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The core promoter of mouse rDNA consists of two functionally distinct domains

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SUMMARY

We have determined the sequences constituting the minimal promoter of mouse rDNA. A very small region immediately upstream of the transcription start site (from -1 to -39) is sufficient to direct correct transcription initiation. Sequences immediately downstream of the transcription start site (+1 to +11) increase the efficiency of transcription initiation. Point mutations within the core promoter have been generated and assayed for their effects on template activity and on interaction with the pol I specific transcription factor TIF-IB. The core promoter element appears to consist of two functionally different domains. The distal sequence motif from position -22 to -16 is recognized by factor TIF-IB. Mutations within this region lead to similar changes of both template activity and binding of TIF-IB. Two point mutations within the proximal sequence motif from -15 to -1 do not affect TIF-IB binding although they severely impair transcription initiation. It is suggested, that this proximal region plays a role in the assembly of functional transcription initiation complexes rather than in the primary binding of TIF-IB.

INTRODUCTION

The transcription of ribosomal genes of several species has been studied in different laboratories during the last few years. Cell free transcription systems capable to initiate transcription on cloned ribosomal DNA have been used to map the sequences that promote transcription of the rRNA genes and to identify the auxilliary protein factors that are necessary for the recognition and utilization of RNA polymerase I (pol I) promoter sequences. These studies have revealed that the transcriptional control signals for pol I appear to consist of at least two functional elements. The core promoter - a region 50 to 60 bp adjacent to and including the 5' terminus of ribosomal precursor RNA - is absolutely required for accurate transcription initiation (1-8). The distal control element is located some 100-200 bp further upstream and appears to influence the efficiency of transcription (4-6). The functional interaction of these two regulatory sequences in directing accurate

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and efficient transcription initiation remains to be elucidated.

Recently we have partially purified two proteins that are required in addition to RNA polymerase I for faithful transcription initiation. The initiation factor TIF-IA mediates the regulation of rRNA synthesis according to the growth rate of the cell (9). The second factor TIF-IB forms stable complexes with mouse rDNA templates in vitro. Exonuclease III protection experiments revealed that TIF-IB binds to the core promoter sequences between positions -21 and -7 (10). If an evolutionarily highly conserved guanine within this core region at position -16 was converted into an adenine, a 90-95% inhibition of transcription (11, 12) and a concomitant loss of both transcription complex formation and TIF-IB binding was observed (10).

In order to define in more detail the nucleotides that specify RNA polymerase I transcription initiation, stable complex formation and binding of factor TIF-IB, respectively, we have introduced single base exchanges in the mouse rDNA promoter and evaluated the transcriptional activity of these point mutants both in the cell-free system, in an in vivo transient expression system and in the exonuclease III footprinting assay. Our data suggest, that two functionally different domains reside within the core promoter. The distal sequence motif 5'CCTATTG-3' (from position -22 to -16) is part of the TIF-IB recognition signal. Mutations within this nucleotide sequence similarily affect binding of TIF-IB and transcriptional activity. Mutations within the start site proximal domain from -15 to -1 have no influence on TIF-IB binding but may exert drastical effects on transcription initiation. Moreover we demonstrate that the first few nucleotides beyond the start site do not contribute to the accuracy of transcription initiation but increase the efficiency of rDNA transcription.

MATERIALS AND METHODS

Wild Type rDNA Templates

The plasmid pMrWT has been described before (11). It contains mouse rDNA sequences from -169 to +155 relative to the transcription start site cloned into pUC9. After truncation with Eco RI 158 nt run-off transcripts are synthesized in the cell free transcription system. The analogous plasmid pMr189 extends 34 bp further into the transcribed region (from -169 to +189) yielding 192 nt transcripts after truncation with Eco RI. Two sub clones were generated containing no sequences downstream of the transcription start site. The plasmid pMr-169-1 extends from the Sal I site position -169 to an Rsa I site at -1. pMr-39-1 contains the Sau 3A/Rsa I fragment from -39 to -1 which has

been cloned into pUC9. The plasmid pMr -39 +11 is similar to pMr-39-1 but extends 11 bp into the transcribed region. The mutant pMr -39 +155 contains a Sau 3A/Sma I fragment (-39 to +155) cloned into pUC9.

