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A Complex Control Region of the Mouse rRNA Gene Directs Accurate Initiation by RNA Polymerase I

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To determine the size and location of the mouse rDNA promoter, we constructed systematic series of deletion mutants approaching the initiation site from the 5′ and 3′ directions. These templates were transcribed in vitro under various conditions with S-100 and whole-cell extracts. Surprisingly, the size of the rDNA region that determines the level of transcription differed markedly, depending on the reaction conditions. In both kinds of cell extracts, the apparent 5′ border of the promoter was at residue ca. −27 under optimal transcription conditions, but as reaction conditions became less favorable, the 5′ border moved progressively out to residues −35, −39, and −45. The complete promoter, however, extends considerably further, for under other nonoptimal conditions, we observed major effects of promoter domains extending in the 5′ direction to positions ca. −100 and −140. In contrast, the apparent 3′ border of the mouse rDNA promoter was at residue ca. +9 under all conditions examined. We also show that the subcloned rDNA region from −39 to +9 contains sufficient information to initiate accurately and that the region between +2 and +9 can influence the specificity of initiation. These data indicate that, although the polymerase I transcription factors recognize and accurately initiate with only the sequences downstream of residue −40, sequences extending out to residue −140 greatly favor the initiation reaction; presumably, this entire region is involved in rRNA transcription in vivo.

The study of transcription of eucaryotic genes is one of the most active areas of research in molecular biology. This interest reflects our understanding that development and differentiation are in large part accomplished through regulation of gene expression. Through the use of in vitro transcription systems, many basic aspects of control of gene expression are being elucidated for all three eucaryotic RNA polymerases. For instance the sequences required for promotion of transcription are being studied by constructing mutant templates in vitro and assaying their ability to direct transcription. The control region of the 5S RNA gene, transcribed by RNA polymerase III, was the first eucaryotic promoter to be precisely mapped. The sequences required for initiation of 5S RNA reside within the gene, between residues +50 and +85, although other upstream sequences are also involved in specifying the precise initiation site (2, 25). In the case of RNA polymerase II, the promoter resides upstream of the gene. When in vitro runoff transcription is used, the TATAA box, common to virtually all mRNA coding genes, is the primary transcriptional determinant (15, 29), but analysis with closed circular templates (10) or in whole cells (6, 22) demonstrates the importance of other sequences, generally located further upstream.

A number of studies have also focused on mapping the sequences that promote transcription of the rRNA genes by RNA polymerase I. The promoter that functions in vitro is reported to reside largely between residue ca. −40 and a position approximately at the initiation site in mouse (12, 13, 32) and in *Drosophila* (16) rDNA. In *Xenopus* rDNA, on the other hand, a major promoter domain extends out to residue −140 (26), and sequences in this region are also found to stimulate transcription of human rDNA (19). However, since rDNA transcription is highly species specific (14), it is not clear to what extent these apparent differences are due to variation between species or to assay differences.

We have studied the control of transcription of mouse rRNA genes in vitro. In an earlier report (23), we have demonstrated faithful transcription of cloned mouse rRNA genes by using an S-100 extract (11, 30) of mouse L1210 cells. In this paper, we characterized the sequences required for initiation in this extract as well as in a whole-cell extract (20) from the same cells. We found that different regions of the rDNA promoter become evident under different transcription conditions. From this information, combined with our studies on *Xenopus* rDNA transcription and results from other laboratories, a clearer picture of rRNA synthesis is emerging.

MATERIALS AND METHODS

Preparation of extracts, rDNA deletions, and templates. The S-100 extract was prepared from L1210 cells rapidly growing in suspension culture as previously described (23). The whole-cell extract was prepared by the procedure of Manley et al. (20) except that in the final resuspension and dialysis, a buffer containing 100 mM KCl, 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [pH 7.9]), 10 mM $MgCl_2$, 2 mM DTT, 0.1 mM EDTA and 20% (vol/vol) glycerol was used. The S-100 and the whole-cell extract contained ca. 20 and 40 mg of protein per ml, respectively.

The 5′ and 3′ rDNA deletions were constructed by standard methods as described in the legend to Fig. 1, starting from the parental plasmids p5′Sal and p5′Sal-Pvu (23). 5′ deletions were cloned into the *Eco*RI (5′Δ) or into the *Bam*HI ($5'\Delta_B$) sites of pBR322, and 3′ deletions were cloned into the *Pvu*II ($3'\Delta_P$) or into the *Bam*HI ($3'\Delta_B$) sites of pBR322; all these constructs transcribe clockwise relative to the normal orientation of pBR322. The minimal promoter

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region (−39 to +9) was inserted into the *Bam*HI site of pBR322 in both orientations.

Resultant 5′ and 3′ deletion mutants were defined by sequence analysis by the chemical method of Maxam and Gilbert (21). Sizes are given as the last nucleotide that matches the rDNA sequence. The only deletions not located by sequence analysis, certain members of the $3'\Delta_P$ series, were sized by relative gel migration of appropriate small restriction fragments.

To prepare templates for runoff transcription, the plasmid DNA (freed from chromosomal DNA by cesium chloride-ethidium bromide centrifugation) was cleaved with the desired restriction endonuclease and purified by phenol extraction and ethanol precipitation. Only complete digests, as monitored by gel electrophoresis with overloaded samples, were used. The concentrations of the stocks of resultant linearized templates were determined by A_{260}; the same preparations of linearized deletions were used as template in all the presented experiments mapping the 5′ promoter border at various KCl concentrations.

Transcription reactions and their analysis. Transcription reactions were performed in 25 μl with extract constituting 5 to 7 μl for the S-100 extract and 3 μl for the whole-cell extract. Final concentrations in the reaction mixture were 25 mM HEPES (pH 7.9), 5 mM $MgCl_2$, 10% glycerol (vol/vol), 1 mM dithiothreitol, 0.1 mM EDTA, 0.5 mM ATP, 0.5 mM GTP, 0.5 mM UTP, and 25 or 50 μM [α-^{32}P]CTP (8 Ci/mmol). The template concentration and KCl concentration that yielded maximal transcription were determined for each extract. The KCl concentrations used for the transcriptions were at optimal (ca. 60 to 80 mM) or at nonoptimal levels, as noted in the figure legends. All reactions were performed at optimal template concentration: 5 to 10 μg/ml for the S-100 extract and ca. 20 μg/ml for the whole-cell extract. In the competition experiments, each template was at this concentration, and the total amount of synthesis was not altered by the additional DNA. After incubation at 30°C for 30 to 60 min, 180 μl of a solution containing 0.15 M NaCl, 50 mM Tris-hydrochloride (pH 8), 0.3 M sodium acetate, 0.5% sodium dodecyl sulfate (SDS), 60 μg of tRNA per ml and 6 mM EDTA was added, and the mixture was phenol extracted, ethanol precipitated, and suspended for electrophoresis in a solution containing 99% formamide, 0.05% bromophenol blue, and 0.05% xylene cyanol. The DNA was resolved on 4% acrylamide–8 M urea gels and detected by autoradiography. Although the gels have been cropped to minimize journal space, no other transcription products were detectable in any of the lanes except for a small amount of end-to-end transcription near the tops of the gels. The intensities of all autoradiographic bands were quantitated by autoradiography.

