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Determination of the promoter region of mouse ribosomal RNA gene by an *in vitro* transcription system

(initiation/S-100 extract/deletion mutant/nuclease S1 mapping)

OSAMI YAMAMOTO, NOBUO TAKAKUSA, YUKIO MISHIMA, RYO KOMINAMI, AND MASAMI MURAMATSU*

Department of Biochemistry, Cancer Institute, Japanese Foundation for Cancer Research, Kami-ikebukuro, Toshima-ku, Tokyo, Japan

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ABSTRACT **Sequences required for a faithful and efficient transcription of a cloned mouse ribosomal RNA gene (rDNA) are determined by testing a series of deletion mutants in an *in vitro* transcription system utilizing two kinds of mouse cellular extract. Deletion of sequences upstream of −40 or downstream of +52 causes only slight reduction in promoter activity as compared with the "wild-type" template. For upstream deletion mutants, the removal of a sequence between −40 and −35 causes a significant decrease in the capacity to direct efficient initiation. This decrease becomes more pronounced when the deletion reaches −32 and the sequence A-T-C-T-T-T, conserved among mouse, rat, and human rDNAs, is lost. Residual template activity is further reduced as more upstream sequence is deleted and finally becomes undetectable when the deletion is extended from −22 down to −17, corresponding to the loss of the conserved sequence T-A-T-T-G. As for downstream deletion mutants, the removal of the sequence downstream of +23 causes some (and further deletions up to +11 cause a more) serious decrease in template activity *in vitro*. These deletions involve other conserved sequences downstream of the transcription start site. However, the removal of the original transcription start site does not abolish the transcription initiation completely, provided that the whole upstream sequence is intact.**

Transcription initiation is certainly one of the major steps of gene regulation, and for this reason much effort has been directed toward the elucidation of its molecular mechanisms. The sequences involved in the regulation of transcription initiation can be analyzed by using artificially altered genes either in *in vitro* transcription systems or in living cells by DNA transfection or microinjection. Thus, the promoters for RNA polymerases II and III already are determined in some detail. The "TATA" box, which is present at about position −30 (+1 being the first nucleotide of transcription) in most eukaryotic genes coding for proteins, is defined as a promoter of RNA polymerase II because this sequence both determines the transcription start site and promotes transcription *in vitro* (1–4). The C-C-A-A-T sequence at about −80 is also important to facilitate transcription, especially *in vivo* (2, 5, 6). Further upstream sequences such as 72-base-pair repeats and others also are shown to enhance the transcription efficiency with circular DNA *in vivo* and *in vitro* (7–15). In the case of RNA polymerase III, one or two regions inside the gene act as a promoter—e.g., a certain internal region of 5S RNA (16, 17) and dihydrouridine and TΨCG stem and loop regions for tRNA genes (18, 19). On the other hand, sequences required for transcription initiation by RNA polymerase I are poorly understood mainly because of the delayed development of both the *in vitro* and *in vivo* transcription systems for this category of genes (20–27). There are, however, reports on the localization of the promoter on relatively large rDNA fragments. Using a microinjection technique into oocytes, Moss found in *Xenopus* that regulatory sequences for RNA polymerase I are present on a DNA fragment between positions −145 and +16, and active promoter elements lie more than 35 base pairs upstream from the initiation site (28). With the aid of an *in vitro* transcription system, Kohorn and Rae reported that in *Drosophila*, whereas the major promoter activity was present within −240 and −30, the shorter DNA stretch between −34 and +30 could support low levels of accurate transcription initiation (29).