In vitro Transcription Assays

The cultivation of Ehrlich ascites cells, the preparation of S-100 and nuclear extracts, the cell free transcription system and the analysis of the synthesized RNA have been described (10). Transcription complex formation was demonstrated in the prebinding transcription assay (13). 200 ng of pMrWT/Eco RI or a derivative mutant were incubated with 30 μl of a mixture of S-100 and nuclear extracts in a buffer containing 5 mM Hepes pH 7.9, 0.1 mM EDTA, 5 mM $MgCl_2$, 75 mM KCl, 10 mM creatine phosphate and 14% glycerol (v/v). The final volume was 48 μl. After 15 min at 30°C the second template pMr +189/Eco RI was added together with the nucleoside triphosphates. Incubation was carried on at 30° C for another 40 min. The RNAs were extracted, precipitated with ethanol and run on 5% polyacrylamide gels.

S1 Mapping of the 5' Ends of RNAs

RNA transcripts derived from in vitro transcription were mixed with $1x10^{5}$ cpm of the 5' labelled Pvu II fragment from pMr-169/-1 (spec. act. 10^{6} cpm/μg). After ethanol precipitation the nucleic acids were dissolved in 25 μl of hybridization buffer (80% formamide, 0.04 M PIPES pH 6.4, 1 mM EDTA and 0.4 M NaCl). The reaction mixture was incubated at 80° C for 10 min and then at 48° C for 3 hr. After dilution with 250 μl of S1 buffer, the hybrids were treated for 30 min at 37° C with 50 units of S1 nuclease and subjected to electrophoresis on a 6% polyacrylamide urea gel along with DNA size markers.

Construction of Point Mutants

The point mutants are derivatives of pMrWT. The G to A transitions at positions -15, -16 and -25 have been generated by the sodium bisulfite mutagenesis procedure which has been described before (11). All other mutants were constructed by oligonucleotide directed mutagenesis according to a modified procedure of Zoller and Smith (14). 3 pmoles of synthetic oligonucleotide were phosphorylated in a solution containing 50 mM Tris- HCl, pH 7.6, 10 mM magnesium chloride, 5 mM DTE, 0.1 mM EDTA, 1 mM spermidine, 0.1 mM ATP and 2 units of T4 polynucleotide kinase in a total volume of 8 μl. After incubation at 37° C for 45 min 0.05 pmoles of single stranded M13 MrWT DNA (which covers mouse rDNA sequences from -169 to +155) and 1 pmole of M13 universal primer were added. The solution was adjusted to 50 mM NaCl, heated for 5 min at 80° C and cooled to room temperature. 10 μl of solution B (20 mM Tris-HCl, pH 7.5, 10 mM magnesium chloride, 10 mM DTE, 1 mM dATP, dGTP, dCTP, dTTP and

1 mM ATP), 2.5 units of T4 DNA ligase and 2 units of the large fragment of E. coli DNA polymerase I were added and the sample was incubated at 14° C overnight. 2 µl of the heteroduplex mixture were used for transfection of E. coli JM 103. The phage DNAs were replica-plated on nitrocellulose filters, hybridized with 5' labelled mutant oligonucleotide and screened by successive washes at progressively higher stringency. Mutant phages were isolated, the nucleotide sequence determined according to Sanger et al (15) and the rDNA insert cloned into pUC9.

Exonuclease III Footprinting

The transcription factor TIF-IB used for the binding experiments was partially purified from cytoplasmic and nuclear extracts by chromatography on DEAE-Sephadex and Heparin-Ultrogel (10). 3-5 µl (ca. 0.3-0.5 µg protein) of a column fraction eluting at 0.6 M KCl from Heparin Ultrogel were incubated with 3 ng of a 343 bp Hind III/Eco RI fragment from pMrWT that had been 5' labelled at the Eco RI site. Incubation was carried out for 15 min at 30° C in 25 µl of a buffer containing 12 mM HEPES, pH 8.0, 90 mM KCl, 5 mM magnesium chloride, 0.1 mM EDTA, 0.5 mM DTE, 0.1 mM ATP, 1 µg yeast tRNA, 0.1 µg pUC9 DNA, and 12% glycerol. After the binding reaction 5 units of exonuclease III (Boehringer Mannheim) were added and the incubation was carried on for another 5 min at 30° C. The reaction was stopped by the addition of 25 µl of 400 mM ammonium acetate, pH 5.5, 0.2 µg/µl yeast tRNA, and 10 mM EDTA. After phenol extraction and ethanol precipitation the DNA was analyzed on a 6% sequencing gel along with size markers (pBR322/Hpa II).

DNA Transfections and RNA Analysis

The rDNA hybrid gene pMrCAT-1 has been described before (16). For the construction of the mutant clones the plasmids pMr -15/-16, pMr -7 and pMr -1 were linearized with Sma I and the blunt-ended Hind III/Bam HI fragment from pSV2-CAT was inserted to generate the plasmids pMr-15/-16CAT, pMr-7CAT, and pMr-1CAT. The transfection of 3T6 cells, the RNA preparation and the analysis of CAT specific transcripts by primer extension analysis was performed as previously described (16).