Transcription analyses with closed circular plasmid DNA as template were performed as with linear template except that the radioactive label was omitted from the reaction and the precipitated, extracted RNA was suspended in 10 mM Tris (pH 7.5)–0.1 mM EDTA. It was then analyzed by hybridization and S1 nuclease digestion as previously described (23). The hybridization probe was prepared by ^{32}P labeling the 5′ ends of rDNA (plasmid p5′Sal-Pvu) linearized with *Sma*I at nucleotide +155 and removing the noncoding strand with exonuclease III digestion.

In reactions with phosphocellulose-fractionated extract (prepared exactly as described elsewhere [24; J. Tower, V. Culotta, and B. Sollner-Webb, in preparation]), 4.5, 2, 1.5, and 3 mg of protein per ml from fractions A, B, C, and D were used, thereby maximizing the transcriptional signal from these fractions.

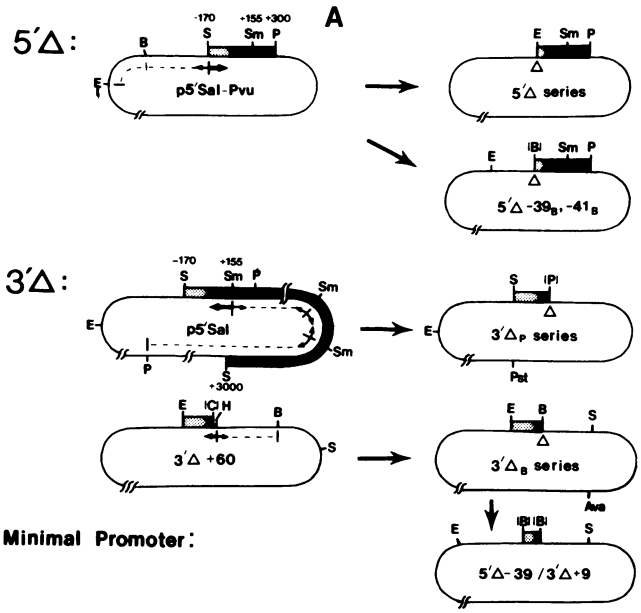
RESULTS

Construction of the mutants. To assess the role of various regions of the mouse rRNA gene in directing transcriptional initiation, we constructed systematic series of deletion mutants approaching the initiation site from the 5′ and 3′ directions. These were made by treatment of a cloned mouse rDNA initiation region with BAL 31 exonuclease or exonuclease III and S1 nuclease. Our earlier work (23) demonstrates that the sequences that promote in vitro transcription reside between residues −168 and +155; hence, it was within this region that the deletions were created. The resultant mutants and the restriction endonuclease sites that were used for runoff transcription are diagrammed in Fig. 1A. Mutants are numbered according to the last nucleotide that matches the sequence of mouse rDNA.

In the course of sequencing the parental rDNA as well as the deletion mutants, we found a number of differences from published mouse rDNA sequences (1, 12, 13, 27). Although some of these differences could be due to natural sequence variation, all of these sequence analyses except that of Urano et al. (27) were performed on derivatives of a single genomic clone (23). Our determination of the sequence of this promoter region is shown in Fig. 1B.

Optimization of transcription conditions. In preliminary experiments, it became clear that the transcriptional capacity of various deletion mutants varied as a function of the salt concentration used in the synthesis reaction. Each cell extract was therefore titrated to determine the dependence of its transcriptional signal on salt concentration. Figure 2 shows examples of such analyses of the amount of runoff transcript directed by a *Sma*I-cleaved wild-type rDNA (plasmid p5′Sal-Pvu) at increasing KCl concentrations in both S-100 and whole-cell extracts. Like the S-100 extract (11, 23), the whole-cell extract (18, 20) accurately transcribes mouse rDNA (Fig. 2). Although there are some differences in transcriptional falloff at KCl concentrations above optimum, all extracts show optima in the range of ca. 60 to 80 mM KCl.

We determined the reaction conditions that give maximal amounts of transcription from the deletion as well as the wild-type rDNA templates. In all cases examined, the salt optima for the deletion mutants, including those that show a diminished signal at elevated salt concentrations, were the same as that seen for the parental plasmid (not shown). Further, we determined the optimum template concentration for transcription in each extract by varying the amount of rDNA added to a reaction. This value, generally 5 to 10 μg/ml for the S-100 extract and ca. 20 μg/ml for the whole-cell extract, was the same for wild-type rDNA and the deletions that have decreased transcription capacity (data not shown) and was used in all the studies reported below. Since the transcribed region of the 5′ deletions is identical to that of wild-type rDNA, any decreased transcriptional signal directed by these deletions was presumably due to impaired initiation. Transcripts of all 3′ deletions, on the other hand, differ in sequence and hence could have differential stabilities; however, pulse-chase analyses on wild-type and selected deleted templates ($3'\Delta+2$, $3'\Delta+9$, and the minimal promoter plasmids) indicate that their signals were not substantially affected by an increased turnover of the detectable rRNA, for the various transcripts were all relatively stable and had comparable half-lives (not shown).



