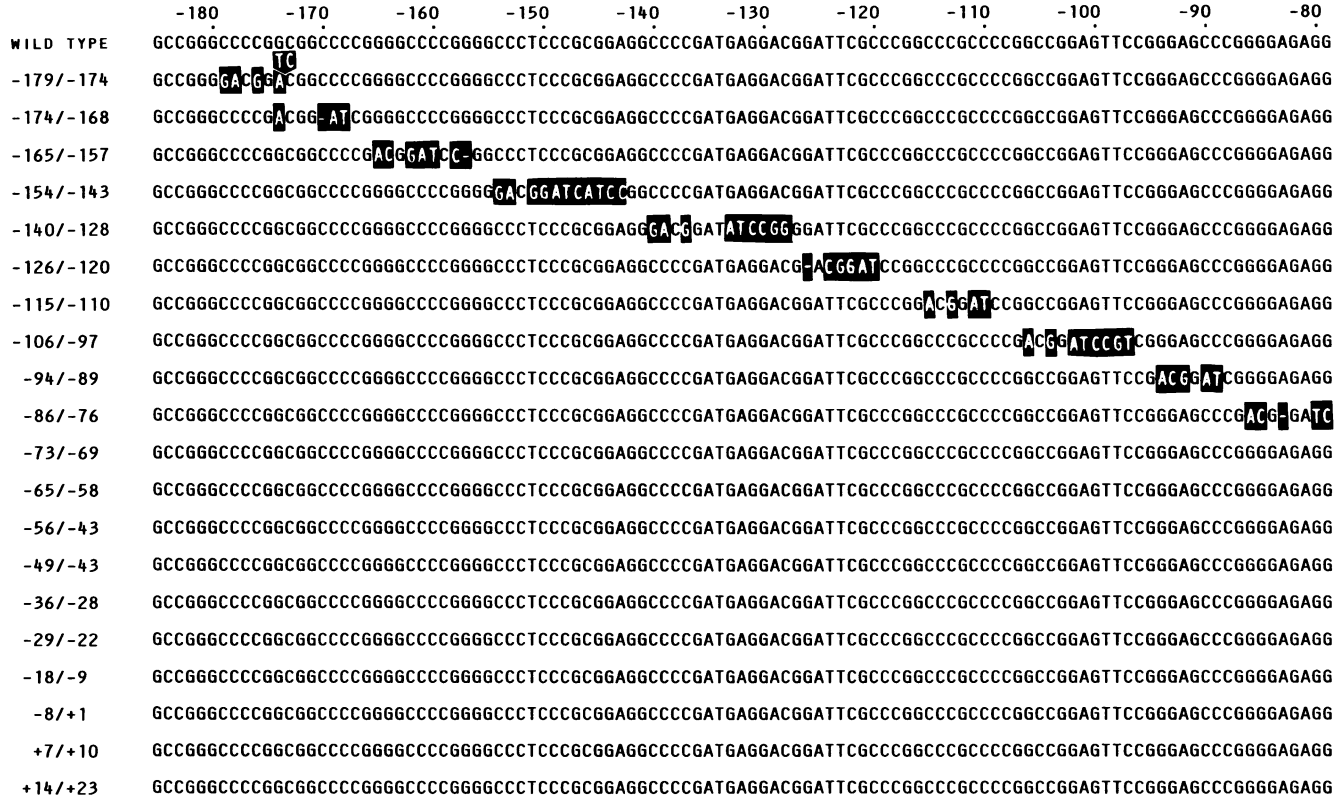


indicate that these upstream domains also act by binding transcription factors (32).

To further characterize and to determine the internal organization of the *X. laevis* rDNA promoter, we constructed a series of linker-scanning mutants across the 200-bp initiation region. Injection of this series of mutant templates into *Xenopus* oocytes revealed two essential promoter domains, a proximal one from residues −36 to +10 and an upstream one from residues −140 to −128. The central, enhancer-cognate region of the promoter does not contain sequences essential for transcription in injected *X. borealis* oocytes, since a linker-scanning mutant in which virtually this entire 42-bp region is replaced by a synthetic polylinker directs wild-type levels of transcription. In contrast, linker-scanning mutants that create spacing changes between the upstream and core promoter domains all drastically decrease the level of transcription, suggesting that a transcription factor binds to the core promoter domain and must precisely interact with a factor that binds to the upstream promoter domains, 100 bp away.

MATERIALS AND METHODS

Construction of deletion mutants and linker-scanning mutants. The starting plasmid for the 5′ deletions was pX1r315, an *X. laevis* rDNA subclone that contains the initiation region from residue −1150 to +115, with a *Bam*HI site at the 5′ end of this region and an *Eco*RI site at the 3′ end (29). Construction of a set of 5′ deletions was reported previously (29) and involved *Bal* 31 digestion from the *Bam*HI site at position −1150, ligation to a 10-bp *Bam*HI linker (effectively adding 5 bp to the site of the deletion), and insertion of the *Bam*HI-*Eco*RI-liberated rDNA into pBR322. Other 5′ deletions were generated by partial digestion with *Sma*I or *Hpa*II and similar ligation to a *Bam*HI linker for cloning.

The 3′ deletions were generated by partial restriction endonuclease cleavage of pXlr14d, a plasmid which contains *X. laevis* rDNA from residues −317 (*Pst*I site) to +115 (*Pvu*I site) subcloned into pBR322 (1). The *X. laevis* rDNA has a high G+C content, and accordingly the promoter region contains a large number (25) of *Hpa*II and *Hae*III sites. Therefore, pX1r14d was partially digested with either *Hpa*II or *Hae*III and then digested to completion with *Pst*I. Fragments of the desired size range were electroeluted from a polyacrylamide gel. The *Pst*I-*Hpa*II fragments were cloned in *Pst*I-*Acc*I-digested pUC9 (33), and the *Pst*I-*Hae*III fragments were cloned in *Pst*I-*Hin*cI-digesting pUC9. The resultant plasmids have a polylinker *Bam*HI site centered 6 bp downstream from the site of the deletion and a *Hin*dIII site 8 bp upstream from the *Pst*I site at −317. Inserts were screened by sizing *Hin*dIII-*Bam*HI-digested plasmid DNA. Other 3′ deletions were similarly generated by digesting rDNA with *Hin*fI or *Hga*I, blunting the end, and cloning the rDNA fragment into a pUC vector.