RESULTS

The Accuracy of Transcription Initiation is Brought about by Sequences Upstream of the Start Site.

Recently we and others have analyzed a series of 5' deletion mutants of mouse rDNA and have shown that removal of sequences to position -39 does not severely affect the template activity in a cell-free transcription system (1-3).

3' deletions beyond position +9 drastically impaired transcriptional activity. It was therefore concluded that sequences within the transcribed region are part of the rDNA promoter (2, 3, 7). To find out whether sequences downstream of the start site are required for faithful transcription initiation we constructed two rDNA subclones which contained only 5' flanking spacer sequences extending from position -169 or -39, respectively, to position -1. In the cell-free system the 3' deletion mutant pMr-169/-1 directs the synthesis of defined 93 nt run-off transcripts after truncation with PvuII (Figure 1B, lane 2). The transcription efficiency of this template, however, is decreased by approximately 50% as compared to the wild type control pMrWT (lane 1). The mutant pMr-39/-1 which contains only 39 bp of spacer sequences is still capable of generating very low levels of correctly initiated transcripts which are visible on longer exposed autoradiograms. The low transcription activity of pMr-39/-1, however, was greatly stimulated if 11 nucleotides downstream of the initiation site were present in the rDNA constructs: pMr-39/+11 which contains rDNA sequences from -39 to +11 (lane 4) is transcribed with 30% of wild type activity while pMr-39+155 (lane 5) reaches near wild type levels under optimal conditions. These results suggest, that a minimal promoter function is exerted by rDNA sequences from -39 to -1, but that some downstream sequences are required for efficient promoter utilization.

To determine whether the transcripts have been initiated at the correct initiation site S1 mapping experiments were performed. A 457 bp PvuII fragment derived from pMr-169/-1 was labelled at the 5' ends and hybridized to transcripts generated from pMr-169/-1 and pMr-39/-1, respectively. As shown in Figure 1C a major S1 resistant 93 bp fragment was obtained, a length which precisely corresponds to the distance of the 5' end of the hybridization probe to the guanine immediately adjacent to the thymine at position -1 of the rDNA insert. This result indicates that the correct positioning of the transcription start site is brought about by a relative ly short region of 5' terminal spacer sequences and that sequences beyond the start point increase the efficiency of initiation.

Transcriptional Activity of Point Mutations within the rDNA Promotor Region

In order to define in detail the functional importance of single nucleotides within the rDNA control region we have introduced single or double base substitutions into the core region between position -1 and -39. The mutants are identical to the wild type both in length and sequence composition of the transcribed region. The sequence of each mutant is shown in Figure 2. _In vitro_