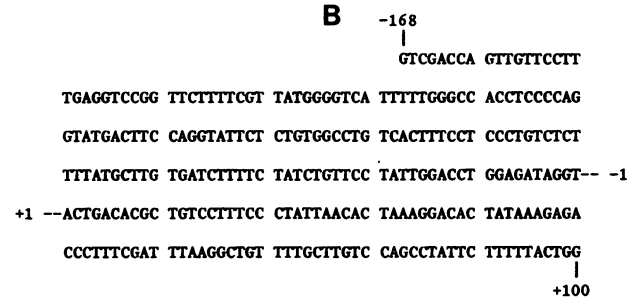


FIG. 1. The rDNA templates. (A) Construction of the deletions. 5′ Deletions were constructed from p5′Sal-Pvu (22), a plasmid which contains the rDNA region from nucleotide −168 (*Sal*I) to +300 (*Pvu*II) inserted at the *Sal*I and *Pvu*II sites of pBR322. For the major 5′ deletion series (top line), p5′Sal-Pvu was digested with *Sal*I and then with BAL 31 exonuclease. Appropriately resected samples were ligated to *Eco*RI linkers (Collaborative Research; GGAATTCC), cleaved with *Eco*RI, and recircularized. Two 5′ deletions were also cloned into the *Bam*HI site of pBR322 (line 2). $5'\Delta-41_B$ was constructed by cleavage of 5′Δ−76 with *Eco*RI, resection with BAL 31 nuclease, and attachment of *Bam*HI linkers (CCGGATCCGG), followed by treatment with *Bam*HI and *Pvu*II and insertion into a similarly cleaved pBR322 vector. Further 5′ deletions were generated from the *Bam*HI site of this plasmid as outlined in line 1. $5'\Delta-39_B$ was formed by insertion of the *Sau*3A (−39)-to-*Pvu*II (+300) rDNA fragment between the *Bam*HI and *Pvu*II sites of pBR322. Two 3′ deletion series were also constructed. For the $3'\Delta_P$ series (line 3), p5′Sal (rDNA nucleotides −168 to +3000) was cleaved with *Sma*I and digested with BAL 31 and then with *Pvu*II, and the large fragment was circularized by blunt end ligation. To construct the 3′Δ+60 parent clone used for the second series ($3'\Delta_B$; line 4), 5′Δ−126 was digested with *Taq*I (residue +59), *Eco*RI (residue −126), and *Hha*I (to destroy the unwanted fragments) and ligated to the large *Cla*I-*Eco*RI fragment isolated from pBR322. From this, 3′ deletions were made by digestion with *Hin*dIII and BAL 31 and exonuclease III and S1, ligation to *Bam*HI linkers, and circularization after digestion with *Bam*HI. To clone the minimal promoter region (bottom line), 3′Δ+9 was digested with *Sau*3A (residues −39 to +9) and ligated into a BamHI-cleaved pBR322 vector in both possible orientations. Abbreviations: E, *Eco*RI; B, *Bam*HI; S, *Sal*I; Sm, *Sma*I; P, *Pvu*II; Pst, *Pst*I; C, *Cla*I; H, *Hin*dIII; Ava, *Ava*I. (B) The sequence of the rDNA region surrounding the promoter was determined and confirmed by the chemical method (21). The *Sal*I site extends from position −168 to −163.

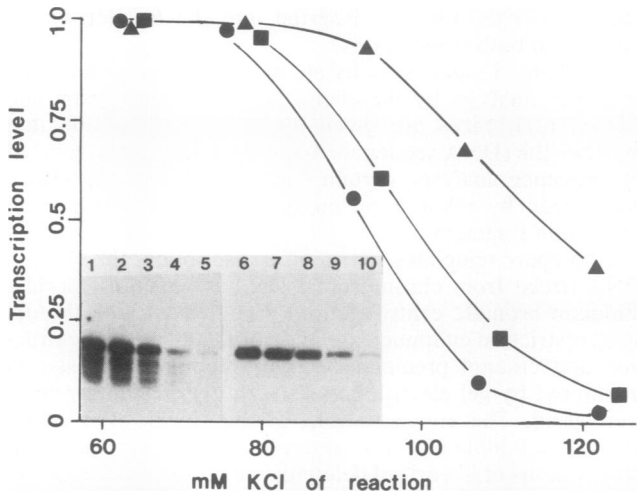


FIG. 2. Relative transcription at various KCl concentrations. Quantitation of transcriptional dependence on KCl concentration of a whole-cell extract (●) and two S-100 extracts (■ and ▲) used in this paper. These data were obtained by densitometric scanning of autoradiographic bands as shown in the insert. For this gel, clone p5′Sal-Pvu, cleaved with *Sma*I at +155, was transcribed in a whole-cell extract (lanes 1 through 5) at 62, 77, 92, 107, and 122 mM KCl total and in an S-100 extract (lanes 6 through 10) at 65, 80, 95, 110, and 125 mM KCl total. Product RNA was resolved on a sequencing gel and detected by autoradiography. The lower bands in lanes 1 through 5 result from a small amount of RNase endogenous in the extract, but since all the transcripts have the identical sequence, they are all equally affected.

The 5′ border: (i) analysis under optimal transcription conditions. Our previous studies (4, 23) demonstrate that closed circular rDNA is a more efficient transcription template than is linearized rDNA. Determination of the sequences required for maximal transcription of closed circular template at optimal salt concentration should thus define the rDNA promoter under conditions most favorable for transcription. This analysis is shown in Fig. 3A by S1 mapping of the transcription products, using wild-type rDNA as the probe. In addition to the 155-nucleotide fragment diagnostic of correct initiation, there was also a strong divergence band resulting from transcripts that had read more than once around the plasmid and thus protected probe out to the site of the deletion. When the 5′ flanking sequences were deleted in to residue −35, synthesis was still as efficient as with the parental rDNA. However, deletion of another eight base pairs of 5′ flanking sequence caused a very marked decrease in transcriptional response. Deletion of yet another 11 base pairs to residue −16 abolished all detectable initiation.

Linearized rDNA plasmids transcribed at optimal salt concentration exhibited the same 5′ border of the promoter. Figure 3B shows the amount of runoff transcript produced when linearized 5′ deletion mutants were transcribed in the whole-cell extract (20) at optimal salt concentration. 5′Δ−35 was the largest deletion that did not significantly impair synthesis, 5′Δ−27 directed a very low level of initiation, and 5′Δ−16 directed none. Similar results can be obtained with the S−100 extract (not shown).

When transcribed at KCl concentrations only slightly above optimal, 5′Δ−39 still directed production of as much rRNA as did all the less extensive deletions or the parental rDNA, but 5′Δ−35 now transcribed at a significantly re-