The linker-scanning mutants were formed by ligating the gel-isolated *Eco*RI-*Bam*HI rDNA fragments containing the 5′ deletion and the gel-isolated *Bam*HI-*Hin*dIII rDNA fragments containing the 3′ deletion to the large *Eco*RI-*Hin*dIII fragment of pBR322. A few of the linker-scanning mutants (LS −56/−43, −36/−28, −29/−22, −8/+1, +7/+10, and +14/+23) were analogously constructed from 3′ deletions which extend upstream to position −245 (which had been converted to a *Sal*I site). They were similarly formed by ligating the isolated *Bam*HI-*Eco*RI 5′ fragments and the isolated *Bam*HI-*Sal*I 3′ fragments to the large *Eco*RI-*Sal*I

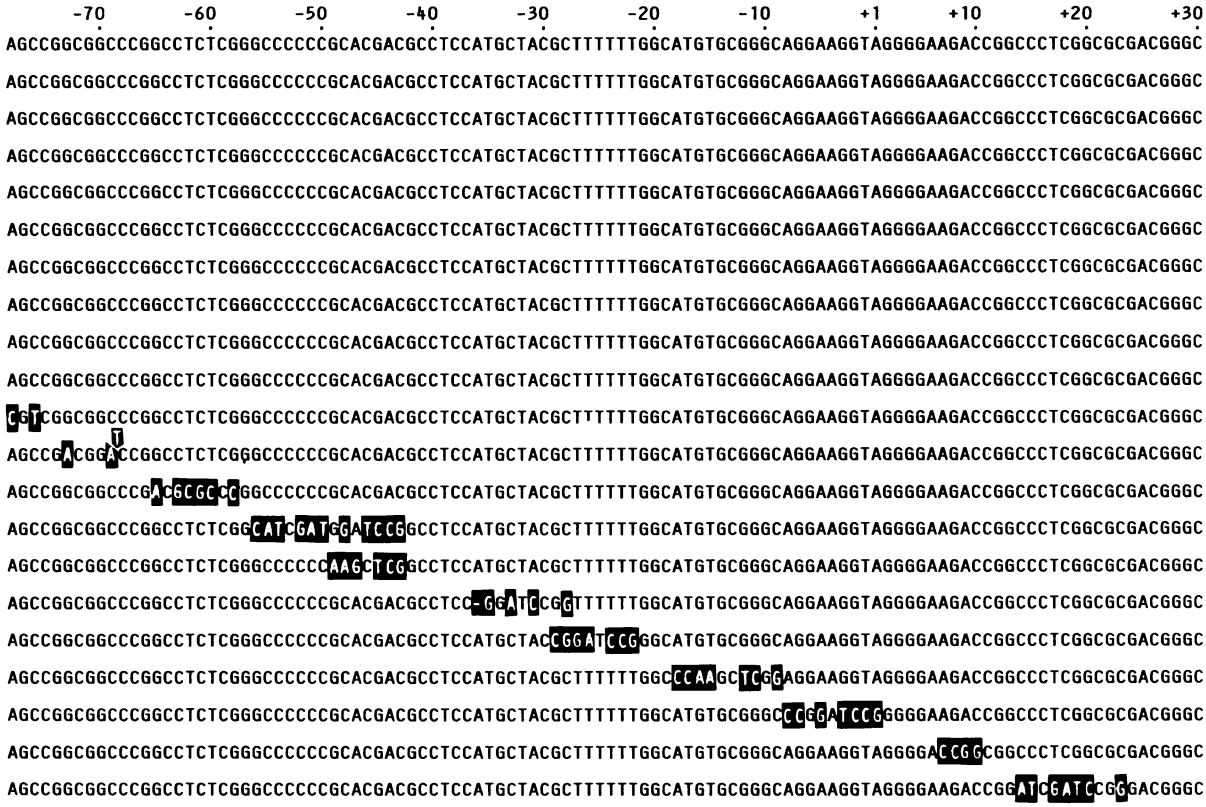


FIG. 1. Nucleotide sequences of linker-scanning mutants of *Xenopus* rDNA promoter. The sequence of the wild-type rRNA gene from residues −185 to +30 is shown on the top line. Each linker scanner is designated by the first and last nucleotide that is mutated, and all nucleotides that are mutated are shaded. In instances in which there are insertions or deletions, the alignment is positioned to provide the best sequence conservation. Insertions are represented by nucleotides above the sequence, and deletions are shown by bars.

fragment of pBR322. To generate the correct spacing be tween the 5′ and 3′ fragments, certain ends were modified before ligation by (i) treatment with Klenow polymerase and deoxyribonucleoside triphosphates to completely or partially fill in the restriction site (17), (ii) treatment with S1 nuclease (10 U/μl for 15 min on ice) to remove the overhanging end, (iii) ligation to a linker, or (iv) combinations of the above.

To obtain the ca. +4- and ca. −4-bp spacing change linker-scanning mutants, the corresponding linker scanners were partially digested with *Bam*HI and either 4 bp were added by treating with Klenow polymerase plus all four deoxyribonucleoside triphosphates or 4 bp were removed by treating with S1 nuclease. Finally, the plasmids were recircularized by treatment with T4 DNA ligase.

The 8-, 9-, or 10-bp spacing change mutants, as well as LS −73/−67 (+3) and LS −65/−58 (+4), were obtained by joining mismatched 5′ and 3′ deletion mutants.

