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Presence of a limited number of essential nucleotides in the promoter region of mouse ribosomal RNA gene

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ABSTRACT

Point mutations are introduced into a mouse rDNA fragment containing the promoter region by a sodium bisulfite method and the mutants are tested for the ability of accurate transcription initiaion in vitro. The results indicate that the change, G to A, at -7 completely eliminates the promoter activity, and those at -16 and at -25 decrease it to about 10% and 50%, respectively. On the other hand, the substitutions at +9, +4, -2, -9 and -39 do not alter the template activity significantly. It is concluded that there are limited but distinct nucleotides that are essential for the transcription initiation of this gene. This sort of absolute requirement for single specific bases is not reported in protein coding genes transcribed by RNA polymerase II. We propose that these rigid recognition signals which we have found are the molecular basis for the strong species-dependency of the transcription machinery of RNA polymerase I system. A model is presented in which a transcription factor interacts with the rDNA promoter from one side of the DNA double-helix with essential contacts at these bases.

INTRODUCTION

Transcription initiation on a specific gene requires specific DNA sequences, termed 'promoter', recognized by the transcription machinery consisting of an RNA polymerase and its cofactors. The promoters for RNA polymerase II and III are relatively well defined by studies with substitution as well as deletion mutants (1-13). For instance, the importance of the 'CAT' and 'TATA' box is well established. In contrast, the promoter structure for RNA polymerase I; i.e. that of ribosomal RNA gene (rDNA), has yet to be clarified, owing partly to the variability of the sequences near the transcription start site among different species of eukaryote (14-21).

In a previous paper (22), we have determined the

promoter region of mouse rDNA by using series of 5'- and 3'-deletion mutants and concluded that it is present over a relatively long stretch of DNA surrounding the transcription start site (+1), between -40 and +53, though the precise boundaries would be localized somewhat inside especially for the 3'-side. Other studies with deletion mutants of the same or different species rather agree with ours (23-27), confirming that the signals and recognition mechanisms for the RNA polymerase I system are distinct from those for the RNA polymerase II and III. The obvious next step is to determine the role of each nucleotide in the promoter region with special attention to the interaction with the transcription factors. Skinner *et al.* (28) reported recently that the change G to A at -16 in the promoter region of mouse rDNA knocked out almost completely its promoter activity. We have also been testing the point mutants produced by *in vitro* mutagenesis. We have introduced point mutations in the promoter region of mouse rDNA by treating the single-stranded DNA with sodium bisulfite (29-31). The mutant genes are tested for their promoter activity by the *in vitro* transcription system using mouse cell extract (32-34). The results indicate that there are specific sites in the promoter region where the nucleotide changes cause a drastic effect in the promoter activity. The three sites that are found to be sensitive to base changes are present at 9 nucleotide-intervals. We suggest that the known species dependency of the RNA polymerase I system (35,36) may be explained by this strong single-base dependence of the promoter function.

MATERIALS AND METHODS

Bacterial strains and reagents

E. coli WU1 (*uvrA*$^-$, *ung*$^-$, *str*, *thy*) was a kind gift from Dr. S. Hirose, National Institute for Basic Biology. BD1528 (*thyA*, *met*$^-$, *nadBF*, *ung*$^-$, *gal*$^-$, *supE*, *supF*, *hsdR*$^-$, *hsdM*$^+$) was provided by Dr. K. Sekikawa, Institute of Animal Health, Tsukuba. Klenow fragment and restriction endonucleases were purchased from Takara Shuzo and used as recommended by the

supplier. [α-^{32}P] dTTP and [α-^{32}P] UTP were obtained from Amersham.

Construction of the point mutants

Point mutants were generated by the procedure described by Kalderon et al. (31, Figure 1). The plasmid pMrSP contains mouse rDNA promoter region encompassing from SalI site to PvuII site (-167 to +291, +1 being the first nucleotide of the rDNA transcription) cloned between the HindIII and PvuII sites of pBR322. pOl18 is identical to pMrSP except for a deletion of 199 bp from -167 to +34. pMrSP and pOl18 were first digested with PstI and HincII, respectively. 1µg portions of both linear plasmid DNAs were mixed together in 18µl H_2O and denatured by adding 2µl 1N NaOH at room temperature for 10min. Reannealing was then carried out by adding 47µl H_2O, 8µl 1M Tris-HCl (pH7.6), 4µl 5M NaCl and 1µl 0.5M EDTA and incubating at 68°C for 3h. Then, the DNA was precipitated with ethanol and finally dissolved in 50µl of 10mM Tris-HCl (pH7.6), 0.1M NaCl and 1mM EDTA.

