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A purified transcription factor (TIF-IB) binds to essential sequences of the mouse rDNA promoter

(cell-free transcription systems/RNA polymerase I/transcription factors/DNA–protein interactions)

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ABSTRACT **A transcription factor that is specific for mouse rDNA has been partially purified from Ehrlich ascites cells. This factor [designated transcription initiation factor (TIF)-IB] is required for accurate *in vitro* synthesis of mouse rRNA in addition to RNA polymerase I and another regulatory factor, TIF-IA. TIF-IB activity is present in extracts both from growing and nongrowing cells in comparable amounts. Prebinding competition experiments with wild-type and mutant templates suggest that TIF-IB interacts with the core control element of the rDNA promoter, which is located immediately upstream of the initiation site. The specific binding of TIF-IB to the RNA polymerase I promoter is demonstrated by exonuclease III protection experiments. The 3′ border of the sequences protected by TIF-IB is shown to be on the coding strand at position −21 and on the noncoding strand at position −7. The results suggest that direct binding of TIF-IB to sequences in the core promoter element is the mechanism by which this factor imparts promoter selectivity to RNA polymerase I.**

Genes transcribed by RNA polymerases II and III have conserved promoter sequences, which are required for faithful transcription (1). This sequence conservation allows class II and III RNA polymerase genes to be transcribed in a wide variety of heterologous *in vitro* systems (2–6). Apparently, a set of general transcription factors, present in the crude extracts, recognize and interact with some conserved promoter elements common to most polymerase II or polymerase III genes. Three factors have been partially purified that are required for accurate transcription of class III genes (7). These factors interact in sequential order with the internal control region of the genes leading to the assembly of stable transcription complexes (8–13). In the case of mRNA coding genes, current studies suggest that the rate of initiation is specified by three types of sequence elements: the TATA-box related sequences, the upstream sequence elements, and the enhancer elements. Each of these structural elements is probably recognized by specific DNA-binding proteins, and additional factors may be necessary for accurate initiation by RNA polymerase II.

The sequences that constitute the RNA polymerase I promoter region have been mapped in several laboratories (14–19). Experimental evidence suggests that the rDNA promoter consists of three sequence domains with distinct functions. One of these functional regions appears to sequester transcription initiation factor(s) (TIF), resulting in the formation of stable preinitiation complexes (19, 20). The proteins that interact with the rDNA promoter are not yet known. We have previously shown that rRNA synthesis is species specific both *in vitro* (21) and *in vivo* (22). This species specificity seems to reside in one of the factors required for initiation (23). Miesfeld and Arnheim (24) showed that one fraction, eluting from phosphocellulose at a high salt concentration, forms a stable preinitiation complex with fragments containing rDNA promoter sequences from homologous, but not from heterologous, species. The accumulating evidence thus strongly suggests that promoter sequences identified by deletion mapping and other mutations are indeed binding sites for proteins.

In this study, we have purified a protein that is responsible for the formation of stable preinitiation complexes and imparts promoter recognition to mouse RNA polymerase I. The highly enriched TIF-IB preparations contain a DNA-binding activity that protects an essential RNA polymerase I promoter domain against exonuclease III digestion.

MATERIALS AND METHODS

DNA Templates. The plasmid pMr600 has been described (20). It contains an approximately 600-base-pair (bp) *Pvu* II fragment from the mouse rDNA initiation region. For *in vitro* transcription, it was cleaved with either *Eco*RI or *Sma* I to yield runoff transcripts of 297 or 155 nucleotides, respectively. pMrWT contains a 322-bp *Sal* I/*Sma* I fragment from the rDNA initiation region (from −167 to +155) cloned into pUC9 (25). The point mutants pMr−15/−25 and pMr−16/−25 are derivatives of pMrWT. They contain two G→A transitions at positions −15 and −25 or at positions −16 and −25, respectively. The transcriptional activity of pMr−15/−25 has been shown to compare to that of the wild type, whereas the template activity of pMr−16/−25 is reduced by 90–95% (25). pMrΔ−39 is a 5′ deletion mutant that extends from −39 to +292 (14).