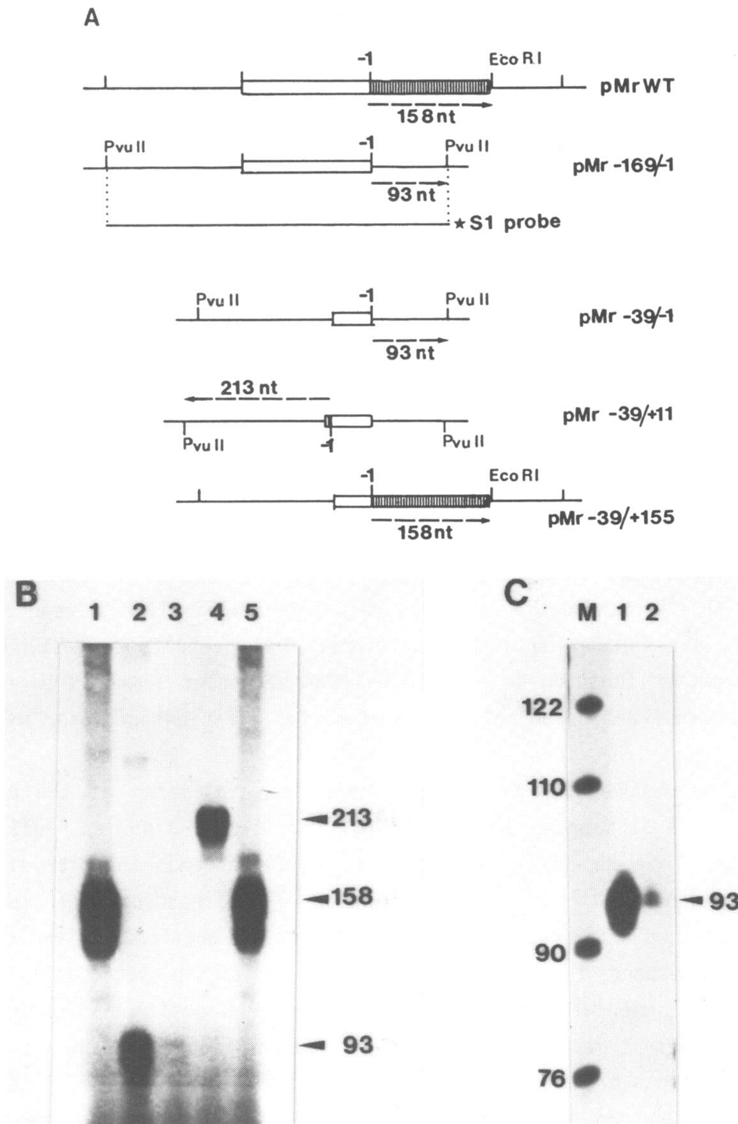

Figure 1: Transcription of the minimal promoter clones.
A) Schematic representation of the recombinant plasmids containing different regions of the mouse rDNA promoter. The transcripts generated in the run-off assay and the DNA fragment used for S1 mapping are shown. The bar represents rDNA sequences, (open bar:5' NTS, hatched area: ETS sequences), the thin line are pUC9 vector sequences.

B) Runoff transcripts: Each of the assays contained 200 ng of template DNA which has been truncated with Eco RI or Pvu II, respectively. Lane 1: pMrWT/Eco RI, lane 2: pMr-169/-1/Pvu II, lane 3: pMr-39/-1/Pvu II, lane 4: pMr-39/+11/Pvu II, lane 5: pMr-39/+155/Eco RI. The numbers indicate

the lengths of transcripts in nucleotides.
C) S1 mapping of 5' ends of transcripts. Cold RNA synthesized from pMr-169/-1 and pMr-39/-1 in a 100 µl transcription assay was hybridized to the 5' labelled 457 bp Pvu II fragment derived from pMr-169/-1. After treatment with S1 nuclease the protected fragments were analyzed on a 6% sequencing gel along with size markers (pBR 322/Hpa II, lane M). Lane 1: pMr-169/-1, lane 2: pMr-39/-1.

transcription directed by each mutant template was evaluated relative to a wild type control template in the runoff assay. Transcripts generated from the wild type rDNA template (pMr189/Eco RI) served as internal control. Transcripts derived from this template are 192 nt long. pMrWT and the point mutants direct the synthesis of 158 nt transcripts after truncation with EcoRI. The mutants were tested under conditions where the ratio of extract proteins to DNA was high, i.e. at low concentrations of template DNAs. Under these conditions the transcription factors are present in excess and no competition should occur. The result of one typical experiment is shown in Figure 3. The intensity of the 192 nt runoff transcript synthesized from the wild-type pMr189/Eco RI template is essentially the same in all samples. However, the phenotypes of the promoter mutants differ in some cases from that of pMrWT (lane 1). There is one mutant, pMr-7 (lane 4) that shows no detectable transcriptional activity at all. In two mutants, pMr-1 (lane 2) and pMr-15/-16 (lane 7), transcription is reduced by at least 90%. pMr-17 and pMr-17/-18 exhibit a 40-50% reduction of template activity (lanes 8, 9).

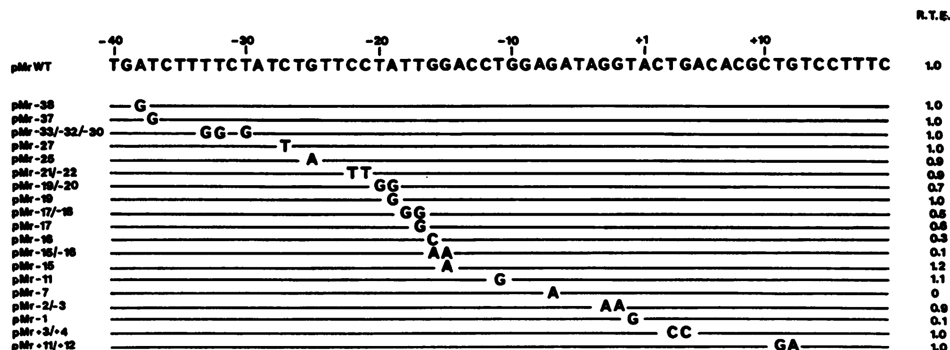

Figure 2: Nucleotide sequence of point mutations of the mouse rRNA promoter. The positions of the base exchanges contained in the mutants are shown below the wild type rDNA sequence. Each mutation is designated by the nucleotides that have been altered. Transcripts generated in run-off assays were quantitated by densitometric scanning of the autoradiograms (see Fig. 3). The relative transcription efficiency (R. T. E.) of each mutant (as obtained from 20 independent experiments) was determined by calculating the ratio of mutant transcripts *versus* transcripts derived from the wild type template pMrWT.