6µl of the annealed products were diluted with H_2O to 200µl and added 4µl 50mM hydroquinone and 200µl freshly prepared 4M sodium bisulfite (pH6.0). Then, the mixture was incubated in the dark with paraffin oil at the top for 4 h at 37°C. Subsequent dialysis was exactly as described by Shortle et al. (29), after which the DNA was ethanol precipitated and resuspended in 20µl of 10mM Tris-HCl (pH7.6), 1mM EDTA.

Transfection into E. coli WU1 or BD1528 was carried out by the RbCl method. Ampicillin resistant independent colonies were selected by streaking three times before analysis.

HindIII digested linear plasmid DNA was end-labeled with [α-^{32}P] dTTP. Then, AccI digested small fragments were eluted from agarose gel and sequenced by the method of Maxam and Gilbert (37).

Construction of the dual promoter fragments containing both wild type and mutant promoters

The wild type HindIII/SmaI fragment (316bp) and a mutant EcoRI/PvuII fragment (485bp) were inserted into EcoRI/HindIII site of plasmid pUC8. When this plasmid DNA was digested with SmaI, a linear DNA fragment was obtained having the wild type

promoter at the one end and the mutant type promoter at the other to the opposite direction, producing 291 and 153 nucleotides transcripts, respectively.

In vitro transcription assays with S-100

The procedure for in vitro transcription was described previously (22). The PstI/PvuII fragment of wild type DNA and the PstI/SmaI fragment of a mutant DNA were incubated simultaneously in the same mixture for 50 min. In some cases, the PstI/SmaI fragment of a mutant DNA or a dual promoter template was incubated alone. The products were analyzed by 4% polyacrylamide gel electrophoresis after glyoxalation.

S1 nuclease mapping of in vitro transcription products

S1 nuclease mapping of in vitro transcription products was carried out as described previously (22). In brief, one-tenth of in vitro transcription products was digested with 25, 50 or 100 units of S1 nuclease for 1h at 30°C, and analyzed on a denaturing acrylamide gel along with a sequence ladder.

RESULTS

Transcription assay of various point mutants

We have introduced point mutations in the promoter region of mouse rDNA by a sodium bisulfite method (see, Figure 1) and isolated 18 independent point mutants (see, Table 1). To test the promoter activity of the point mutants, the plasmids were digested with PstI and SmaI and the fragments containing the rDNA initiation region separated by gel electrophoresis (Figure 2-A). In vitro transcription assays were carried out as described previously (22; see, MATERIALS AND METHODS), but including a half or one-fourth amount of wild type template cut with PstI and PvuII (Figure 2-A). Figure 2-B shows that there is a competition between the two templates. Increasing the amount of PstI/SmaI fragment having the wild type promoter sequence resulted in a decreased transcription on the wild type template cut with PstI and PvuII (Figure 2-B, lanes Wt (0-0.3)). This phenomenon suggests that some of the factors in transcription machinery is limited in this system.