***In Vitro* Transcription.** S100 extracts were prepared according to Weil *et al.* (2), and nuclear extracts were prepared according to Dignam *et al.* (27) from cultured Ehrlich ascites tumor cells. The extracts were extensively dialyzed against buffer A (20 mM Hepes, pH 7.9/5 mM $MgCl_2$/20% (vol/vol) glycerol/0.2 mM EDTA/1 mM dithioerythreitol) containing 100 mM KCl (A-100). The standard transcription reaction was in a 25-μl final volume containing 12 mM Hepes (pH 7.9); 0.12 mM EDTA; 0.5 mM dithioerythreitol; 10% (vol/vol) glycerol; 5 mM $MgCl_2$; 85 mM KCl; 0.6 mM each ATP, UTP, CTP; 12.5 μM GTP and 2 μCi of [α-^{32}P]GTP (1 Ci = 37 GBq); 20–100 ng of linearized plasmid DNA; and 3–15 μl of column fractions. After incubation for 45 min at 30°C, the RNA was isolated and analyzed by electrophoresis on 4% polyacrylamide gels.

Competition Prebinding Assay. The prebinding assay for the identification of components involved in transcription complex formation has been described (20). When purified

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Abbreviations: TIF, transcription initiation factor; bp, base pair(s).
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fractions were assayed, the reaction volume was 25 μl and the amount of DNA required to bind 3 μl of a TIF-IB preparation was determined before each experiment. Usually, 10–20 ng of the first template was incubated for 10 min at 30°C with 3 μl of column fractions under standard transcription conditions in the absence of nucleotide triphosphates. After addition of the second template (30–50 ng) and other column fractions, the reaction was started by the addition of nucleoside triphosphates.

Purification of TIF-IB Activity. A mixture of S100 and nuclear extracts derived from cultured cells (250 ml) was applied onto a 500-ml DEAE-Sephadex column equilibrated with buffer A-100. The column was washed with 2 vol of A-100 and the active fractions were eluted with 200 mM KCl. Fractions from the DE200 step were applied directly to a heparin Ultrogel column equilibrated with buffer A-250. The column was washed with 2 vol of A-250 and bound protein was eluted with three salt steps containing 400, 600, and 1000 mM KCl. The fractions eluting at 600 mM KCl from heparin Ultrogel (H-600) were pooled, diluted 1:2 with buffer A, and chromatographed on Blue Sepharose. The bound proteins were step-eluted with buffer A containing 600 and 1000 mM KCl. The BS-1000 fraction that contained the TIF-IB activity was subjected to chromatography on Bio-Rex 70. After elution with 600 and 1000 mM KCl, TIF-IB activity was found in the BR-1000 fraction. At this stage, the factor has been purified ≈100,000-fold. The purification scheme of TIF-IB is shown in Fig. 1*A*.

Assay for TIF-IB Activity. The 400 mM KCl eluate from the heparin Ultrogel column (H-400) contained RNA polymerase I and TIF-IA. The H-400 pool was dialyzed against buffer A with 100 mM KCl and was frozen in aliquots at −80°C to be used as the source of polymerase I and TIF-IA in the reconstitution assays. For identification of TIF-IB activity, individual fractions were tested for their ability to restore promoter-specific transcription of the H-400 fraction.

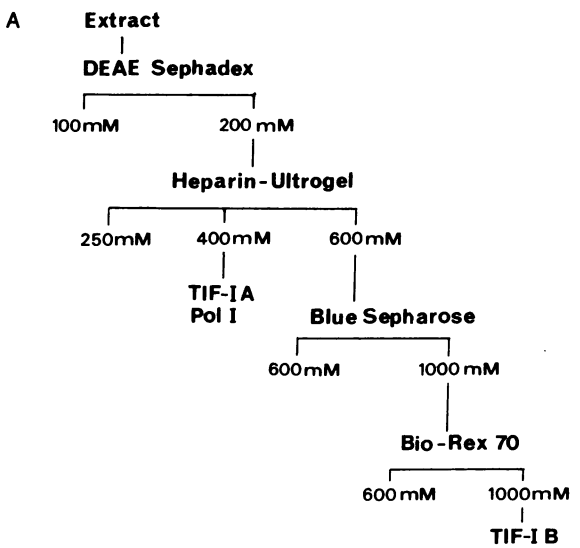
Exonuclease III Footprinting Reactions. The 5′-labeled restriction fragments (3–5 ng) were incubated with or without protein fractions in a buffer containing 100 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 10% glycerol, and 12 mM Hepes (pH 8.0) in a total vol of 25 μl. After 10 min, 5 units of exonuclease III (Boehringer Mannheim) was added and incubation was carried on for another 5 min. The reaction was stopped with 25 μl of stop buffer (400 mM NH_4 acetate, pH 5.5/0.4% sodium dodecylphosphate/yeast tRNA (0.2 mg/ml)/1 mM EDTA). After phenol extraction and ethanol precipitation, the probes were separated on a 6% polyacrylamide urea gel and autoradiographed.