To construct LS −115/−77, the rDNA sequences upstream of the *Bam*HI site of LS −115/−110 and the rDNA sequences downstream of the *Bam*HI site of LS −86/−76 (blunted by treatment with S1 nuclease) were joined by using the *Bam*HI-*Hae*III fragment from pUC8 (33).

All linker-scanning and spacing change linker-scanning mutants were sequenced by the method of Maxam and Gilbert (18). For certain templates, including LS −140/−128, two different plasmid preparations were used as templates.

Construction of control plasmids. Plasmid −245B was formed from 3′Δ+13 (29), which contains rDNA from positions −245 to +13 and is cloned 5′ → 3′ between the *Sal*I and *Bam*HI sites of pBR322. The *Bam*HI-*Hin*dIII vector fragment of this plasmid was replaced with the pBR322 region from the *Bam*HI site to the *Hae*II site at position 417. Plasmid −317B is identical except that it contains rDNA to position −317 and is cloned by using the *Hin*dIII instead of the SalI site of pBR322. Thus, −245B and −317B are cloned into pBR322 in the same context as the corresponding linker-scanning mutants.

Transcription in microinjected oocytes and S1 nuclease analysis. Microinjection into *X. borealis* oocytes was performed as described previously (27, 35). Approximately 30 oocytes were injected per DNA sample. The linker-scanning mutants were injected at a concentration of 25 μg/ml with an equal amount of their corresponding control plasmid, either −245B or −317B. In the experiment of Fig. 3, the control plasmid was maxi-5S, a derivative of a *X. borealis* 5S gene plasmid that contains a duplication of sequences +77 to +115 3′ to the internal control region (4).

The hybridization probe for transcripts from the linker-scanning mutants was the "*Hin*fI probe" (27), a 5′-end-labeled coding strand fragment extending from rDNA positions +55 to −125. The "B" probe for transcripts from the −245B and −317B genes was the isolated *Eco*RI-*Ava*I fragment (+89 to −60 relative to the transcription initiation site) from −245B, 5′ end labeled and strand separated. The probe for the maxi-5S gene was the isolated 5′-end-labeled coding strand extending from the *Eco*RI site to the *Bam*HI site (+115 to −79) of the maxi-5S plasmid.

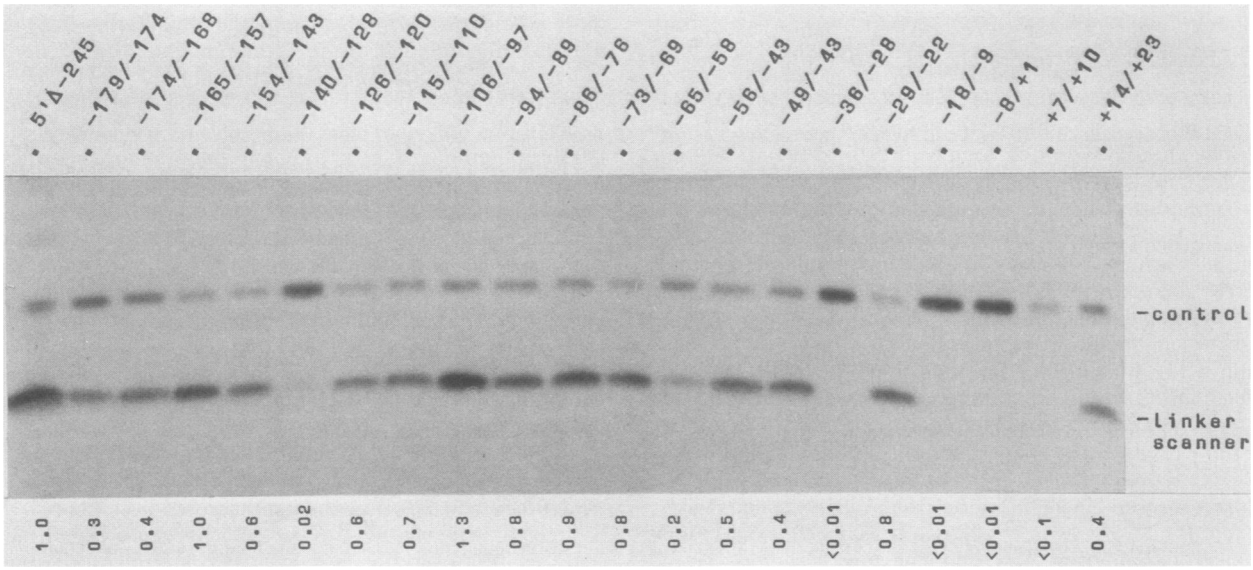


FIG. 2. Effect of linker-scanning mutations on rDNA transcription in microinjected oocytes: coinjection with a control rRNA gene. *X. borealis* oocytes were microinjected with ~0.25 fmol each of the indicated plasmid and a wild-type control gene. The resultant RNAs were detected by hybridization to a mixture of the *Hin*f probe (which hybridizes specifically to transcripts from the linker-scanning mutants and 5'Δ−245 and yields a 55-nucleotide protected fragment) and the B probe (which hybridizes specifically to transcripts from the control genes and yields an 89-nucleotide protected fragment). After treatment of the hybrids with S1 nuclease, the protected fragments were resolved by electrophoresis and visualized by autoradiography. The intensity of the band representing the linker scanner transcript relative to the intensity of the band representing the control transcript is shown below each lane, normalized to 1.0 for the parental gene.

For S1 analysis of rRNA, 2 oocyte equivalents of RNA were hybridized to 0.01 pmol of probe as described previously (27). Both the *Hin*fI and B probes were contained in the same reaction mixture. For S1 analysis of maxi-5S RNA, 0.5 oocyte equivalent was separately hybridized to 0.05 pmol of probe. All hybridizations were conducted in probe excess, and S1 nuclease digestion, polyacrylamide gel electrophoresis, and autoradiography were performed as described previously (27, 29). The intensity of the autoradiographic bands was determined by densitometry of exposures within the linear range.