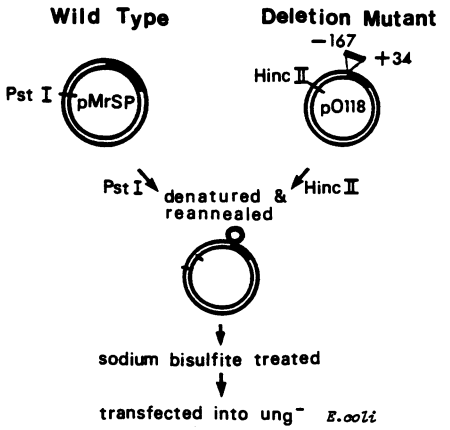
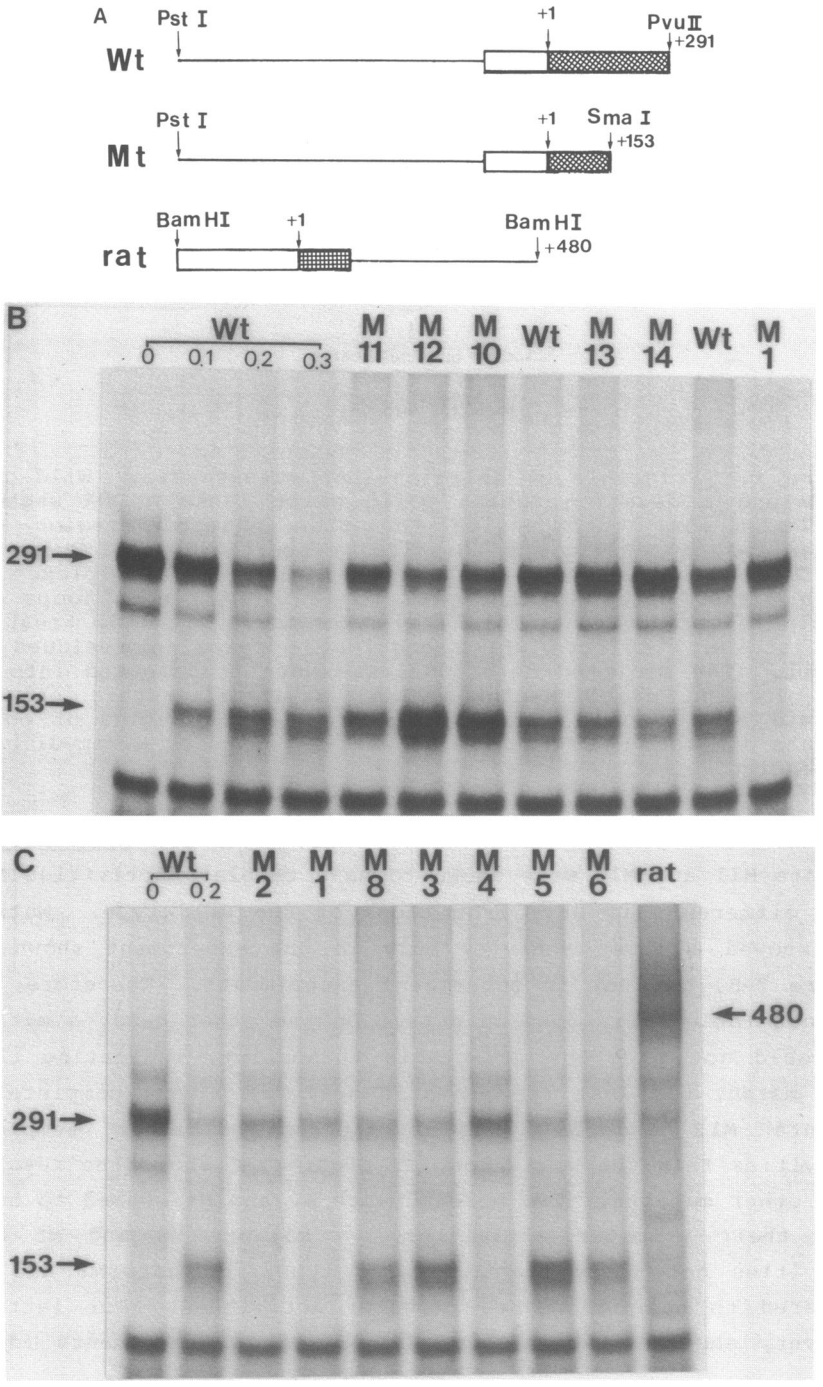


Figure 1. Principle of deletion-loop mutagenesis. Wild-type pMrSP and a deletion mutant pO118 which lacks a DNA segment (-167 to +34) encompassing the rDNA promoter region are linearized by PstI and HincII, respectively. They are denatured together and reannealed to make heteroduplexes in which either strand of the -167 to +34 rDNA region loops out as single-strand DNA. This region is mutagenized by treating with sodium bisulfite which deaminates cytosine residues to uracil. The modified heteroduplexes were transfected into E. coli lacking uracil-N-glucosylase, a uracil repair enzyme. Plasmid clones were randomly picked up and a series of point mutants due to GC→AT transition were selected by Maxam-Gilbert sequencing.

When the sizes of the transcripts were considered, mutants M11 and M13 were shown to have template activities not much different, if any, from those of the wild type. Mutant M14 showed a lower activity only in the experiment shown in Figure 2-B, but not in subsequent experiments. Therefore, it was not included in down mutants. On the other hand, a mutant M1 could not make any detectable transcript, indicating that this mutant had lost its promoter activity almost completely. Mutants M12 and M10 showed apparently higher template activities than the wild type. Figure 2-C shows the results with other mutants. Two more mutants M2 and M4 seemed to have lost their promoter activities. Mutants M3 and M5 had activities not lower than the wild type. Mutants M8 and M6 appeared to have decreased promoter activities. The latter, however, showed a normal activity in other experiments (data


A
Pst I
+1
PvuII
+291
Wt
Pst I
+1
Sma I
+153
Mt
BamHI
+1
BamHI
+480
rat
B
Wt
0 0.1 0.2 0.3
M 11
M 12
M 10
Wt
M 13
M 14
Wt
M 1
291
153
C
Wt
0 0.2
M 2
M 1
M 8
M 3
M 4
M 5
M 6
rat
480
291
153

not shown). The lane of the rat indicates that the mouse extract can utilize the rat rDNA promoter as described previously (36), though the efficiency appears to be about a half of the homologous template.