We have compared the initiation regions of mouse, rat, and human ribosomal RNA genes (rDNAs) which have little homology with those of *Xenopus* and *Drosophila*, indicating that at least six regions are well conserved among these mammalian species; namely, A-T-G-G-G-G-T-C-A-T-T-T-T-T-G-G-G-C-C-A (positions −129 to −110), C-C-T-C-C-C (−63 to −58), A-T-C-T-T-T (−38 to −33), T-A-T-T-G (−20 to −16), T-A-C-T-G-A-C-A-C-G-C-T-G-T-C-C-T-T-T (−1 to +18), and T-A-A-A-G-G-A-C-A-C-T (+31 to +41) as written and numbered in mouse sequence (21). With these structural features of rDNA in mind, we set out to study the DNA sequences required for the faithful and efficient transcription initiation of rDNA, using an *in vitro* transcription system. In the mean time, Grummt used several 5′-deletion mutants to make an estimation of the 5′ boundary of the required sequence and reported that the efficiency of transcription decreased drastically between −39 and −34 and was lost completely between −34 and −13 (24). The exact boundaries of the promoter sequences, however, remained to be determined.

Here, we report the results of the experiments in which a number of artificial deletion mutants were tested in an *in vitro* transcription system utilizing either the S-100 extract or the whole-cell extract for the faithful transcription initiation. The data show that although a short DNA stretch upstream of the transcription start site, −22 to −2, is absolutely necessary for transcription initiation at a minimal level, a longer DNA stretch surrounding the transcription start site, −40 to +52, is required for full activity as the template *in vitro*. The quantitative data are discussed in terms of the conserved sequences present in this region that may have important roles in transcription initiation.

MATERIALS AND METHODS

Reagents. Restriction endonucleases were purchased from Bethesda Research Laboratories or Takara Shuzo. T4 DNA ligase was from Takara Shuzo. BAL-31 nuclease and synthetic *Hin*dIII linker were obtained from Bethesda Research Laboratories; bacterial alkaline phosphatase and deoxyribonuclease I, from Worthington; and polynucleotide kinase, from Boehringer Mannheim. [γ-^{32}P]ATP and [α-^{32}P]UTP were purchased from Amersham.

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*To whom reprint requests should be addressed at: Department of Biochemistry, The University of Tokyo Faculty of Medicine, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113, Japan.

Construction of the Deletion Mutants. The recombinant plasmid pMrSL-II (20, 30) containing a mouse rDNA fragment—i.e., the *Sal* I 3.2-kilobase fragment (−167 to about +3,000 of rDNA, +1 being the first nucleotide of the rDNA transcription)—was the starting material. In order to construct the 5′-deletion mutants, the pMrSL-II DNA was cleaved at the *Sal* I site (−167) and then digested with BAL-31 nuclease (4 units/40 pmol of DNA ends) in 400 μl of BAL-31 buffer (12 mM $CaCl_2$/12 mM $MgCl_2$/600 mM NaCl/26 mM Tris·HCl, pH 8.0) at 30°C. Aliquots were withdrawn at 30-sec intervals and poured into EDTA solution to stop the reaction. The treated DNA was phenol-purified, to which a phosphorylated *Hin*dIII linker (4 nmol) was ligated in 80 μl of 66 mM Tris·HCl, pH 7.5/6.6 mM $MgCl_2$/10 mM dithiothreitol/1 mM ATP/6 units of T4 DNA ligase for 16 hr at 12.5°C. The DNAs were phenol-purified and then digested with *Hin*dIII and *Pvu* II. The smaller fragments were separated with a 4% polyacrylamide gel and ligated to the larger fragment of pBR322 digested with *Hin*dIII and *Pvu* II. The recombinant plasmids were introduced into *Escherichia coli* HB101. The extent of deletion of each mutant was determined by DNA sequence determination by the method of Maxam and Gilbert (31). Plasmid DNA was truncated with *Hin*cII and *Pvu* II to make a template for *in vitro* transcription.

In order to construct the 3′-deletion mutants, pMrSS, a self-circularized plasmid of the larger fragment produced by *Sma* I digestion of pMrSL-II (20), was cleaved at the *Sma* I site (+152), digested with BAL-31 nuclease, and ligated to the phosphorylated *Hin*dIII linker as described above. After digestion with *Hin*dIII and *Hin*cII (−165), the smaller fragments were fractionated, ligated to the larger fragment produced by *Hin*dIII/*Pvu* II digestion of pBR322, and transfected into *E. coli* HB101. Plasmid DNA was truncated with *Acc* I and *Hin*cII before use as the template.