RESULTS

Effect of linker-scanning mutations on rDNA transcription. We previously demonstrated that *Xenopus* rDNA sequences extending from positions ca. −142 to ca. +6 are required for efficient transcription of genes microinjected into *Xenopus* oocytes at ≤1 fmol per oocyte (35). However, when higher concentrations of template are microinjected, a much smaller region (from −7 to +6) is sufficient for accurate and efficient initiation, and upstream sequences do not augment this level of transcription (29). This suggests that the promoter consists of at least two domains and that the upstream one is dispensable under certain assay conditions. To further define the elements that constitute the *Xenopus* rDNA promoter and to discern its internal organization, we constructed a series of linker-scanning mutants spanning the initiation region from residues −179 to +23. These clustered point mutations are formed by combining pairs of matched 5' and 3' deletion mutants so that a segment of ~10 bp is replaced by a synthetic oligonucleotide linker of equal length (20). Thus, any effect on transcription can be attributed to the base substitutions and not to spacing changes. The sequence of each of these mutants is shown in Fig. 1. Most of the linker-scanning mutants exactly preserve the original spacing, whereas a few contain a 1-bp insertion or deletion.

Each linker-scanning mutant was microinjected into *X. borealis* oocytes under conditions in which the upstream promoter region is required to direct transcription. An equal amount of a control (B) gene containing a wild-type rDNA promoter fused at residue +13 to procaryotic sequences was coinjected with each template. Transcription of this internal control gene can be selectively detected after hybridization to a probe specific for this (B) gene. Assay of the transcriptional level directed by these linker-scanning mutants revealed two distinct essential promoter domains, a small upstream domain and a larger core domain surrounding the initiation site (Fig. 2).

The upstream domain is defined by LS −140/−128. Its 5' border coincides with the 5' border of the promoter defined by transcription of 5' deletion mutants of *Xenopus* rDNA both in injected oocytes (35) and in vitro (29). Although the essential region of the upstream promoter domain is quite small (Fig. 2), relevant sequences evidently extend beyond the boundaries of LS −140/−128, since adjoining linker-scanning mutants also directed reduced levels of transcription. Reproducibly, LS −179/−174, −174/−168, −154/−143, −126/−120, and −115/−110 were less active than was the wild-type gene. Moreover, this result is consistent with our previous observation that 5'Δ−154 can be much less active than 5'Δ−170 in injected oocytes from certain frogs (35).

The downstream promoter domain extends from residues ca. −36 to ca. +10 (Fig. 2). This region is appreciably larger than the small promoter sequence (residue −7 to +6) that is sufficient to direct efficient transcription when higher concentrations of rDNA are microinjected into oocytes (29). This larger downstream promoter segment is interrupted by LS −29/−22, which has virtually wild-type activity. We cannot yet distinguish whether this downstream promoter region consists of two closely positioned functional domains or whether it represents a single functional domain in which the nucleotides that are altered in LS −29/−22 are simply not essential. The transcriptional activity of LS −29/−22 is

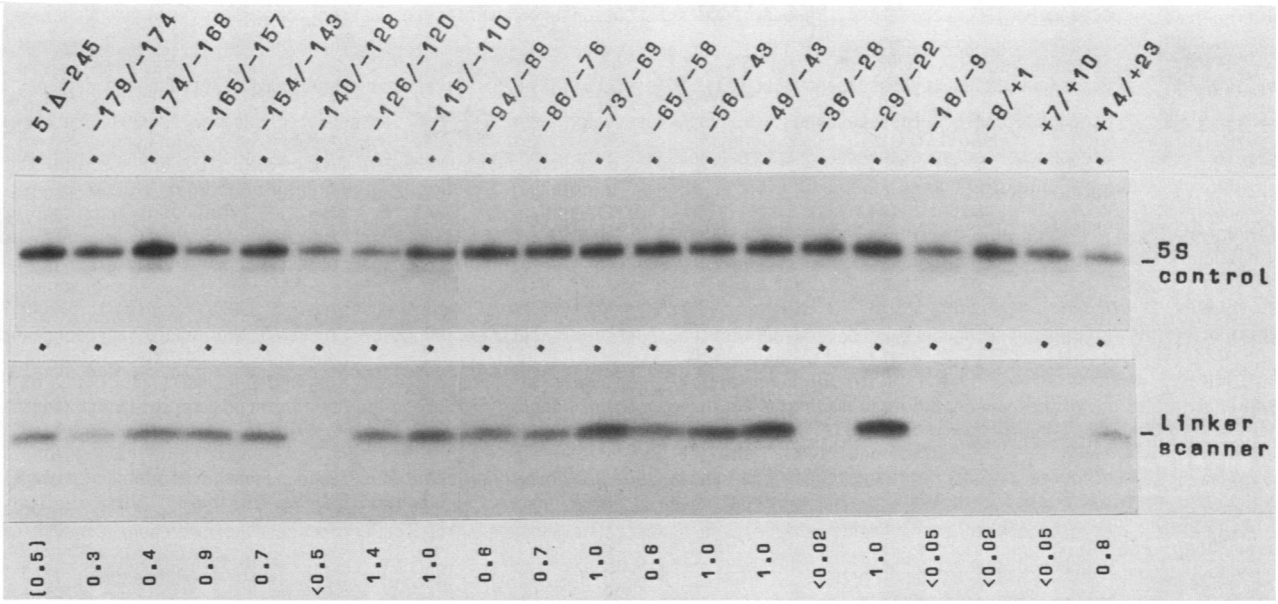


FIG. 3. Effect of linker-scanning mutations on rRNA transcription: coinjection with a control 5S gene. The linker-scanning mutants were assayed by microinjection as described in the legend to Fig. 2, except that a 5S gene (maxi-5S) was coinjected as the internal control. For detection of linker scanner transcription, 2.0 oocyte equivalents of RNA were hybridized to the *Hin*f probe as described in the legend to Fig. 2. For detection of 5S transcription, 0.5 oocyte equivalent of RNA was hybridized in a separate reaction mixture to 0.05 pmol of the maxi-5S probe. Quantitation was performed as described in the legend to Fig. 2 and is shown below each lane.

particularly interesting since the cluster of six T residues that occurs within an otherwise highly G+C-rich promoter and is conserved in the promoter region of the rDNA of other species has been eliminated in this mutant (30).