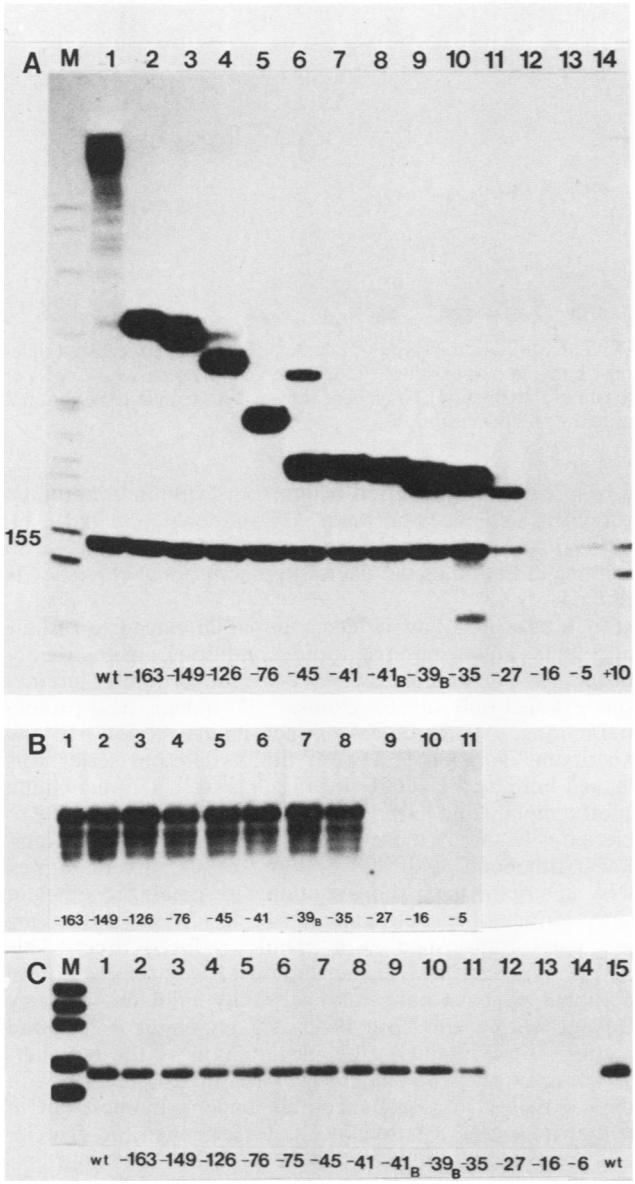


FIG. 3. 5′ boundary of the promoter at optimal KCl concentration. (A) The designated closed circular 5′ deletions were transcribed at optimal KCl concentration in an S-100 extract. RNA products were analyzed by S1 nuclease mapping after hybridization to a 5′ end-labeled probe made from the parental rDNA; the protected probe regions were resolved on a 4% sequencing gel and detected by autoradiography. Transcripts which read into the rDNA region from upstream, in particular those that initiated correctly and transcribed more than one time around the plasmid, differ in sequence from the probe beyond the site of the deletion and form a divergence band whose size varies according to the deletion endpoint. Wt, Wild-type clone, a −168 deletion; M, *Hpa*II-cleaved pBR322 markers. (B) The designated 5′ deletions, linearized at position +155, were transcribed in the whole-cell extract at optimal KCl concentration. The RNA products were fractionated on sequencing gels and detected by autoradiography. S1 analysis (not shown) demonstrates that the bands below the major ones are due to transcripts that initiated correctly but lack a few nucleotides at the 3′ terminus. (C) The designated 5′ deletions were transcribed in an S-100 extract at KCl concentration very slightly above optimal (93 mM in the extract designated ▲ in Fig. 2) and RNA products were resolved as in part B. M is *Hpa*II-cleaved pBR322 marker.

duced level, and 5′Δ−27 generated only barely detectable amounts of rRNA product. This is illustrated in Fig. 3C by runoff transcription in an S-100 extract at ca. 10 mM KCl above the concentration that would elicit maximal rRNA synthesis. Identical results were also obtained in the whole-cell extract at a salt concentration slightly above optimal (not shown).

Thus, under conditions that produce the greatest amount of transcription, all the information required to direct efficient initiation is encoded downstream of residue −36, whereas at near-to-optimal conditions, an additional ca. four base pairs are used; only under the most favorable conditions is information downstream of residue −27 sufficient to obtain appreciable amounts of synthesis.

(ii) Transcription under nonoptimal conditions. When the salt concentration of the runoff transcription reaction was raised from optimal by ca. 30 mM to a level at which synthesis became significantly impaired, the role of sequences still further upstream became apparent. This is shown in Fig. 4 with an S-100 extract, and a whole-cell extract behaved identically (not shown). In both cases, transcription of parental rDNA was reduced to about one-third of that obtained at optimal salt concentration, and the 5′ borders of the regions required for full and partial synthesis moved upstream by ca. 10 base pairs. Transcription levels equal to those of the parental plasmid were obtained only with 5′Δ−45 and less extensive 5′ deletions, and now 5′Δ−41, 5′Δ−40, and 5′Δ−39 initiated at markedly reduced levels.

This variation in relative initiation efficiency between the large and small 5′ deletions according to the overall level of synthesis was further studied by transcribing two templates alone and in competition at increasing salt concentrations. In this experiment, 5′Δ−41 was cleaved with *Pvu*II to generate a 300-nucleotide runoff transcript while rDNA with a larger 5′ flanking region was cleaved with *Sma*I to generate a 155-nucleotide runoff. The primary data of Fig. 5A were quantitated by densitometric scanning of the autoradiographic bands in Fig. 5B. At each KCl concentration, the transcriptional level of the control rDNA is shown in lanes 2; this is plotted as the *X* axis in Fig. 5B. The efficiency of the 5′Δ−41 deletion (shown in lanes 1) relative to the separately transcribed control plasmid (lanes 2) is plotted by circles in Fig. 5B. This confirms the results of Fig. 3 and 4, showing that 5′Δ−41 becomes a relatively less efficient template when transcribed at KCl concentrations that greatly inhibit overall synthesis. Lanes 3 show the relative efficiency of the two plasmids when transcribed under competition conditions in the same reaction; these data are plotted by squares

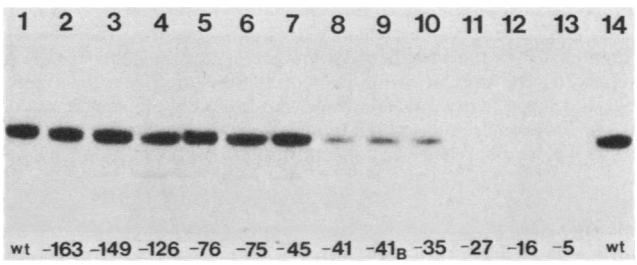


FIG. 4. 5′ boundary of the promoter at above optimal KCl concentration. The designated 5′ deletions were transcribed in an S-100 extract at KCl concentrations that yield about one-third as much transcription as that observed at optimal KCl. Analysis was as in Fig. 3B.