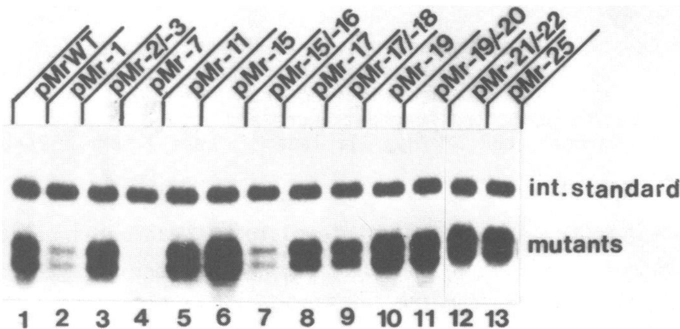

Figure 3: Effect of base substitutions within the core promoter on rRNA transcription. 40 ng of wild type template pMrWT or point mutants (truncated with Eco RI) were incubated in the standard runoff transcription assay. Prior to phenol extraction an internal standard, i.e. an aliquot of a transcription assay containing pMr+189/Eco RI as template was added to each probe to allow quantitation of the transcripts. Shown is an autoradiogram of RNA products after electrophoresis on a 5% polyacrylamide gel. Transcripts derived from the control are 192 nt long and from the mutant templates 158 nt. The double band which is consistently observed in runoff transcripts from pMrWT and derivatives thereof appears to be due to some heterogeneity at the 3' ends. The 5' ends of the transcripts map at a unique point.

pMr-19/-20 and -21/-22 are transcribed at almost wild type levels (about 90%). Two mutants pMr-11 and pMr-15 (lanes 5 and 6) exhibit a 10-20% higher template activity as compared to the wild type. On the other hand, there is a number of nucleotides that can be exchanged without affecting transcriptional activity (pMr-2/-3, pMr-19, pMr-25). Similarly, templates bearing point mutations at positions -38, -37, -33 and -30 or in the transcribed region at positions +3, +4, +11 and +12, were transcribed at wild type levels (data not shown). The relative transcription efficiencies (R.T.E.) of the point mutants are summarized in Figure 2. Interestingly, the three nucleotides which are most sensitive against mutation, a thymine at -1, a guanine at -7 and at -16, are highly conserved in vertebrates (see ref. 10). This strong conservation of a few essential nucleotides within the rDNA promoter region suggests an important role in the initiation process.

Transcription Complex Formation on Mutant rDNA Templates.

The following experiments were performed to study the function of defined nucleotides within the core promoter in the formation of preinitiation complexes. Wild type (pMrWT) and mutant templates were incubated for 10 min in the standard transcription assay before adding the reference template pMr+189 as the second template. Fig. 4 shows the result of one typical experiment.

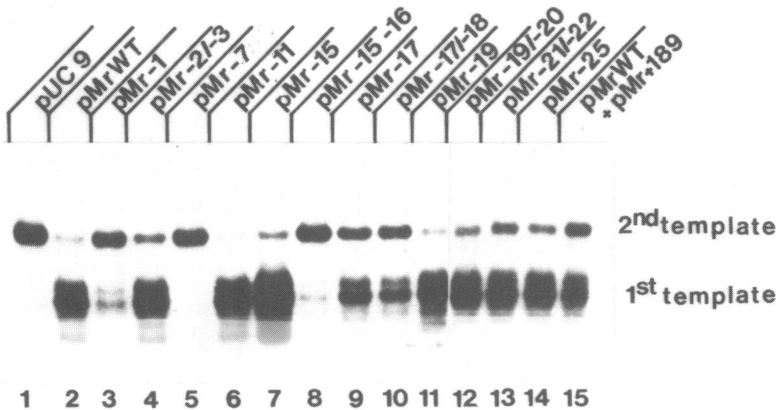

Figure 4: Prebinding competition between mutant templates and wild type DNA. 120 ng of pMrWT mutant templates or pUC9 were linearized with Eco RI and were preincubated in the absence of nucleoside triphosphates with extract proteins in the standard transcription assay. After 10 min at 30°C 80 ng of pMr189/Eco RI and NTPs were added and incubation was continued for another 40 min. Lane 1: Preincubation with pUC9/Eco RI. Lanes 2-14: Preincubation with pMrWT or the mutant templates. Lane 15: pMrWT/Eco RI and pMr189/Eco RI were both present during preincubation.