Analysis of promoter activity of mutants M8 and M12 with the dual promoter fragments

To ascertain that the mutants M8 and M12 have, in fact, altered promoter activities, the dual promoter fragments were prepared (Figure 3-A) and used as templates. The advantages of using the dual promoter are that 1) this fragment assures the exact equimolarity of the wild type and the mutant promoters in the transcription mixture and that 2) the condition of the DNA preparation is identical for the wild type and the mutant promoters, therefore the wild type transcript (291 nucleotides) being a reliable internal marker.

A dose-response experiment was carried out on the dual promoter fragment as described in the previous section. As

Figure 2. In vitro transcription assay of point mutants of mouse rDNA. (A) Organization of the template DNAs for in vitro transcription assay of the mouse wild type (Wt), point mutants (Mt) and rat rDNA (rat). These templates were cut out from respective plasmids and isolated by gel electrophoresis before transcription. □, 5'-flanking region; ■, transcribable mouse rDNA region; ■, transcribable rat rDNA region; ———, pBR322 DNA. (B) Autoradiography of gel electrophoresis of the run-off products with S-100 extract. The arrow 291 indicates transcripts (291 nucleotides) of PstI/PvuII fragment of wild type pMrSP DNA. The arrow 153 indicates transcripts (153 nucleotides) of PstI/SmaI fragments carrying different point mutations excepting the lanes Wt (0-0.3) which carry no mutation. Both DNAs were incubated simultaneously in the same mixture and the products analyzed on 4% polyacrylamide gel after glyoxalation. Templates used are, in addition to 0.1μg wild type PstI/PvuII fragment, lanes Wt (0-0.3), 0, 0.1, 0.2 and 0.3μg, respectively, of pMrSP PstI/SmaI fragment without mutation; lanes M11 to M1, 0.2μg PstI/SmaI fragments from wild type or mutants, M11, M12, M10, Wt, M13, M14, Wt and M1, respectively. (C) In vitro transcripts of other point mutants. PstI/PvuII and PstI/SmaI fragments used as templates give rise to 291 and 153 nucleotides transcripts, respectively. Templates used are, in addition to 0.05μg wild type PstI/PvuII fragment, Lanes Wt (0,0.2), 0 and 0.2μg pMrSP PstI/SmaI fragment without mutation; lanes M2 to M6, 0.2μg PstI/SmaI fragments from mutants, M2, M1, M8, M3, M4, M5, and M6, respectively. Lane rat contains 0.2μg rat rDNA BamHI fragment containing promoter region giving a 480 nucleotides transcript.