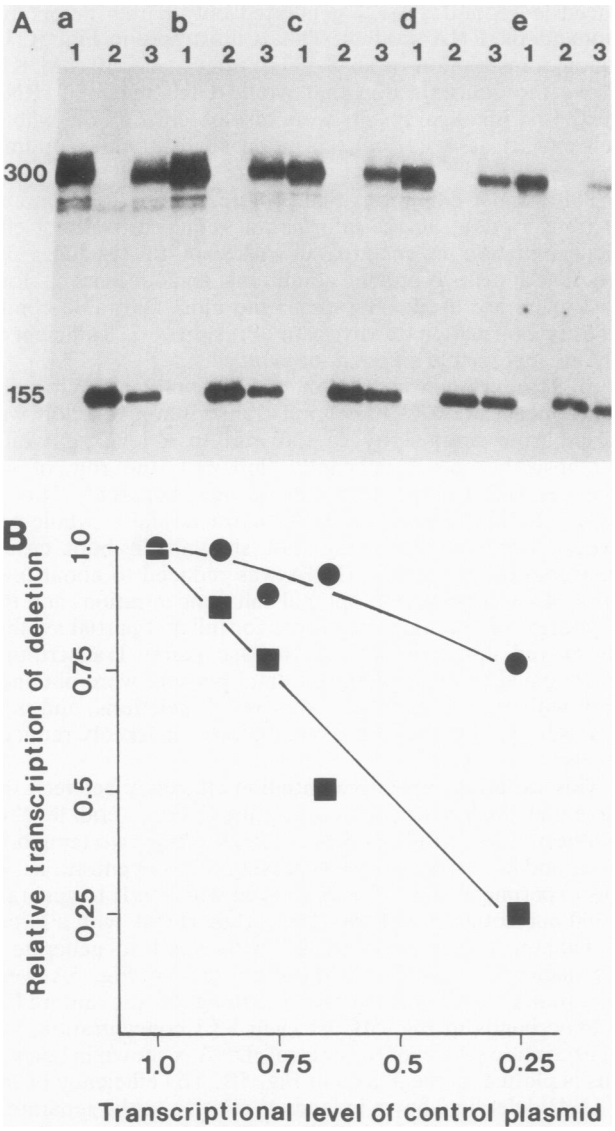


FIG. 5. Competition increases the effect of the 5′ promoter domain. (A) Plasmids 5′Δ−41, cleaved at position +300, and 5′Δ−126, cleaved at position +155, were transcribed individually (lanes 1 and 2) and together in the same reaction (lanes 3) at the following KCl concentrations: 70 mM (a lanes), 90 mM (b lanes), 110 mM (c lanes), 120 mM (d lanes), and 130 mM (e lanes). (This S-100 extract maintains transcription at relatively high KCl concentrations.) (B) The results of (A) were quantitated by densitometry. Closed circles denote the amount of 300-nucleotide RNA from 5′Δ−41 (lanes 1) relative to the amount of 155-nucleotide transcript from the control plasmid (lanes 2) when transcribed individually. Squares denote the amount of 300-nucleotide transcript from 5′Δ−41 relative to the amount of 155-nucleotide transcript from the control plasmid when the two DNAs were transcribed in the same reaction (lanes 3). Data are plotted relative to the transcriptional level of the control plasmid at that KCl concentration, transcribed alone in lanes 2.

in Fig. 5B. It is obvious that competition accentuates the differential initiation efficiency of the two plasmids, for 5′Δ−41 was a considerably poorer template when the two plasmids competed in one reaction (squares) than when they were transcribed individually (circles).

From the studies presented above, we conclude that the 5′ border of the mouse rDNA promoter region changes by 10 to

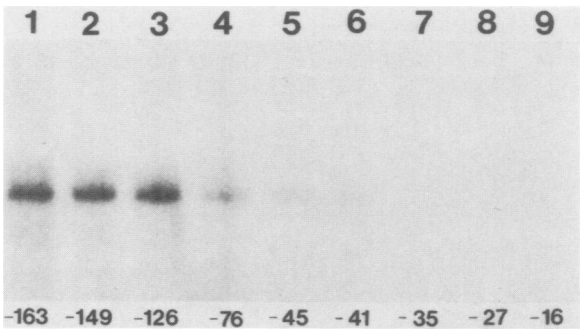


FIG. 6. Initiation requires 5′ flanking DNA. The indicated deletions, linearized at residue +155, were also cleaved with *Eco*RI at the site of the deletion. They were transcribed in an S-100 extract at optimal KCl concentration.

15 base pairs as a function of the transcription conditions. Evidently, sequences between −27 and −35, −35 and −39, and −41 and −45 became sequentially more important in directing initiation as the overall transcriptional efficiency is reduced.

(iii) Interactions that influence initiation extend to residue ca. −100. Under optimal ionic conditions, when the 5′ border of the detectable sequence-specific promoter interactions extends only out to residue −35, nucleic acid-protein interactions extends over a much larger region. In the experiment depicted in Fig. 6, the 5′ deletion series was cleaved both at the *Sma*I site to generate a 155-nucleotide runoff template and in the cloning linker that demarks the 5′ deletion site. With these small rDNA fragments as templates, fragments with 128 or more base pairs of duplex DNA upstream from the initiation site catalyzed efficient transcription, whereas fragments with 78 or fewer 5′ flanking base pairs transcribed at a greatly reduced level. This residual synthesis was further diminished by removal of an additional 31 base pairs and was fully inhibited only by cleavage within the core promoter region at or beyond residue −37. Evidently, efficient initiation by the polymerase I transcription machinery requires the interaction with DNA, possibly in a sequence-independent manner, out to residue ca. −100, a border not detected when the 5′ deletions are attached to vector DNA.

(iv) A 5′ promoter border extends upstream to residue ca. −140. Under a variety of other inhibitory conditions, the 5′ border of the rDNA promoter was seen to extend yet farther upstream. This was most evident in transcription reactions with reconstituted polymerase I transcription factors derived by phosphocellulose chromatography of the S-100 cell extract. These reactions accurately and specifically transcribed mouse rDNA (24; Tower et al. in preparation), but unless rather large amounts of the limiting factors were used in reconstitution, the overall level of synthesis was reduced relative to that of the unfractionated extract. In this case, transcription was hampered not by ionic conditions but evidently by decreased availability of the necessary factors (Tower et al., in preparation). When the various 5′ deletion mutants were transcribed with these reconstituted phosphocellulose fractions, a 5′ border of the promoter region between residues −126 and −149 was highly evident (Fig. 7A); deletions extending in to or beyond residue −126 transcribed with ca. 1/10 of the efficiency of deletions extending to residue −149. Consistent with the experiments reported above, removal of sequences beyond residue −35 abolished the residual level of synthesis.