Simultaneous incubation of both wild type DNAs results in transcription from both templates (lane 15). Preincubation of pMrWT precludes transcription of the subsequently added pMr+189 at limiting factor concentrations (lane 2). Drastic differences are observed in the ability of the different mutants to stably bind transcription factors which results in a more or less efficient transcription of the reference template pMr+189. In most cases complex formation as measured in the prebinding assay, correlates with transcriptional activity. Mutants, the template activity of which compared to the wild type, stably bound transcription factors thus efficiently repressing transcription of the second template. Point mutants with a decreased template activity usually showed a reduced ability to form stable complexes which results in clear transcription of the second template. However, there appear to exist functional differences. Mutants pMr-15/-16, pMr-17, Mr-17/18, pMr-19/20 and pMr-21/-22 show a reduction of complex formation which compares to their transcriptional activities. On the other hand both mutant pMr-7 which is transcriptionally inactive and pMr-1, the template activity of which is reduced by 90-95% appear to sequester transcription factors which results in a suppression of transcription of the second wild type DNA by about 30%. Thus, the severe effects on expression of these mutants obviously are probably not

due to a failure of transcription factors to bind to the rDNA promoter but may involve reactions following the primary factor-DNA interaction which finally lead to the assembly of stable transcription complexes.

This view is supported by the observation that the inactive mutants pMr-1 and pMr-7 also compete for transcription factors when challenged simultaneously with a wild type template. If pMr-7 or pMr-1 were incubated together with wild type DNA at limiting factor concentrations, i.e. at relatively high amounts of template DNA, both mutants were able to reduce transcription from the wild type template (data not shown). We infer from these results that a different molecular mechanism impairs the transcription of the start site proximal mutants pMr-1 and pMr-7 as compared to the mutants containing base substitutions between position -16 and -22.

Binding of Transcription Factor TIF-IB to the Promoter Mutants

Recently we have isolated a transcription factor from mouse cells that specifically binds to the core element of the mouse rDNA promoter and forms stable preinitiation complexes with an rDNA template. Exonuclease III footprinting with highly purified TIF-IB preparations placed the 3-borders of the exonuclease III resistant region at position -21 on the coding strand and position -7 on the noncoding strand. The colinearity of the sequences protected by TIF-IB and the core promoter element of mouse rDNA suggests that binding of TIF-IB to these sequences may play an important role in transcription initiation. To further correlate promoter activity with the observed factor-DNA interactions we have tested the ability of partially purified TIF-IB preparations to specifically bind to the mutant rDNA templates.

The result of an exonuclease III protection experiment using six selected point mutants is shown in Figure 5. In the absence of TIF-IB the exonuclease III produced 5' labelled single stranded DNA fragments of about half the original size (lane 1). In the presence of purified TIF-IB an additional band was observed representing a stop of exonuclease III digestion due to protein binding (lane 2). This stop signal at position -21 on the coding strand was also generated with the point mutants pMr-1 (lane 3), pMr-7 (lane 4), pMr-15(lane 5) and pMr-25 (lane 8). These mutants, the transcriptional activities of which differ considerably (see Figure 2), appear to interact with TIF-IB with approximately the same efficiency. On the other hand, two other mutants pMr-15/-16 and pMr-17/-18, the template activities of which are strongly impaired do not show this exonuclease III resistant band (lanes 6 and 7) indicating that these base substitutions prevent the binding of TIF-IB to the mutant DNAs. At present we cannot explain why pMr-17/-18