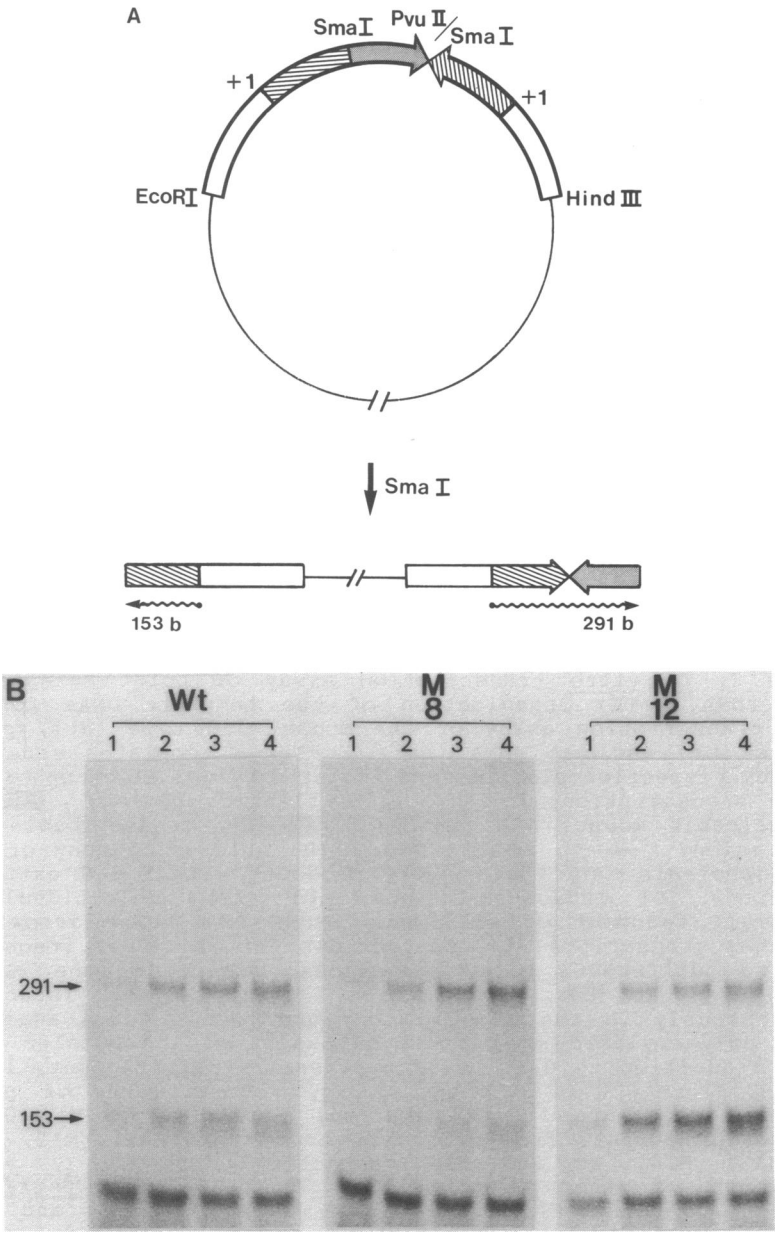


Figure 3. In vitro transcription assay of the dual promoter fragments. (A) Construction of the dual promoter fragments containing both wild type and mutant promoters. The wild type HindIII/SmaI fragment (316 bp) and a mutant EcoRI/PvuII fragment (485 bp) were inserted into the EcoRI/HindIII site of

plasmid pUC8. When this plasmid DNA was digested with SmaI, a linear DNA fragment was obtained having the wild type promoter at the one end and the mutant type promoter at the other to the opposite direction producing 291 and 153 nucleotides transcripts, respectively. (B) Transcription of the dual promoter fragments. The template fragment has a wild type promoter on one end and a wild type or a mutant promoter derived from either mutant M8 or M12 on the other. Lanes 1-4; 0.05, 0.1, 0.2 and 0.4 μg template DNA, respectively.

shown in Figure 3-B, there is a competition phenomenon between the wild type and the mutant promoter. Therefore, the difference of the mutant promoter activity from the wild type promoter activity is amplified in this assay. It is clear from these data that the mutants M8 and M12 are real down and up mutants, respectively.

S1 nuclease mapping of mutants M8 and M12

To see if these point mutations give rise to products transcribed from different initiation sites, the *in vitro* products were analyzed by S1 nuclease protection mapping. The S1 nuclease protected transcripts from the down mutant M8 and from the up mutant M12 are shown in Figure 4. Apparently, the transcription of these mutants starts from the same initiation site as the wild type template.

Effect of template dose on transcription efficiency of mutants M1, M4 and M8

In order to characterize the altered promoter activity of the mutants M1, M4 and M8 in more detail, a dose-response experiment was carried out in which different amounts of template DNA were used in the transcription assay system with a fixed amount of S-100 extract. In this experiment, mutant DNA's were used without the internal marker of wild type DNA to exclude the competition effect. Figure 5-A shows that the mutant M1 has no detectable transcript at all at any DNA concentration, whereas the mutant M4 can produce up to 10% amount of transcripts as compared with the wild type template at higher DNA concentrations. In a similar manner, nearly the same amount of transcripts as the wild type may be obtained with mutant M8, when sufficiently high DNA concentrations are used (Figure 5-B). These kinetics, when plotted (data not shown), suggest that the binding constant ($1/K_m$) of M8 to the

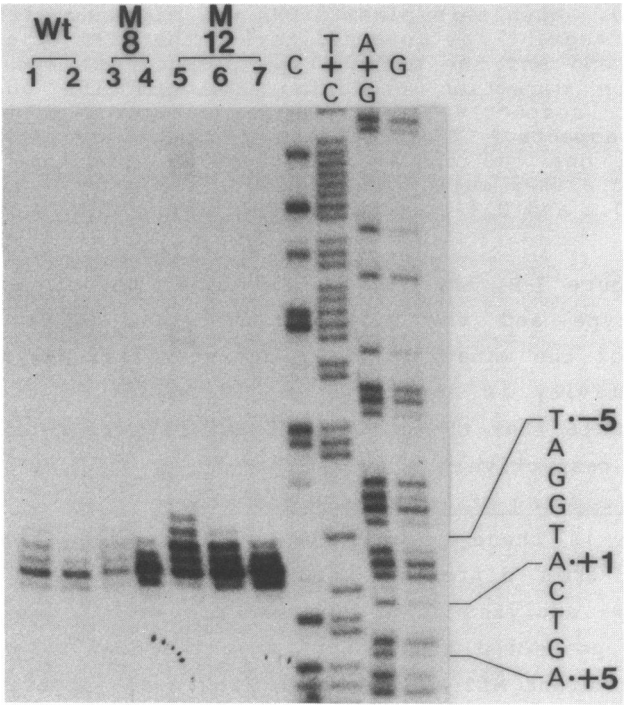


Figure 4. S1 nuclease mapping of *in vitro* transcription products. S1 nuclease mapping was carried out as described (22). Capital letters represent rDNA sequences. Nucleotide +1 is the transcription start site. Lanes : 1 and 2, 3 and 4 and 5 to 7 show the bands protected by the products made by wild type (Wt), mutants M8 and M12, respectively. In case of down mutant M8 (lanes 3 and 4), the radioactivity applied on the gel was increased to show protected bands clearly. Concentrations of S1 nuclease were 25u. (Lane 5), 50u. (Lanes 1, 3 and 6) and 100u. (Lanes 2, 4 and 7). C, T+C, A+G and G are DNA sequence markers derived from the single stranded *Hin*dIII/*Ava*I probe of wild type DNA.