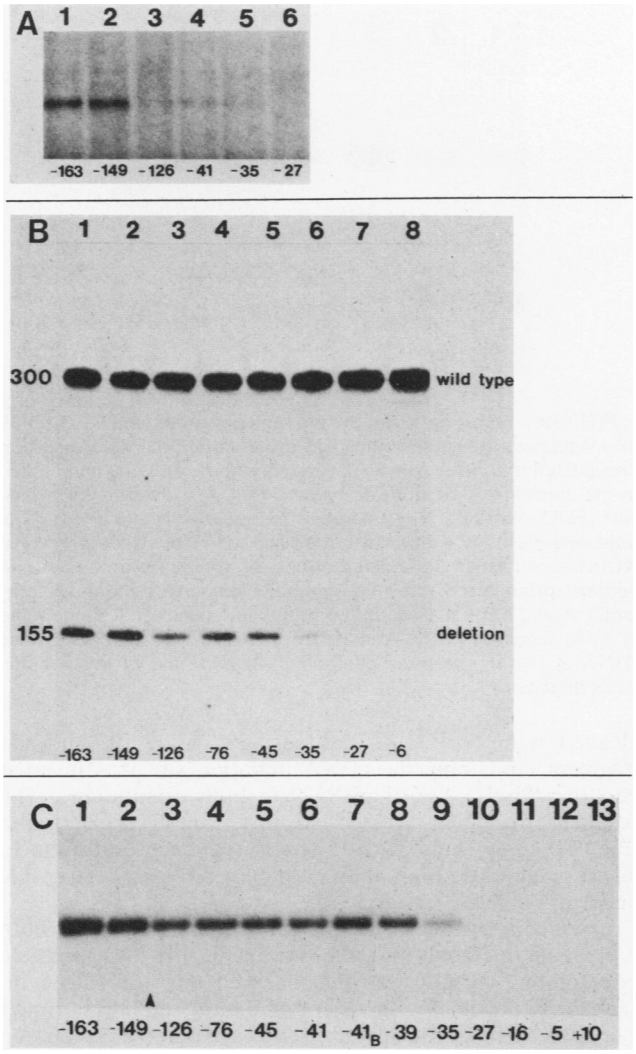


FIG. 7. A distal 5′ promoter domain. (A) The indicated 5′ deletions were transcribed at optimal KCl concentration in reactions catalyzed by the factors of the S-100 extract, recombined after fractionation on phosphocellulose (see the text). (B) p5′Sal-Pvu, linearized at residue +300, and the indicated 5′ deletions, linearized at residue +155, were transcribed in the same reaction at slightly above the optimal KCl concentration. (C) The indicated 5′ deletions were transcribed in an S-100 extract at a KCl concentration slightly above optimal (105 mM in the extract indicated by the triangles of Fig. 2).

This promoter region extending out to residue −149 was also observed with unfractionated extracts. Figure 7B represents an experiment in which the 5′ deletions were transcribed in competition with an undeleted rDNA plasmid at slightly above optimal KCl concentration. In this case, deletions extending beyond the border between −149 and −126 transcribed approximately twofold less efficiently in competition with wild-type rDNA than did plasmids that contained this upstream promoter domain. In fact, the border between −149 and −126 could also be discerned when members of the 5′ deletion series were transcribed individually at such slightly inhibitory ionic strength. For example, 5′ deletions extending to and beyond residue −126 transcribed at ca. 80% the efficiency of 5′Δ−149 and less extensive deletions (Fig. 7C). Although the upstream promoter domain had only a small effect in such uncompeted reactions, densitometric scans of autoradiograms from several separate experiments demonstrate that it is a highly reproducible effect.

The 3′ border: transcription of the 3′ deletion series. Two series of deletions approaching the initiation site from the 3′ direction were constructed; one was cloned into the *Bam*HI site and one was cloned into the *Pvu*II site of pBR322 (Fig. 1), and their ability to promote runoff transcription was assayed (Fig. 8). Since the restriction endonuclease cleavage site used to linearize these templates for runoff transcription is in the pBR322 region beyond the cloning site of the deletion, the size of the resultant runoff transcripts varied according to the extent of the deletion. With templates that produced long runoffs, this effect appeared minor (Fig. 8A), but with templates cleaved closer to the initiation site, the length variation became quite pronounced (Fig. 8B).

With both deletion series, the 3′ boundary of the promoter resided between residues +9 and +2 (Fig. 8A). This same 3′

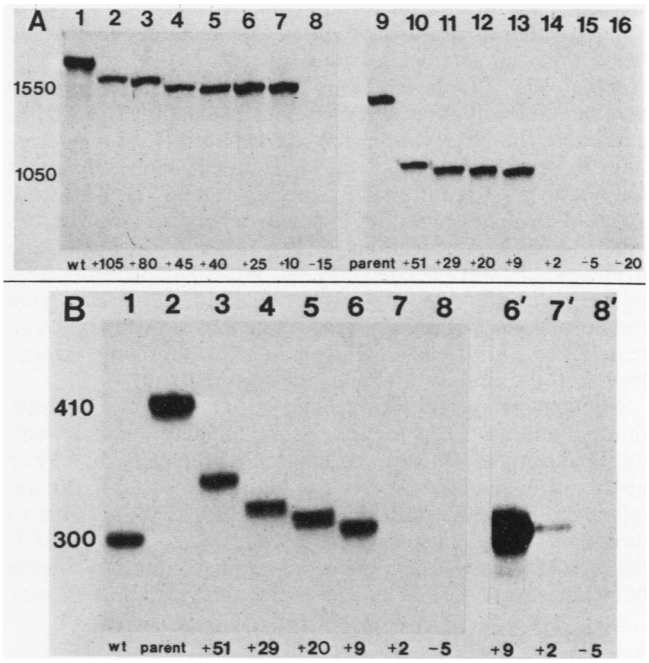


FIG. 8. Transcription of 3′ deletions. (A) The indicated 3′ deletions were transcribed in an S-100 extract. Lanes 1 through 8 represent transcripts of the $3'\Delta_P$ series cloned into the *Pvu*II site of pBR322 and linearized at the *Pst*I site in the vector; lanes 9 through 16 represent transcripts of the $3'\Delta_B$ series, cloned into the *Bam*HI site of pBR322 and linearized at the *Ava*I site of the vector. Wt designates p5′Sal-Pvu and parent designates the 3′Δ+60 parent of this deletion series. See Fig. 1 for the maps of these plasmids. (B) The indicated members of the $3'\Delta_B$ series were linearized at the *Sal*I site in the vector and transcribed in an S-100 extract. Lanes 6′ through 8′ are a longer exposure of lanes 6 through 8. Parent designates the 3′Δ+60 parent clone. The template in lane 1 was p5′Sal-Pvu cleaved with *Pvu*II at residue +300. To confirm that the transcription of 3′Δ+2 could not simply result from its contamination with a very small amount of 3′Δ+9, a second 3′Δ+2 preparation was grown from a single colony transformed with the original DNA, and it behaved identically. Generally both series of 3′ deletion templates yield only about three-fourths as much RNA as the wild-type plasmid. However this does not indicate the existence of a distal 3′ promoter border, because the 3′Δ+60 cloned into the *Hin*dIII site of pBR322 yields as much transcript as the wild-type plasmid yet contains fewer rDNA sequences than several of the 3′ deletions. Instead, RNA elongation or accumulation may be somewhat influenced by the nature of the 3′ flanking sequences.

promoter border is observed under all the different transcription conditions that have been investigated. These conditions include using short and long runoff templates (Fig. 8A and B) as well as optimal and inhibitory salt concentrations and transcription in both the S-100 and the whole-cell extract. Further, this same border between +2 and +9 was observed upon competition of the 3′ deletions with undeleted templates (data not shown).