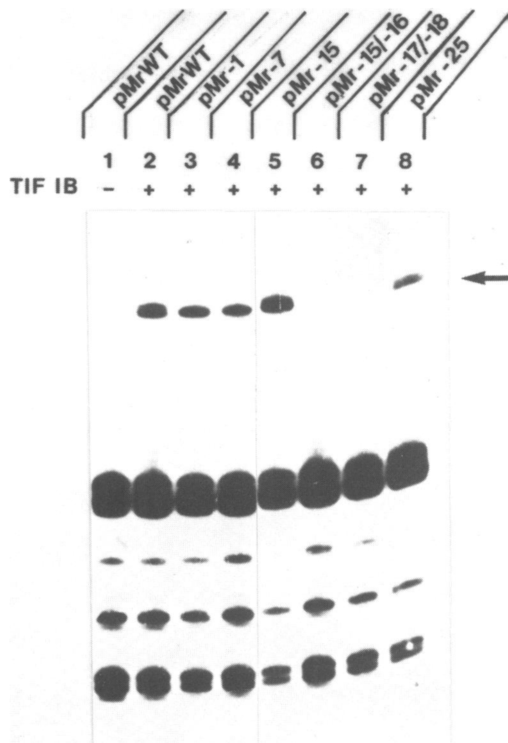

Figure 5: Exonuclease III footprinting. 343 bp Hind III/Eco RI fragments from pMrWT and seven point mutants were 5' labelled at the Eco RI site. 5 ng of the fragments were incubated with 100 ng of a TIF-IB preparation that was purified on DEAE-Sephadex and Heparin Ultrogel. After 10 min at 30°C 5 units of exonuclease III were added and incubation continued for another 5 min. The digestion products were separated on a 6% polyacrylamide urea gel. Lane 1: Wild type fragment incubated in the absence of TIF-IB. Lanes 2-8: Wild type or mutant fragments incubated with TIF-IB before exonuclease III digestion. The arrow indicates the TIF-IB mediated extra stop at position -21. The smaller DNA bands represent half-size DNA fragments generated by exonuclease III digestion in the absence of specifically bound proteins.

which is transcribed at 50% of wild type level is not protected by factor TIF-IB. We assume that the primary factor-mutant DNA complex is very labile and is stabilized by interaction with other proteins present in crude extracts which are required to assemble functional transcription complexes.

Transcriptional Activity of the Point Mutants in vivo

To address the question whether the nucleotides that affect rDNA transcription in vitro are also required for specific transcription in vivo, a transient expression assay was used. 3T6 cells were transfected with re-

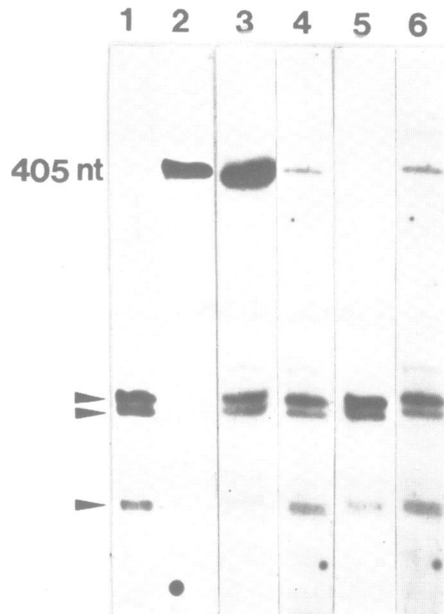

Figure 6: Primer extension analysis of RNA isolated from 3T6 cells transfected with rDNA-CAT fusion genes. Cellular RNA (50 µg) was hybridized to a labelled 102 bp Pvu II/Eco RI fragment derived from the CAT gene, and the hybrids were transcribed by reverse transcriptase. The cDNAs were electrophoresed on a 6% polyacrylamide urea gel. DNA transcripts of RNA from cells transfected with 20 µg pSV2-CAT (lane 1) and pMrCAT (lane 2) are shown. The next lanes show the reverse transcripts from cells that had been co-transfected with 2 µg pSV2-CAT and 20 µg of rDNA-CAT plasmids, pMrCAT (lane 3), pMr-15/-16CAT (lane 4), pMr-7CAT (lane 5), and pMr-1CAT (lane 6). The 405 nt band corresponds to transcripts initiated at the rDNA promoter. The arrows mark SV40-specific transcripts.

combinant plasmids containing mouse rDNA sequences from -169 to +155 fused to the bacterial marker gene coding for chloramphenicol acetyltransferase (CAT). The expression of the hybrid genes was monitored by primer extension analysis of cellular RNA extracted 44 hours after transfection. The activity of the wild-type promoter present in the recombinant plasmid pMrCAT was tested and compared to the activity of the promoter mutants pMr-15/-16CAT, pMr-7CAT, and pMr -1CAT respectively. To quantitate the levels of CAT-specific transcripts, the cells were cotransfected with pSV2-CAT which served as an internal marker for the transfection efficiency. RNA from cells transfected with pSV2-CAT yielded three CAT- specific transcripts that were initiated at the SV40 cap sites (Figure 6, lane 1). The reverse transcripts of RNA initiated at the rDNA promoter are 405 nt long (lane 2). The transcriptional activities of wild-

type *versus* the mutant promoters are shown in lanes 3 to 6. It is evident, that the *in vivo* activities of the rDNA mutants quantitatively compare to their template activities *in vitro.* Exchange of the guanine at position -16 as well as a T to G transversion at -1 reduces the transcription rate 10-20 fold (lanes 4, 6). The mutant pMr-7CAT (lane 5) is incapable of directing RNA synthesis at a detectable level. This result suggests that the factor- DNA interactions occuring *in vitro* actually serve a role in controlling rRNA transcription initiation *in vivo.*