***In Vitro* Transcription Assays with S-100 and the Whole-Cell Extract.** The preparation of S-100 extract from FM3A mouse cells has been described (20). The whole-cell extract was prepared by the procedure of Manley *et al.* (31). *In vitro* run-off transcription assays were carried out as described (19, 31). For S-100, 0.7 pmol of template DNA was incubated for 50 min at 30°C in a 25-μl reaction mixture containing 10 mM Hepes (pH 7.9), 80 mM KCl, 2 mM $MgCl_2$, 0.06 mM EDTA, 0.33 mM dithiothreitol, 6% glycerol, 100 μg of α-amanitin per ml, 0.67 mM each NTP (ATP, GTP, and CTP), 0.026 mM UTP, 5 μCi (1 Ci = 37 GBq) of [α-^{32}P]UTP, and 7.5 μl of S-100 (19). In the case of whole-cell extract, 0.2 pmol of template DNA was incubated for 60 min at 30°C in a 25-μl reaction mixture containing 12 mM Hepes (pH 7.9), 60 mM KCl, 7 mM $MgCl_2$, 0.2 mM EDTA, 1.3 mM dithiothreitol, 10% glycerol, 100 μg of α-amanitin per ml, 0.67 mM each NTP (ATP, GTP, and CTP), 0.026 mM UTP, 5 μCi [α-^{32}P]UTP, and 15 μl of whole-cell extract (31). *In vitro* transcription products were glyoxalated and analyzed by 4% polyacrylamide gel electrophoresis.

S1 Nuclease Mapping of *in Vitro* Transcription Products. *In vitro* synthesized RNA was treated with 0.04 unit of DNase I in 200 μl for 30 min at 30°C. RNA and 5′-end-labeled DNA were hybridized in 10 μl of 50% formamide/0.4 M NaCl/40 mM Pipes, pH 6.4/1 mM EDTA for 16 hr at 42°C. The solution was diluted 1:20 with S1 buffer (250 mM NaCl/30 mM sodium acetate, pH 4.6/1 mM $ZnCl_2$) containing 1 μg of heat-denatured calf thymus DNA and treated with 50 units of S1 nuclease for 1 hr at 30°C. The samples were run on an 8% polyacrylamide/7 M urea gel.

RESULTS

5′ Boundary of the Promoter as Determined with S-100. The *Sal* I (position −167)–*Sma* I (position +153) fragment of the mouse rDNA is known to serve as a template for accurate transcription initiation *in vitro* by RNA polymerase I with a cellular extract, S-100 (20–22). To examine the functional significance of the upstream sequences in transcription initiation by RNA polymerase I, we made a series of deletion mutants progressing from the *Sal* I site (−167) toward the transcription start site (+1) as described. After incubation with S-100, the run-off transcript produced with each mutant template was analyzed by acrylamide gel electrophoresis (Fig. 1*B*). The *Sal* I–*Pvu* II rDNA fragment supported an efficient synthesis of the faithful transcript of 291 nucleotides as demonstrated (20, 21). This template designated 5′Δ−166 is tentatively called "wild-type" because this is the control DNA without deletion in this experiment. This figure shows that the 5′-deletion mutants designated 5′Δ−92, −68, −47, and −41 have efficiencies not readily distinguishable from that of the wild-type template. The efficiency of transcription was estimated by monitoring the absorbance of each band with the aid of a scanning densitometer and schematically presented in Fig. 2 with other data, which will be described below. This procedure provides us at least rough estimates