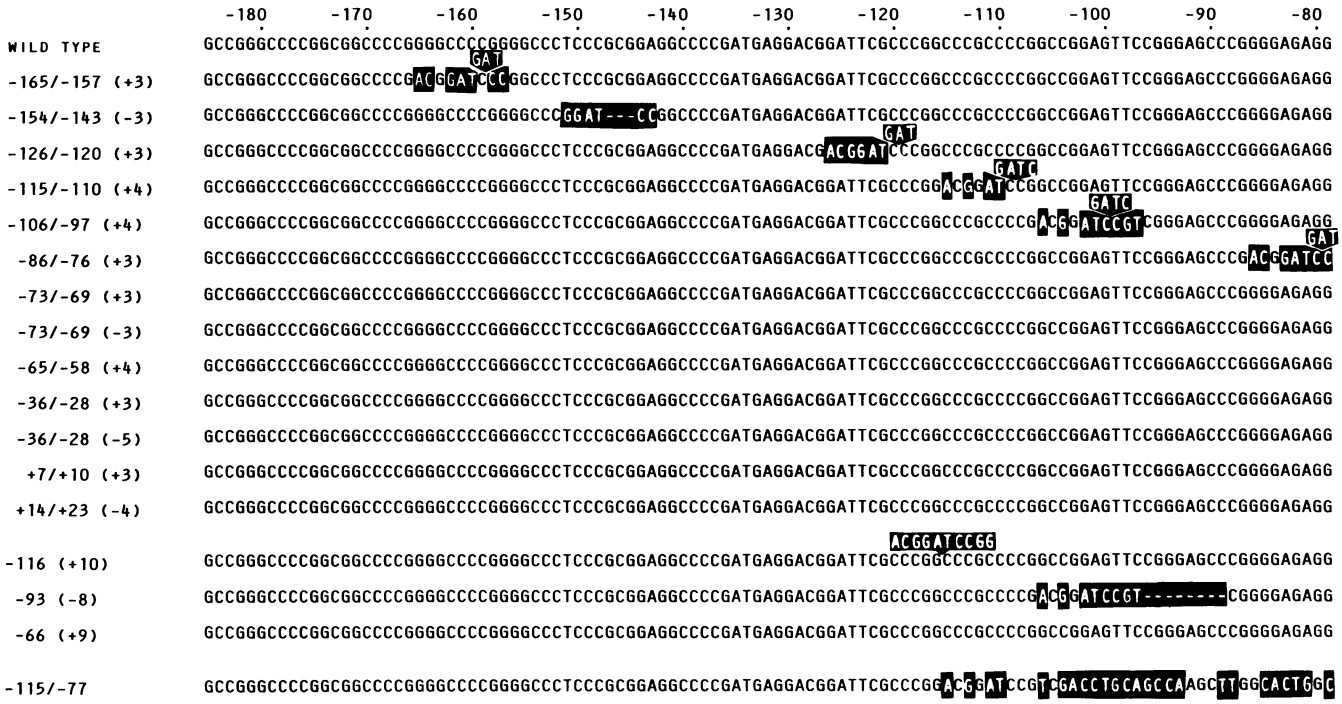
Linker-scanning mutations that fall between the upstream and downstream major promoter domains also affected the level of transcription, although less dramatically (Fig. 2). This was most obvious for LS −65/−58, which exhibited an ca. fivefold reduction in the transcriptional level, but was also reproducibly observed for a number of the other linker-scanning mutants. This indicates that an appreciable portion of the rDNA promoter region is involved in making sequence-specific contacts, presumably with essential or stimulating transcription factors.

Most of the linker-scanning mutants which abolished transcription (LS −140/−128, −36/−28, −18/−9, and −8/+1) caused the coinjected control gene to be transcribed at a correspondingly higher level (Fig. 2). This demonstrates that there is competition between the two coinjected genes, a fact which may accentuate the observed effect of a mutation. Therefore, to assay the promoter efficiency of the linker-scanning mutants in the absence of any intentional competition, each template was instead coinjected with a 5S gene-containing plasmid as the control (maxi-5S; 4). The results (Fig. 3) qualitatively confirmed those shown in Fig. 2. The same core and upstream promoter domains were required for transcription, whereas other linker-scanning mutants across the entire promoter region had a less dramatic but significant effect on the level of transcription.

Effect of spacing change mutations on transcriptional efficiency. To determine whether the precise spacing between the upstream and downstream promoter domains is critical, a number of insertion and deletion mutants were constructed (Fig. 4). Most of these were formed by filling in or digesting away the single-stranded ends after *Bam*HI digestion within the linker, resulting in the insertion or deletion of 4 bp relative to the parental linker-scanning mutant. The activity of the +4 spacing change mutants is shown in Fig. 5A. The gene bearing an insertion upstream of the major 5′ promoter boundary [LS −165/−157 (+3)] transcribed as well as the parental linker-scanning mutant. However, all of the six ~4-bp insertion mutations between the upstream and downstream promoter domains greatly diminished the level of transcription. A similar result was obtained with the ~4-bp deletion mutants (Fig. 5B). Those just upstream and downstream of the major promoter borders, LS −154/−143 (−3) and +14/+23 (−4), had a minimal effect, whereas the deletion linker-scanning mutants between these promoter boundaries directed virtually no transcription.