DISCUSSION

Previous analysis of deletion and base substitution mutants revealed that the control signals for RNA polymerase I consist of multiple, complex sequence elements. The upstream control element (UCE) which is located 100-200 bp upstream of the transcription start site appears to modulate the efficiency of promoter function, whereas the start site proximal core promoter element is indispensable for accurate and specific initiation. In this study we have defined in more detail the core promoter element of mouse rDNA by introducing single base substitutions into the region between -1 and -25 relative to the transcription start site and assaying the template activity of these point mutants in a cell-free transcription system. The mutation-sensitive region revealed by these experiments contains the nucleotide sequence CCTATTG which extends from position -22 to -16. Previous exonuclease III protection experiments have indicated that these sequences in the mouse core promoter element are recognized and bound by a partially purified transcription factor TIF-IB (10). This transcription factor is probably responsible for the species-specific rDNA promoter recognition that is intrinsic to pol I transcription (17). We recently obtained preliminary evidence that partially purified TIF-IB is able to reprogram human extracts to transcribe mouse rDNA (C. Schoneberg, unpublished results). Thus the sequence CCTATTG within the core promoter appears to be the target for TIF-IB which confers species-specificity to the mouse pol I transcription machinery. This conclusion is supported by the fact that this sequence is also present at the same position in rat rDNA (which is transcribed in the mouse system) but is not conserved in human rDNA (18). Mutations within this control region weaken or eliminate both TIF-IB binding and promoter activity. A G to A transition at residue -16 impairs template activity and factor binding by more than 90% (10), whereas the exchange of nucleotides -17, -18, -20 and -21 decreases promoter function by 10-60%. This suppression of promoter activity correlates with a reduced affinity of transcription factor(s) to the rDNA template. Thus the effect of

mutations within the binding region becomes more pronounced at decreasing DNA concentrations (data not shown). Similar observations have been made when the template activity of 5' deletion mutants of rDNA have been assayed in either a cell-free system (3) or after microinjection into oocyte nuclei (19). The transcription capacity of these mutants have been shown to strongly depend on the transcription conditions and DNA concentration. These results suggest a complex association of several factors with different domains of the rDNA promoter which function cooperatively to activate transcription initiation.
Our data are in accord with this hypothesis. The substitution of a guanine at position -7 by an adenine (pMr-7) eliminates transcription completely, a T to G transversion at -1 impairs transcription by more than 90%. This suppression of promoter activity was not only observed in vitro but also in vivo after transfection of rDNA-CAT gene fusions into 3T6 cells. Both mutations at -7 and -1 reside outside of the TIF-IB binding site and apparently do not affect the primary binding of TIF-IB to the rDNA promoter. They are able to compete to some degree for transcription factors in template committment experiments but fail to assemble functional initiation complexes. We suggest that the start site proximal part of the core promoter serves a different function than the distal region. After binding of TIF-IB to the distal region, factor TIF-IA, RNA polymerase I or another yet unidentified protein interacts with the proximal sequence element. These cooperative interactions are probably mediated by protein- protein contacts. Thus, the association of at least two factors with the minimal core promoter (extending from -39 to -1) imparts promoter specificity to polymerase I and directs accurate initiation. Miller et al. (3) have reported that deletion of downstream sequences up to position +2 results in incorrect initiations. This conclusion was based on the apparent length of runoff transcripts and not on 5' end mapping. Our results clearly demonstrate that sequences from -39 to -1 suffice to promote transcripts that start one nucleotide beyond the thymine at position -1. To achieve efficient rDNA transcription, both sequences downstream of the transcription start site and upstream control elements are required. In human rDNA the upstream control element has been shown to serve as a target for binding cellular trans-activating proteins (20). One of these factors SL1, has been extensively purified and shown to be a species-specific protein required to reconstitute in vitro RNA synthesis (21). Thus the different sequence elements in the rRNA promoter, which exhibit distinct structural and functional characteristics, appear to operate in concert with one another to direct accurate and efficient transcription. Understanding of the role of these elements in promoter func-

tion will require the isolation of the trans-acting factors and the identification of the protein-DNA and protein-protein interactions involved in rRNA transcription initiation.

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