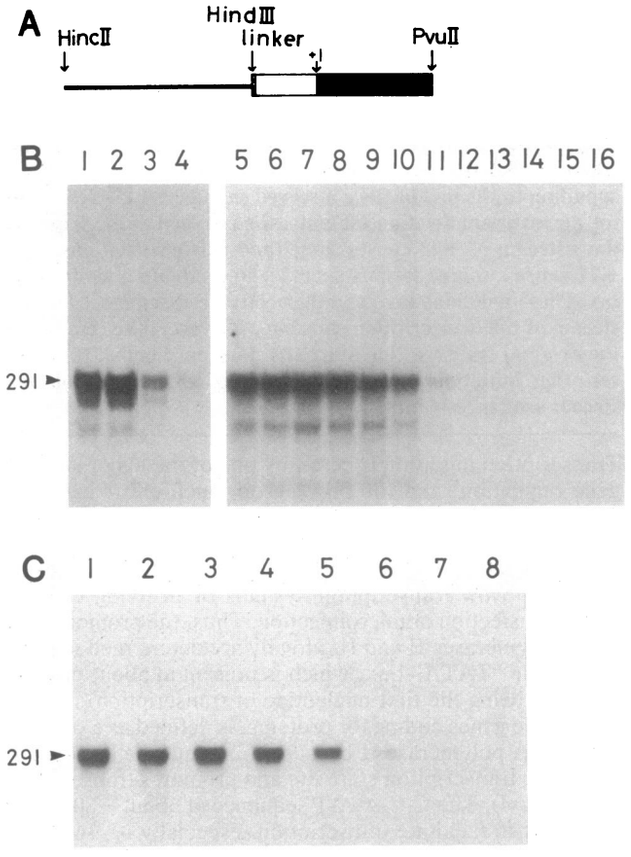

FIG. 1. *In vitro* transcription assay of the 5′-deletion mutants of mouse rDNA. *In vitro* transcription of wild-type and mutant rDNA was carried out as described. (*A*) Organization of the template DNA from wild type and 5′-deletion mutants. □, 5′ flanking region; ■, transcribable rDNA region; ▨, *Hin*dIII linker; —, pBR322 DNA. (*B*) Autoradiography of gel electrophoresis of the run-off products with S-100 extract. Transcripts from wild type (5′Δ−166) and 5′-deletion mutants 5′Δ−92, −32, −28, −92, −68, −47, −41, −35, −32, −28, −26, −23, −17, −10, and −4 are shown in lanes 1–16, respectively. The origin of the weaker band with a lower molecular weight is not clear but may represent some processing product or product from some downstream start site. (*C*) Autoradiography of electrophoresis of the products with the whole-cell extract. Transcripts from wild type (5′Δ−166) and 5′-deletion mutants 5′Δ−47, −41, −35, −32, −28, −26, and −23 are shown in lanes 1–8, respectively. The arrowheads in *B* and *C* show the expected products of 291 nucleotides.