transcription machinery is decreased by 2 to 3 folds, while the V_{max} is maintained rather normally.

Analysis of the effect of point mutations on the promoter activity

In Table 1 are summarized the promoter activities of the mutants with the point mutations in the relevant region. Mutations found outside this region (-40 to +40) are not listed here. Our previous work on 5'-deletion mutants has

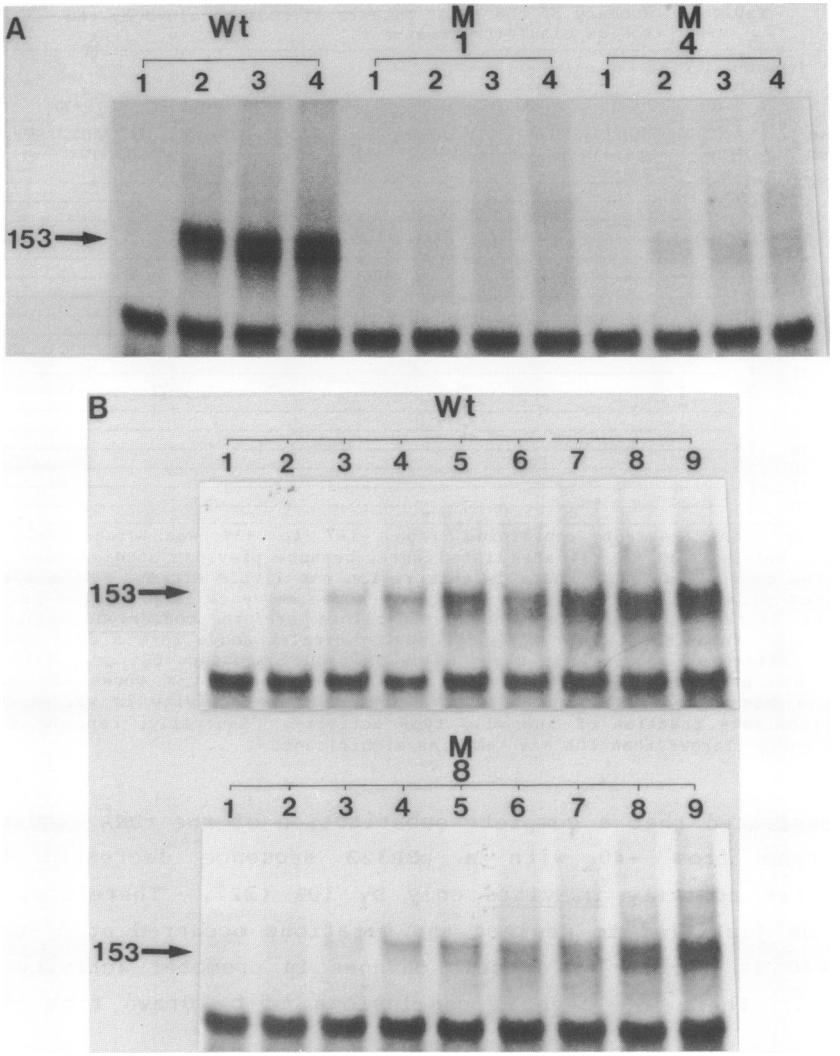
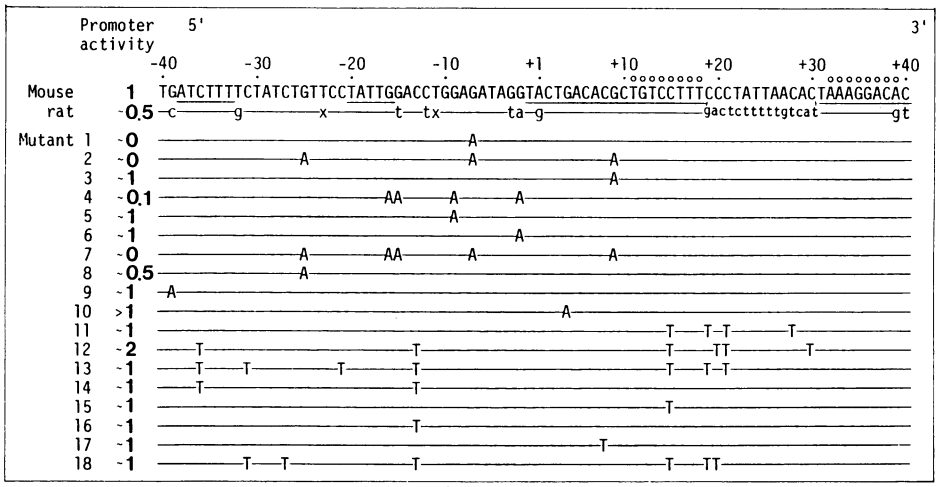