On long exposures of gels that resolve the transcripts directed by the 3′ deletions, it was clear that certain deletions extending beyond the promoter boundary between +9 and +2 can promote synthesis at a low level. 3′Δ+2 can direct production of appreciable amounts of RNA (Fig. 8B, lane 7′) and 3′Δ−5 can direct extremely low levels (data not shown). 3′Δ+2, however, produced a transcript that appears to be one to two nucleotides larger than that from 3′Δ+9; had 3′Δ+2 initiated accurately, its transcript would have been seven nucleotides shorter. This 3′Δ+2 deletion thus initiated inefficiently and ca. 8 to 9 nucleotides upstream of the normal start site. From the rDNA sequence we know that if 3′Δ+2 indeed initiated at nucleotide −8 it would begin with an adenine residue, whereas if it initiated at residue −7, −9, or −10 it would begin with a guanine residue. The initiation site of 3′Δ+2 is examined in Fig. 9, exploiting the observation that the apparent K_m for nucleotides at the initiation step was substantially higher than that for nucleotides required only in elongation (31). The transcript of 3′Δ+2 required ATP in high concentration, whereas much lower concentrations of the other three nucleoside triphosphates were needed (Fig. 9). We conclude that 3′Δ+2 initiates with the A residue eight nucleotides upstream from the normal starting site; hence, sequences residing between residues +2 and +9 evidently can affect both the overall level and the precision of the initiation reaction.

Minimal promoter region. The above data demonstrate that polymerase I can accurately and efficiently initiate with only the rDNA sequences downstream of nucleotide −39 or upstream of nucleotide +9. To determine whether the sequence contained within these boundaries is in fact able to direct initiation by itself in the absence of additional 5′ or 3′ flanking rDNA, we subcloned the minimal promoter region between residues −39 and +9. To this end, 3′Δ+9 was cleaved with *Sau*3A at residues −39 and +9, and the fragment containing the rDNA initiation site was inserted into the *Bam*HI site of pBR322 in both orientations. The resultant plasmids directed accurate runoff transcription (Fig. 10, lanes 1 and 3) and, hence, sequences sufficient to direct polymerase I initiation on mouse rDNA indeed reside between residues −39 and +9.

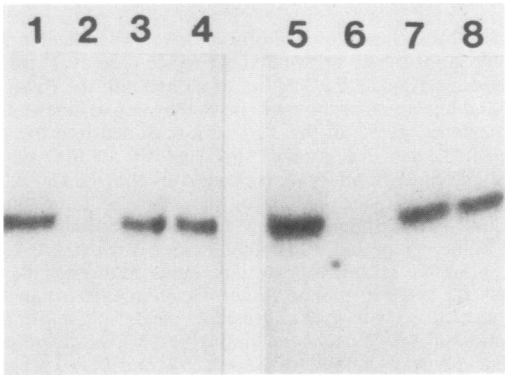


FIG. 9. Initiation of 3′Δ+2. Plasmids 3′Δ+9 (lanes 1 through 4) and 3′Δ+2 (lanes 5 through 8) were transcribed in an S-100 extract. Reaction mixtures contained 50 μM CTP plus the following: 500 μM ATP, 500 μM GTP, and 500 μM UTP (lanes 1 and 5); 100 μM ATP, 500 μM GTP, and 500 μM UTP (lanes 2 and 6); 500 μM ATP, 500 μM UTP, and 100 μM GTP (lanes 3 and 7); 500 μM ATP, 500 μM GTP, and 100 μM UTP (lanes 4 and 8). The autoradiogram of lanes 5 through 8 was exposed ca. 10 times longer than that of lanes 1 through 4.

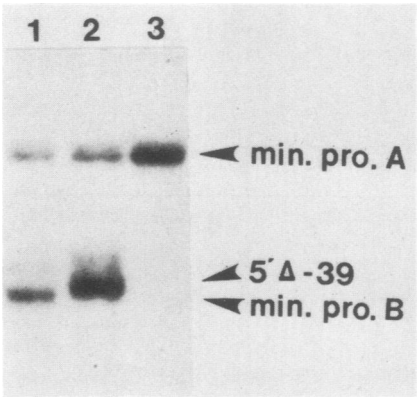


FIG. 10. Transcription of the minimal promoter clones. The −39 to +9 minimal promoter cloned in the counterclockwise direction (designated min. pro. A) was linearized with *Eco*RI, the one cloned in the clockwise direction (designated min. pro. B) was linearized with *Sal*I, and the 5′Δ−39 parent was linearized with *Pvu*II. The following plasmids were transcribed in an S-100 extract at optimal KCl concentration: equimolar amounts of minimal promoter A and minimal promoter B (lane 1), equimolar amounts of minimal promoter A and 5′Δ−39 (lane 2), and minimal promoter A alone (lane 3). (The sequence of the A clone from −42 to −44 matches mouse rDNA at two of the three positions, whereas that of the B clone lacks matches.)

Upon competition of the plasmids containing the −39 to +9 region inserted into the vector in the two possible orientations, we consistently observed that the rDNA inserted in the clockwise orientation directed more runoff synthesis than did the one inserted in the reverse orientation (Fig. 10, lane 1). This may be attributed to a greater number of fortuitous matches in the juxtaposed pBR322 sequences of the former mutant with the natural rDNA sequence between residues −42 and −45, a region shown above to contribute to promoter efficiency. It is clear, however, that neither of the subcloned small rDNA promoter regions directed as much transcription as did the 5′Δ−39 parent (Fig. 10, lane 2). Control experiments demonstrate that transcription from these various plasmids had the same DNA concentration optimum and the same KCl concentration optimum and that their transcripts appeared equally stable by pulse-chase analysis (data not shown). It is possible that sequences 5′ of residue −39 and sequences 3′ of residue +9 might have a synergistic effect on initiation.

DISCUSSION

We have identified the DNA sequences important for initiation of transcription of mouse rRNA by constructing systematic series of 5′ and 3′ deletion mutants around the initiation site and assaying their transcriptional efficiency in both S-100 and whole-cell extracts under various reaction conditions. Although small amounts of transcription can be directed by 5′Δ−27 and 3′Δ+2, sequences downstream of residue −35 and sequences upstream of residue +9 are required to obtain efficient initiation. These sequences are the only promoter region detected under optimal transcription conditions with both closed circular (Fig. 3A) and linear

(Fig. 3B and 8) 5′ and 3′ deletion templates and in both the S-100 and the whole-cell extracts. Under conditions very near to optimal for transcription, a slightly larger region between residues −39 and +9 is used (Fig. 3C). These sequences form the core of the mouse rDNA promoter. Confirming that this core is sufficient to direct initiation, a template that only contains the rDNA sequences between residues −39 and +9 is accurately transcribed (Fig. 10).