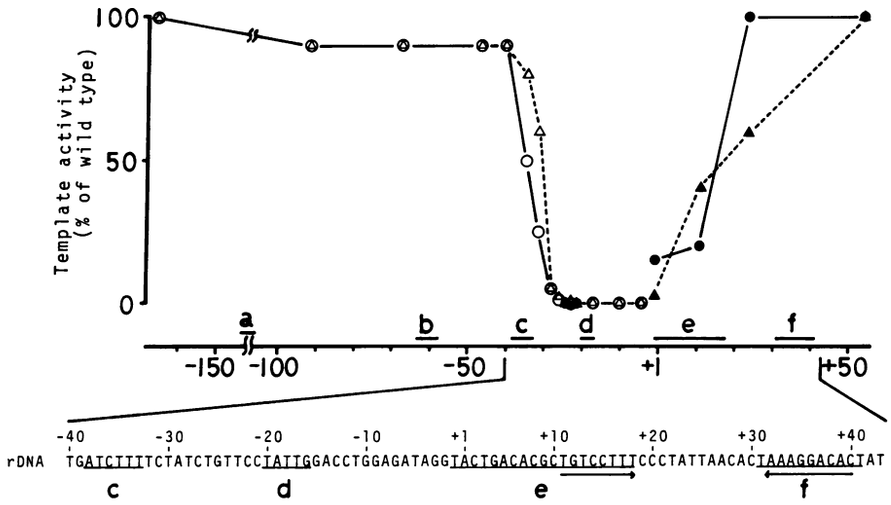

FIG. 2. Efficiency of transcription initiation *in vitro* of different deletion mutants of rDNA with S-100 extract and the whole-cell extract. *In vitro* transcription assay was carried out three or four times, and the difference between S-100 and the whole-cell extract was found to be reproducible. The efficiency of *in vitro* transcription was determined by scanning densitometry of the specific bands on the gels as shown in Figs. 1 and 3. Average percentages of the control (wild type) value are plotted for each mutant. ○, 5′ deletions with S-100; △, 5′ deletions with whole-cell extract; ●, 3′ deletions with S-100; ▲, 3′ deletions with whole-cell extract. Solid circles and triangles representing 3′Δ−1 and 3′Δ−21 are not connected by lines because of the lack of mutants between them. Lines a, b, c, d, e, and f under the map show the conserved regions between mouse, rat, and human rDNAs (23): a, A-T-G-G-G-G-T-C-A-T-T-T-T-T-G-G-G-C-C-A (−129 to −110); b, C-C-T-C-C-C (−63 to −58); c, A-T-C-T-T-T (−38 to −33); d, T-A-T-T-G (−20 to −16); e, T-A-C-T-G-A-C-A-C-G-C-T-G-T-C-C-T-T-T (−1 to +18); and f, T-A-A-A-G-G-A-C-A-C-T (+31 to +41). Regions c–f are also underlined on the sequence. Sequences indicated by converging arrows form a palindrome.

of promoter efficiency, although linearity of density with cpm is not warranted outside a certain range of radioactivity. The deletion mutant 5′Δ−92 appeared to have a slightly lower template activity than that of the wild-type DNA. Although this activity was maintained up to deletion mutant 5′Δ−41, it was reduced to 50% of the wild type at 5′Δ−35. A more pronounced decrease in template activity (25% of wild-type activity) was noted for 5′Δ−32, which had lost a conserved sequence A-T-C-T-T-T reported previously (23). Further decrease in template activity (5% of wild-type activity) was seen in 5′Δ−28, with a trace amount of the correct transcript (1% of wild type) being detected when 5′Δ−26 was used. However, templates having upstream sequences shorter than that of 5′Δ−23 could not produce detectable amounts of accurate transcript.

5′ Boundary of the Promoter as Determined with the Whole-Cell Extract. Although S-100 has so far been used principally for *in vitro* transcription experiments by RNA polymerase I (20–27), we have tested the whole-cell extract to obtain more information, if any, concerning the behavior of the altered templates. Indeed, somewhat different results were reported with respect to the effect of upstream sequences when transcription by RNA polymerase II was carried out with different extracts (33). As shown in Fig. 1*C* and Fig. 2, the effects of the removal of the 5′ side of the gene were similar to those obtained with S-100, though not identical. Only, the whole cell extract did not respond with the partial deletion as much as did S-100. It should be noted that, with the whole-cell extract, 5′Δ−23 showed a positive signal of correct initiation, though very faint (1% of the wild-type signal), which was not seen with the S-100 system.

3′ Boundary of the Promoter as Determined with S-100. Next, we tested the transcription activity of the 3′-deletion mutants with S-100. The 3′ deletion 3′Δ+53 showed a template activity indistinguishable from the wild-type DNA (−165 to +153) (data not shown). Further deletion up to 3′Δ+23 did not change the template activity (Figs. 2 and 3*B*). The efficiency of faithful transcription decreased from that of 3′Δ+11, in which 8 base pairs at the 3′ side were removed from the conserved 19 nucleotides adjacent to the initiation point (−1 to +18) (Fig. 3*B*). Here, 20% of the wild-type activity was retained. Interestingly, however, further deletion up to −1 involving complete removal of the transcribed region (+1 to downstream), including the conserved sequence