Since each of these ~4-bp insertion and deletion mutations changes the spacing between the upstream and downstream promoter domains by approximately one-half of a helix turn, it is possible that the transcriptional decrease was not due to the change in distance between these promoter elements per se but that these mutations cause the promoter domains to be oriented on the opposite face of the DNA helix relative to each other. To address the effect of spacing changes in the absence of helix face considerations, three mutants which alter spacing by approximately one helix turn were constructed: LS −116 (+10), LS −66 (+9), and LS −93 (−8) (Fig. 4). Notably, these mutants also abolished correct transcription (Fig. 5C), although their corresponding linker-scanning mutants (Fig. 2) were actively transcribed. Thus, it appears that the precise spacing between the upstream and downstream promoter elements is critical for rDNA promoter function.

Effect of enhancer-cognate region on transcriptional efficiency. We found it striking that none of the linker-scanning mutations between rDNA positions ca. −125 and ca. −70 decreased transcriptional efficiency by more than about 50%. This region bears a 90% sequence homology to the repetitive 60/81-bp elements of the rDNA spacer that con-



stitute the polymerase I enhancer sequence (24). It has been generally hypothesized that this enhancer acts by initially binding a transcription factor and thereby increasing the availability of this factor for binding at the analogous sequence within the rDNA promoter (23–25). This model suggests that the enhancer-cognate region of the promoter might be vital for transcription. Our data with the linker-scanning mutants (Fig. 2) could be reconciled with this prediction if the factor that binds to this sequence makes a large number of contacts, so that none of the individual linker-scanning mutations is severe enough to abolish this interaction. To test this hypothesis, LS −115/−77 was constructed. This mutant contains synthetic polylinker sequence in place of virtually the entire enhancer-cognate region (Fig. 4). LS −115/−77 promoted a wild-type level of transcription upon coinjection into oocytes with a control gene (Fig. 6, lanes 2 and 4). Thus, if this region does in fact interact with a transcription factor, the interaction cannot be of critical importance for promotion of transcription.

DISCUSSION

We used a series of genes bearing clustered point mutations (linker-scanning mutants) to analyze the sequence requirements for efficient transcription of the *X. laevis* rRNA gene in injected oocytes. The analysis revealed two essential promoter elements, a core domain and an upstream domain. The core domain surrounds the initiation site and extends from residues ca. −36 to ca. +10; the upstream domain encompasses a relatively small region, from residues ca. −140 to ca. −128. Mutations of residues within these regions caused a >50-fold reduction in the transcriptional level. In addition, sequences upstream of and between these regions had a lesser but reproducible effect.

These results confirm and considerably extend the data previously available on rDNA promoter organization. First, the rDNA region that is spanned by the essential segments of the promoter (residues −140 to +10; Fig. 2) corroborates the extent of the essential rDNA promoter region determined from analysis of 5′ and 3′ deletion mutants (residues −142 to +6; 29, 35). Furthermore, analysis of both linker-scanning mutants (Fig. 2) and 5′ deletion mutants (35) indicated that a region between residues ca. −180 and ca. −140 can have an ca. threefold stimulatory effect on the level of rDNA transcription.

It is noteworthy that the segment between the upstream and core promoter domains showed only a limited effect in our analyses. This segment includes a region of rDNA (residues −114 to −72) that is adjacent to, but not overlapping with, the upstream promoter domain and is duplicated from 20 to 80 times in the "nontranscribed" spacer that separates adjacent rRNA coding regions. This repetitive region has been shown to be a *cis*-acting enhancer of polymerase I transcription, whereas in *trans* this repetitive region can abolish transcription of an rRNA gene lacking the enhancer sequence (24, 25). The logical hypothesis is that the duplicated −114 to −72 sequences attract a limiting component that is essential for transcriptional initiation by the rDNA promoter. Thus, it was quite surprising that none of the linker-scanning mutations that traverse this −114 to −72 enhancer-cognate region caused more than an ca. twofold decrease in the transcriptional level. This result was verified and extended by the fact that LS −115/−77, in which the sequence of 28 of 40 residues within this segment is changed, transcribed with virtually the same efficiency as did a gene with the intact promoter when reacted in competition with a control gene (Fig. 6). Thus, under conditions in which the enhancer has a large effect, its cognate region in the promoter is quite dispensable. (It should be noted that our linker-scanning mutants lack the duplicated enhancer.) These data suggest the rather unprecedented conclusion that if the −114 to −72 enhancer sequence acts by binding an essential transcription factor, this factor must also bind efficiently to the promoter in the absence of the −114/−72 sequence. Other studies (B. Sollner-Webb, J. Windle, S. Henderson, J. Tower, V. Culotta, S. Cass, and N. Craig, *in*