Figure 5. Template dose-response without internal marker (wild type) DNA for in vitro transcription. Another batch of mutant DNA than the one used in Figure 2 was used in this experiment. The consistency of the data will eliminate the possible differences in template activity of different DNA preparations due to damages on DNA and/or impurities. (A) Template dose-responses of the wild type (Wt) and mutants M1 and M4. 7.5µl of S-100 and the indicated amount of template DNA were incubated in the standard mixture at 30°C for 50 min. Lanes 1-4; 0, 0.2, 0.4 and 0.8µg template DNA, respectively. (B) Template dose-responses of the wild type (Wt) and a down mutant (M8). Lanes 1-9; 0.012, 0.023, 0.047, 0.094, 0.19, 0.38, 0.75,.1.5 and 3 µg template DNA, respectively.

Table 1. Summary of the point mutants of rDNA obtained by the sodium bisulfite treatment.



Although rDNA fragment containing from -167 to +34 was mutagenized, the changes between -40 to +34 are listed here, because previous studies have shown that the complete deletion outside this region has little effect (≤ 10%) on in vitro promoter activity (22). Wild type mouse sequence is presented in capital letters on the top line. Underlined are the conserved sequences previously reported .(20). Sequences having circles above form a dyad symmetry. Corresponding rat sequence is shown below the mouse sequence but only different bases are presented by lowercase letters. The character x shows the place where a base is deleted in the rat. The template activity is expressed as an approximate fraction of the wild type activity. Generally, reproducible differences larger than 10% are taken as significant.

demonstrated that a complete substitution of the rDNA sequence upstream from -40 with a pBR322 sequence decreased the promoter activity in vitro only by 10% (22). Therefore, it may be justified to neglect the mutations occurred at 5'-ward of -40 as a cause of drastic changes in promoter activity in vitro. There are several conclusions to be drawn from this table.

First, it may be noted on the second line that the rat rDNA, though base-substitutions are plentiful, could be transcribed by the mouse extract at a lower but significant efficiency (36), suggesting that many of the base changes are really without profound effect. Second, the mutant M1 which has only one substitution in this region does not have any detectable template activity. The change G to A at -7 is, therefore, responsible for this total loss of template

activity. The data of mutants M2 and M3, which indicate the substitution at +9 alone does not alter the activity at all but the involvement of the base at -7 leads to a complete loss of template activity, is consistent with the above conclusion. The substitution at -25 causes only a decrease in promoter activity which will be discussed later. Third, mutant M4, a strong down mutant, suggests another important site for promoter activity. A comparison of sequences of M4, M5, M6 and the rat points to the vital role played by G at -16. We are aware that this type of 'exclusion' logic is not always correct. But, it appears valid in this case, since Skinner et al. (28) came to the same conclusion using different mutants. The result with mutant M7 which has substitutions both at -7 and at -16 is also compatible with the above conclusions. Fourth, the important role of G at position -25 is demonstrated by a careful examination of mutant M8 as described in the previous section. Fifth, there are at least two mutants (mutants M10 and M12) which show apparently higher template activities in vitro. The dual promoter experiments reveal that at least mutant M12 is a real up-mutant of the promoter. Sixth, other mutants, many of which have multiple mutations, C to T, do not show any change in template activity at least in this in vitro transcription system.

DISCUSSION

Absolute requirement for single specific bases

We have shown in the present study that there are a limited number of nucleotides that are essential for rDNA promoter function. The G at -7 is indispensable for this function and those at -16 and at -25 are required for full activity. These three nucleotides are in contrast with many others whose changes do not affect appreciably the promoter activity. These include G's at +9, +4, -2, -9 and -39 and C's at more than 10 sites in the promoter region (Table 1). It is interesting that two guanine nucleotides such as -16 and -15 (see mouse sequence), even adjacent, have completely different effects: one almost essential and the other not.