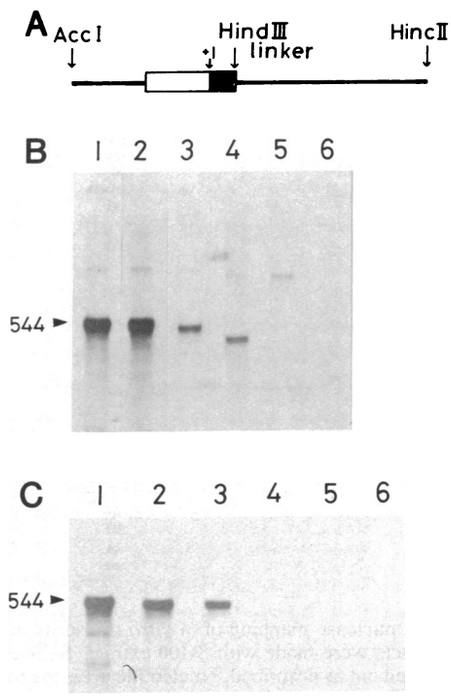

FIG. 3. *In vitro* transcription assay of 3′-deletion mutants. *In vitro* transcription of wild-type and mutant rDNA was carried out as described. (*A*) Organization of the template DNA from 3′-deletion mutants. □, 5′ flanking region; ■, transcribable rDNA region; ▨, *Hin*dIII linker; —, pBR322 DNA. (*B* and *C*) Autoradiography of gel electrophoresis of the run-off products with S-100 extract (*B*) and with the whole-cell extract (*C*). Transcripts from 3′-deletion mutants 3′Δ+53, +23, +11, −1, −21, and −24 are shown in lanes 1–6, respectively. Major bands in lanes 1–4 correspond to the expected products with decreasing sizes from 544 nucleotides (lane 1).

mentioned above, exhibited only a small additional effect—i.e., 3′Δ−1 showed 15% of the wild-type activity (Fig. 2). In deletion mutants 3′Δ−21 and 3′Δ−24, no faithful transcripts could be demonstrated (Fig. 3*B*).

3′ Boundary of the Promoter as Determined with the Whole-Cell Extract. For further analysis, the whole-cell extract was utilized to determine the 3′ boundary of the promoter. As seen in Figs. 2 and 3*C*, the whole-cell extract showed a higher sensitivity than did S-100 to the 3′ deletions—i.e., 3′Δ+23 exhibited a 40% decrease in efficiency as compared with 3′Δ+53. This effect was not detected with S-100. This deletion involves removal of the latter half of the palindrome sequences A-A-A-G-G-A-C-A, which could form a stem-and-loop structure at 11 nucleotides downstream of the transcription start site (ref. 23; Fig. 2). Further deletion, 3′Δ+11, involving the former half of the palindrome T-G-T-C-C-T-T-T and the latter half of the conserved sequence downstream of the initiation site (ref. 23; Fig. 2), caused a serious decrease in template activity with both extracts. The complete removal of the transcribed region up to −1 depressed the template activity more drastically than with S-100, but not completely (2% of wild-type activity). 3′Δ−21 and 3′Δ−24 did not show any template activity with this extract, too.

S1 Nuclease Mapping of the *in Vitro* Transcription Products. In order to prove that the transcription was a faithful one, S1 nuclease mapping was carried out for certain representative transcripts. The results in Fig. 4 indicate that, with deletion mutant 5′Δ−32, the transcription actually started from the same site as did wild-type template, which was known to start correctly (20). With the mutant 3′Δ−1, which had lost the real initiation site of rDNA, the transcription started from an adenosine residue at either +1 or +2, which most probably corresponded to the correct initiation site.