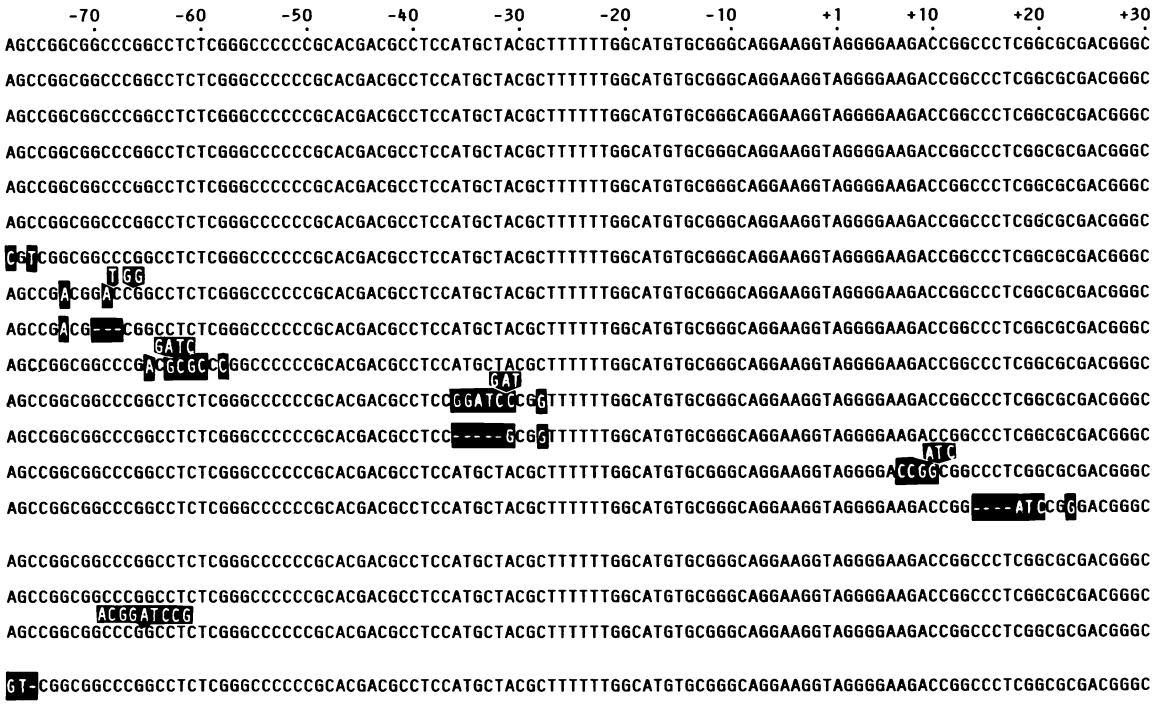


FIG. 4. Nucleotide sequences of spacing change linker-scanning mutants and LS −115/−77. The sequences are designated as in Fig. 1.

D. Granner, G. Rosenfeld, and S. Chang, ed., *Transcriptional Control Mechanisms*, in press) have shown that the enhancer acts in close conjunction with the upstream domain of the promoter, so it is possible that the same factor may be initially attracted to the enhancer sequence and then binds to the adjacent upstream promoter domain.

Our results nicely complement a recent study by Dunaway et al. (7) in which the proteins of an *X. laevis* oocyte extract protect distinct regions of the rDNA promoter in a DNase I footprinting assay. Their protected region III (residues −140 to −120) correlates well with our upstream promoter domain. Moreover, their protected region I (residues −10 to +15) correlates well with the 3′ portion of our core promoter domain. However, their protected region II (residues −100 to −70, in the 3′ portion of the enhancer-cognate sequence) is a region that we detected as only of lesser importance.

In this study we mapped the core domain of the *X. laevis* rDNA promoter to residues ca. −36 to +10. This is very similar in both size and position to the minimal promoter of the mouse rRNA gene (ca. −39 to +9; 21), a result which underscores a growing body of evidence that rDNA promoters of different eucaryotic species are organized similarly (28). In both mouse and *Acanthamoeba* rDNA, the upstream portion of the minimal promoter (upstream of ca. residue −15) has been shown to interact with the species-specific transcription factor, whereas the downstream portion (ca. −10 to +10) may interact more closely with the active polymerase (2, 32). Most likely, the corresponding frog promoter regions (which can direct specific transcription in a mouse cell extract; 34) function similarly. In this regard, it is noteworthy that transcription of *Xenopus* rDNA upon injection into oocytes at a high promoter concentration requires only the downstream portion of the core promoter domain (from residues ca. −7 to +6; 29). Although it remains to be determined why the upstream portions of the *Xenopus* promoter are dispensable at high rDNA concentrations, we have found that under these conditions (i) the upstream promoter domain does not inhibit transcription in *trans* and (ii) LS −36/−28 and LS −18/−9 are active templates (unpublished observations). The possibility that at a high template concentration rRNA transcription is directed in the absence of the binding site for the normally essential species-specific transcription factor must be considered. Indeed, one cannot even rule out the possibility that RNA polymerase I might be sufficient to catalyze accurate synthesis under these specialized transcription conditions.

We also analyzed the distance requirements between the upstream and core promoter domains by inserting and deleting small numbers of base pairs in several of the linker-scanning mutants. These spacing change mutants demonstrated that the distance between the two promoter elements is extremely critical, since any insertion or deletion between these two domains that changed spacing by 3 or more bp virtually abolished the transcriptional signal (Fig. 5). This was true whether the mutations altered the spacing by ca. one-half or ca. one helix turn and suggests a model in which each promoter domain represents a protein-binding site and the upstream binding protein interacts directly with the core binding protein in a stereospecific manner. This precise spacing requirement between two domains that are separated by ~100 bp is notable. In most other instances that have been studied, the requisite relative spacing of *cis*-acting domains does not appear to be as precise (12, 15, 19, 31).

Haltiner et al. (11) recently reported a linker scanner analysis of the human rDNA promoter. Consistent with our results with *Xenopus* rDNA, they also observed an essential core domain containing the initiation site and an upstream domain centered on ca. residue −130. However, in the human system, it appears that spacing increases between these two domains of up to 28 bp do not reduce the level of transcription. This is in contrast to the precise spacing requirement we observed in the *Xenopus* system. It is,