Although Skinner et al. (28) reported that the substitution

of the G at -16 with an A residue decreased the promoter activity to less than 5%, our results of DNA dose-response experiment indicate that the mutant has about one-tenth of the wild-type promoter activity (Figure 5-A). This discrepancy may be explained by the different sensitivity of the extracts used. Indeed, we have shown that different extracts have different sensitivity to various deletions (22). Or, they might have failed to detect the transcripts of low level due to the experimental conditions. In any event, it is clear from our results that this mutation does not knock out completely the promoter activity, while the substitution at -7 really does.

Competition experiments with these mutants indicate that the mutant M1 (-7 mutation) can compete effectively with the wild type template for binding a transcription factor, whereas the mutant M4 (-16 mutation) does not compete with the wild type template in agreement with Skinner et al. (28). Thus, these sites appear to be functionally different (in preparation).

The molecular basis for the strong species-dependency of the transcription machinery of RNA polymerase I system

In the case of the protein-coding genes transcribed by the RNA polymerase II, such a strong single-base dependence as has been found here was not observed (2,5,6). For instance, in the fibroin gene, a single-base change of the TATA box and its downstream sequence produced a maximum of only 70-80% decrease in promoter activity (5). Ten base pairs between -17 and -30 were examined in the above-mentioned study.

In the case of rDNA promoter described here, one base change at either -7 or -16, among 11 base pairs studied between -2 to -36, caused a drastic decrease ($\geq$ 90%)of the promoter activity. These findings suggest that the RNA polymerase I system has a more rigid recognition signal than the RNA polymerase II system. Perhaps, more flexibility is required for the RNA polymerase II system, since it has to transcribe a number of different genes unlike the RNA polymerase I system which is solely engaged in rDNA transcription.

This notion seems important in relation to the strong species-dependency of the transcribing machinery of RNA

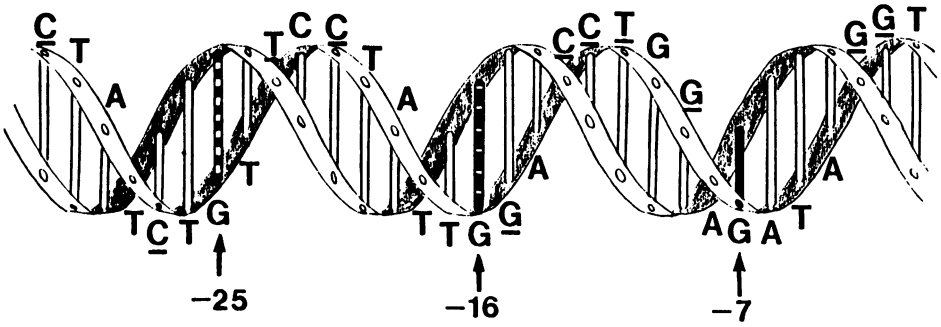


Figure 6. Schematic three dimensional model of the DNA double-helix around the promoter region of mouse ribosomal RNA gene. Two ribbons and the vertical rods represent sugar-phosphate backbones and base pairs, respectively. The wild type, non-coding sequence is presented. Filled rods at -7 indicate essential or unchangeable base pairs for rDNA transcription initiation, whereas the striped rods at -16 and at -25 represent base pairs which affect the efficiency of transcription significantly. Underlined are the bases whose changes do not affect the transcription efficiency significantly.

polymerase I (35,36,38-40) which is also revealed by the nucleolar dominance phenomenon (41-45). Mouse cell extract is known to transcribe mouse rDNA but not human rDNA in vitro and vice versa (35). We have shown that a factor which is indispensable for an accurate transcription initiation of rDNA is responsible for this phenomenon but also have shown that mouse extract (factor) can transcribe rat rDNA (36).

This species-dependency of transcribing machinery may be explained by the strong single-base dependence of the promoter function demonstrated in this study. From the sequence divergence among mouse, rat and human rDNA promoter region (20), it may be predicted that probability of losing template activity to mouse cell extract is much greater in the human than in the rat rDNA, which appears really happened.

It is reported that HeLa cell extract can transcribe Rhesus monkey rDNA (39) and Tetrahymena thermophila cell extract can transcribe T. pyriformis rDNA (T. Higashinakagawa, personal communication). It will be interesting to compare the divergence of the promoter sequence and compatibility of transcription machinery in these and other related animal species.

Nine base-pair intervals found between the bases essential for the promoter activity

We note that the two almost essential G's at -7 and -16 are 9 nucleotides apart from each other and another important G at -25 is also 9 nucleotides apart from the latter. This is reminiscent of the models that have been put forward for the interaction of the fibroin gene TATA box and RNA polymerase II machinery (5) and for that of prokaryotic promoters and E. coli RNA polymerase (46). A hypothetical picture may be drawn that at least a part of the transcription machinery interacts with the promoter region of rDNA from one side of the DNA double-helix, having some essential contacts with these G's (Figure 6).

ACKNOWLEDGEMENTS

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