By deliberately varying the conditions of the assay, we detect additional upstream sequence domains that affect the amount of transcription. At higher than optimal KCl concentrations (to substantially inhibit initiation), the region extending just upstream from the core to residue −45 greatly augments the level of initiation (Fig. 4). The effect of this region is further revealed by competition analysis (Fig. 5). In addition, sequences extending for an additional ca. 100 base pairs in the 5′ direction can also markedly increase the level of transcription. The effect of this upstream domain is most clearly demonstrated under competition conditions (Fig. 7B) and by transcription with fractionated extract components (Fig. 7A), but this border between −126 and −149 is also discernible in salt-inhibited standard transcription assays (Fig. 7C). Further, an interaction involving DNA, possibly in a sequence-nonspecific manner, occurs within the region between −126 and −76 (Fig. 6). It should be emphasized that promoter mapping, especially under optimized initiation conditions, does not necessarily detect all the specific interactions between promoter regions and transcription factors, for it only identifies those domains whose loss depresses the overall level of synthesis. It is likely that the upstream polymerase I promoter regions act at optimal as well as at inhibitory reaction conditions but that in the former circumstance, the core region of the promoter is sufficient to obtain maximal levels of transcription; hence, the upstream domains are not detected. Thus, mapping promoters only under optimal initiation conditions can preclude detection of domains that may be crucial under other conditions. In fact, results of preliminary experiments in which the 5′ deletion mutants were assayed in vivo by transient expression (unpublished data) suggest that all the promoter interactions observed under the different conditions in vitro actually do serve a role in initiating or controlling transcription in vivo.

The 3′ boundary of the rDNA promoter appears far more rigidly defined. Under all conditions in which our 3′ deletion series have been examined, the region extending 3′ of residue +9 can be removed without affecting transcription, but deletion into residue +2 greatly reduces initiation efficiency and causes synthesis to begin eight nucleotides upstream from the normal initiation site. The action of 3′Δ+2 is somewhat reminiscent of transcription of *Xenopus* rDNA in the mouse cell extract, where synthesis initiates specifically but several nucleotides upstream of the in vivo initiation site (26); accordingly, sequence homology between *Xenopus* and mouse rDNA initiation regions also only extends up to residue +2. These results suggest that interactions between mouse transcription factors and rDNA upstream of residue +2 direct RNA polymerase to the correct general region of the template and that additional interactions with sequences between +2 and +9 can modulate the overall level of transcription and the exact initiation site.

The fact that a number of different rDNA promoter borders are observed gives a minimum estimate for the extent of important contacts between the rDNA and the transcription factors: such relevant contacts must take place within the regions −149 to −126, −126 to −76, −45 to −41, −39 to −35, −35 to −27, −27 to −16, −5 to +2, and +2 to +9. Although it has been suggested that promoter activity or inactivity of deletion mutants might be appreciably influenced by the particular nucleotide sequences that are brought up to adjoin the remaining DNA at the cloning site, we observe no major artifacts of this kind. In the experiments reported in this paper, 5′ deletions ending precisely at a major boundary of the promoter are cloned into both the *Eco*RI (5′Δ−41, 5′Δ−40) and *Bam*HI (5′Δ−41$_B$, (5′Δ−39$_B$) sites of pBR322, and they behave identically, exhibiting maximal activity at optimal conditions and impaired initiation capacity at elevated salt concentrations (Fig. 3 and 4). Similarly, 3′ deletion series cloned into the *Bam*HI and the *Pvu*II sites of pBR322 both identify the same boundary, between +9 and +2 (Fig. 8). Thus, the borders of the mouse rDNA promoter we have defined appear to reflect true promoter interactions and are not affected to a detectable degree by the identities of juxtaposed bacterial sequences.

Despite the species specificity of rRNA transcriptional initiation (14, 18), we suggest that the promoter is organized in much the same manner in different species and that a large (ca. 140 base-pair) or only a small 5′ promoter region is observed depending on the conditions chosen for the assay. In the mouse system, Grummt (13) has mapped 5′ borders of the rDNA promoter between residues −39 and −12, for a 5′Δ−39 was active, a 5′Δ−34 was partly active, and a differently constructed 5′Δ−12 was inactive. Yamamoto et al. (32) have also reported that 5′ boundaries of the mouse rDNA promoter resides between residues −41 and −26. However, using competition studies, Grummt (13) finds that mouse rDNA sequences upstream of residue −45 can offer a selective advantage; possibly this reflects the border at ca. −140 identified in Fig. 7. In *Xenopus*, we mapped two 5′ rDNA promoter borders, one between −141 and −126 by using in vitro analysis and one just upstream of the initiation site by using the considerably more efficient transcription system of oocyte microinjection (26). In the human system, Learned et al. (18) also find an absolute border between residues −52 and −26 and a quantitative border between residues −158 and −132, the effect of which increases somewhat upon competition with a wild-type template. Thus, the 5′ borders of the mouse, *Xenopus*, and human rDNAs may reflect similarly organized domains of sequences, proximal ones that are essential and more distal ones that are stimulatory for initiation.

The 3′ border of the rDNA promoter has also been examined in several species. Like the mouse case reported here, the 3′ boundary of the *Xenopus* rDNA promoter is located immediately adjacent to the initiation site and does not appear to vary with the transcription conditions (26). The 3′ border of the *Drosophila* rDNA promoter is also located a few nucleotides 3′ to the initiation site (17). Curiously, mouse and human rDNA have an identical sequence between residues +2 and +18 (8), and this conserved region appears to be part of the human rDNA promoter (18). Moreover, it has also been recently reported (32) that the mouse 3′ border extends progressively between residues −1 and +53, but interpretation of these data may be somewhat complicated by the unexpected relative electrophoretic migration of the transcripts of certain of the 3′ deletions. It should also be noted that since the transcripts of 3′ deletion series differ in sequence, they may well also differ in RNA stability (9, 26); unless specifically excluded, this effect could artificially create apparent 3′ promoter borders.

In conclusion, the mouse rDNA promoter may be thought of as a nested set of control regions, with the core region

specifying accurate initiation and sequences extending ca. 100 base pairs further upstream greatly aiding in the efficiency of this process. How might the promoter region function? Unlike procaryotes, in which the promoter is the direct binding site for the RNA polymerase, eucaryotes use stable transcription complexes, involving both DNA and additional proteins, as the template for synthesis catalyzed by class I (4, 28), II (5), and III (3) RNA polymerases. Both for 5S RNA transcription by RNA polymerase III (7) and for transcription by RNA polymerase II (5), the sequences involved in stable complex formation appear to precisely coincide with the in vitro-defined promoter, the region from +50 to +90 and the TATAA region, respectively. Recent work in our laboratory has demonstrated that the regions involved in the formation of the rDNA stable complex also correspond precisely to the various domains of the rDNA promoter (Tower et al., in preparation). The segment between residues −149 and +9 is needed for maximal stability of the transcription complex, and 5′ deletions which extend beyond each of the above defined internal borders of the rDNA promoter form successively less stable complexes. Apparently, the major role of the promoter region of higher eucaryotes is to stably bind the components that allow initiation by RNA polymerase.

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