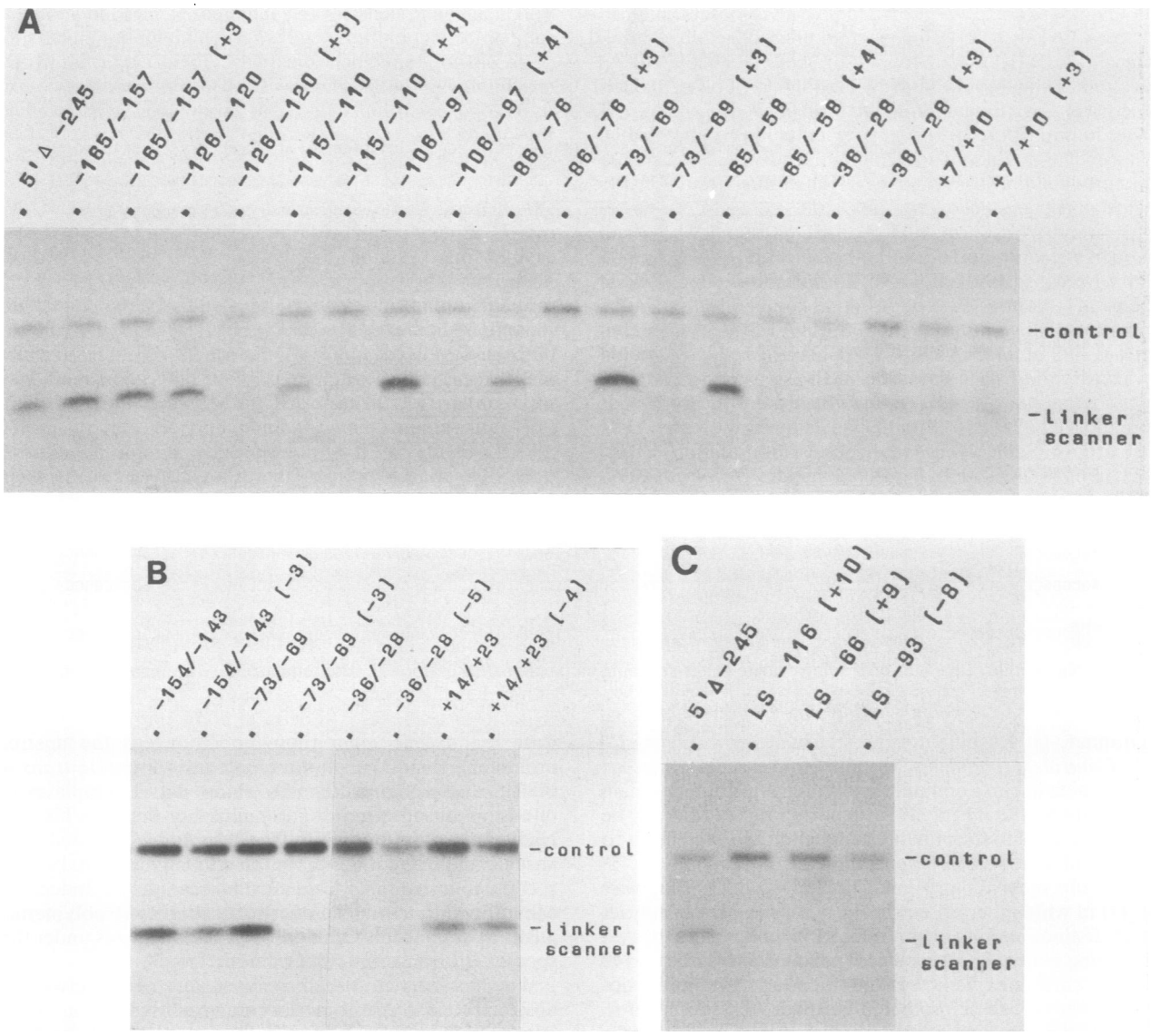
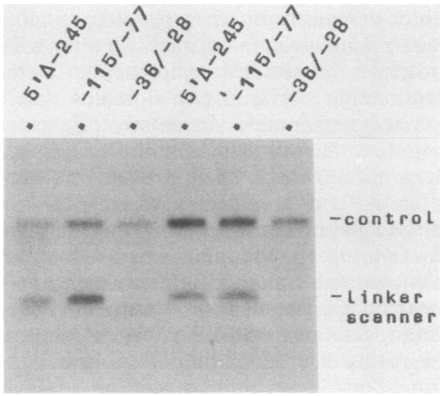


FIG. 5. Effect of ~4-bp and ~10-bp insertion and deletion mutations on transcriptional efficiency. Linker-scanning mutants bearing insertions of ~4 bp (A), deletions of ~4 bp (B), or spacing changes of 8 to 10 bp (C) were assayed as described in the legend to Fig. 2. The spacing changes are indicated in parentheses.



however, possible that this apparent difference might not reflect a basic difference between the action of the frog and human upstream and core domains but may be due to the fact that the human rDNA mutations were assessed under in vitro conditions in which the upstream sequences have only an ca. fourfold stimulatory effect. If assessed under conditions in which the upstream domain has a larger effect, it is possible that the spacing of the human rDNA promoter domains would appear to be more critical.

Taken together, the linker-scanning mutant and deletion mutant analyses of rDNA genes of several species demon-

FIG. 6. Effect of replacement of enhancer-cognate region of the promoter. The transcriptional activity of LS −115/−77 (lanes 2 and 5) was assessed in competition with a control rRNA gene as described in the legend to Fig. 2. Control lanes show analogous coinjections of 5′Δ−245 (lanes 1 and 4) or LS −36/−28 (lanes 3 and 6). The results of two independent sets of injections (lanes 1 to 3 and 4 to 6) are shown.

strate a basic similarity in promoter organization. The rDNA promoter consists of an essential core domain surrounding the initiation site and a stimulatory domain ~130 bp upstream of the initiation site. The effects of this upstream domain can range from negliglible to essential depending on the transcription conditions. Under conditions in which the upstream domain is essential for transcription, its spacing relative to the core domain is also crucial, presumably reflecting a close association of factors that interact with these two domains. An apparently unique feature of the *Xenopus* rRNA gene is that it contains an enhancer element whose sequence is homologous to that of the central segment of the rDNA promoter. However, our data indicate that the enhancer-homologous region of the promoter is not important to the level of transcription catalyzed in injected *X. borealis* oocytes. Whether it serves a role in mediating the effect of the enhancer remains to be determined.

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