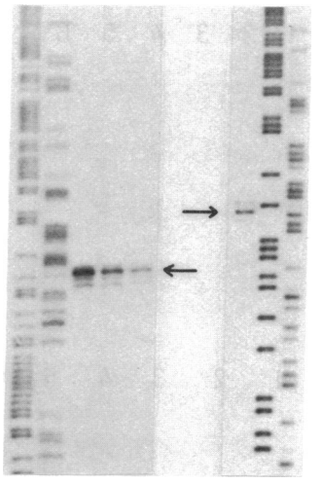

FIG. 4. S1 nuclease mapping of *in vitro* transcription products. *In vitro* products were made with S-100 extract. S1 nuclease mapping was carried out as described. Nucleotide +1 is the transcription initiation site. Capital and small letters represent rDNA and pBR322 DNA sequences, respectively. Arrows indicate the protected fragments. (*A*) Lanes: 1–3, bands protected with the products made on wild type (5′Δ−166) and 5′-deletion mutants 5′Δ−47 and −32, respectively; G+A and T+C, DNA sequence markers derived from the single-stranded *Hin*dIII/*Ava* I probe of wild-type DNA. The ladder in lane T+C is delayed by 1.5 bases, probably because of salt contamination. (*B*) Lanes: 1, band protected with the product made on 3′-deletion mutant 3′Δ−1; G+A and T+C, DNA sequence markers derived from the *Hae* III single-stranded probe of 3′-deletion mutant 3′Δ−1.

DISCUSSION

We have attempted in this study to define the 5′ and the 3′ boundaries of the promoter region of the mouse ribosomal RNA gene, using an *in vitro* transcription system derived from mouse cellular extracts. In spite of accumulating knowledge in the promoters of other genes, this gene system merits special investigation because it is transcribed by RNA polymerase I, which is engaged almost exclusively in ribosomal RNA synthesis, and is apparently under different control than other classes of genes, including mRNA, tRNA, and 5SRNA, which are transcribed by RNA polymerase II or III.

The present study has revealed a rather precise picture as to the promoter region of mouse rDNA. It was shown that the DNA stretch from −40 to +52 was sufficient for near full activity as template in this *in vitro* system. This region contains three sequences conserved among mouse, rat, and human rDNA—A-T-C-T-T-T (−38 to −33), T-A-T-T-G (−20 to −16), and T-A-C-T-G-A-C-A-C-G-C-T-G-T-C-C-T-T-T (−1 to +18)—and a sequence that forms a palindrome with the final eight nucleotides of the last conserved sequence described above (23). The two conserved sequences found further upstream, −129 to −110 and −63 to −58 (23), had only a minor effect, at least in this *in vitro* system. However, this finding does not exclude the possibility that these sequences have some important regulatory functions *in vivo* that could not be detected with the present *in vitro* systems. Destruction of further inside sequences resulted in a serious impairment of the template capacity. Thus, a conserved sequence, A-T-C-T-T-T, especially the latter half of this sequence, was found to be an essential part of the promoter for the RNA polymerase I. Another conserved sequence T-A-T-T-G with a few more upstream nucleotides could support a very minimal transcription start from the correct site. Estimations on mouse rDNA by Grummt (24), observations on human rDNA by Learned *et al.* (34), and similar results for *Drosophila* (35, 36) are in general accord with the present more precise determination of the 5′ boundary of the promoter. Our experiment also shows that the upstream region of rDNA, without the transcription start site (+1) and downstream transcribed region, is sufficient for setting the correct transcription start site for RNA polymerase I. At the same time, the destruction of the conserved sequence at, and downstream of, the start site resulted in a significant decrease in the template activity, indicating that this region also contributes to the efficiency of transcription initiation.

The different behavior of the deletion mutants to S-100 extract and the whole-cell extract deserves further consideration. In general, S-100 appears more sensitive to 5′ deletions—i.e., this extract cannot work efficiently without the A-T-C-T-T-T sequence, whereas the whole-cell extract could overcome this deletion at least partially. The reverse was found for 3′ deletions—namely, the whole-cell extract seems more sensitive to downstream deletions. The reason for these differences is not clear at the present time. However, the possibility may exist that S-100 contains more of the factors that recognize the upstream promoter sequences, and the whole-cell extract has more of the factors interacting with the downstream promoter sequences, thus resulting in a higher efficiency in each case. The functions of these sequences including nonconserved regions have to be determined more rigorously by using more deletion and substitution mutants with *in vivo* as well as *in vitro* systems.

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