RESULTS

Fractionation of RNA Polymerase I Transcription Factors. Recently, we have shown that after chromatography of transcriptionally active extracts on sizing columns and DEAE-Sephadex, the fractions that contained the RNA polymerase I were able to specifically initiate transcription on cloned mouse rDNA (32). This finding indicates that at early steps of purification the transcription initiation factors may be associated with RNA polymerase I. When the active fractions were further purified by chromatography on heparin Ultrogel, the RNA polymerase I activity was eluted from the column by a 200 to 400 mM KCl salt step (H-400). These fractions contain, in addition to RNA polymerase I, an essential initiation factor, TIF-IA, which is present or active only in extracts derived from rapidly growing cells. This H-400 alone was unable to accurately initiate RNA synthesis from the rDNA promoter (Fig. 1*B*, lane 1). The fractions eluting at 600 mM KCl did not contain measurable amounts of RNA polymerase I and did not direct any specific transcription (lane 2). The ability of RNA polymerase I to initiate transcription at the correct site was restored by mixing the H-400 and the H-600 fraction (lane 3). This suggests that the H-600 fraction contains factor(s) that confers promoter recognition to RNA polymerase I. This factor is designated TIF-IB. For further purification of TIF-IB, the H-600 fractions were subjected to chromatography on Blue Sepharose and Bio-Rex 70 as shown in Fig. 1*A*. The individual fractions were assayed for their ability to reconstitute promoter-specific transcription by the H-400 fraction. At both columns, the factor activity eluted at a high salt concentration, which resulted in highly purified TIF-IB preparations.

TIF-IB Forms Stable Preinitiation Complexes on Mouse rDNA. Because TIF-IB was bound to heparin Ultrogel and has been found to be required for promoter recognition by RNA polymerase I, we assumed that this factor may be a sequence-specific DNA-binding protein. We therefore tested the H-600 fraction for factors with specific DNA-binding activity, using the competition prebinding assay. In this assay, one template DNA is preincubated with transcriptional components prior to the addition of a second DNA. During the preincubation period, the first DNA template sequesters all the available TIF into a preinitiation complex. If a second DNA is subsequently added, it is unable to form a transcriptionally active complex, and addition of nucleoside triphosphates results in transcription of only the first DNA (20).

As shown in Fig. 2*A*, the RNA polymerase I- and TIF-IA-containing H-400 fraction alone randomly transcribed the



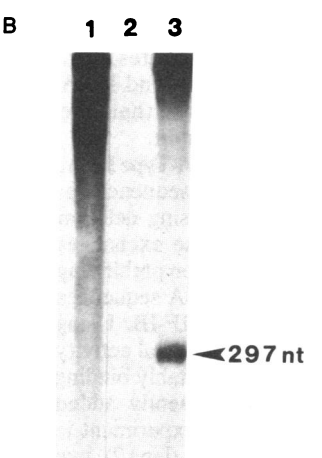


FIG. 1. Fractionation of polymerase I transcription factors. (*A*) Scheme for fractionation of nuclear extracts from Ehrlich ascites cells. (*B*) Transcription activity of heparin Ultrogel fractions. Template DNA (30 ng of pMr600/*Eco*RI) was incubated in a 25-μl standard transcription assay containing 3 μl of H-400 fraction (lane 1), 3 μl of H-600 fraction (lane 2), or 3 μl of each fraction (lane 3). nt, Nucleotides.

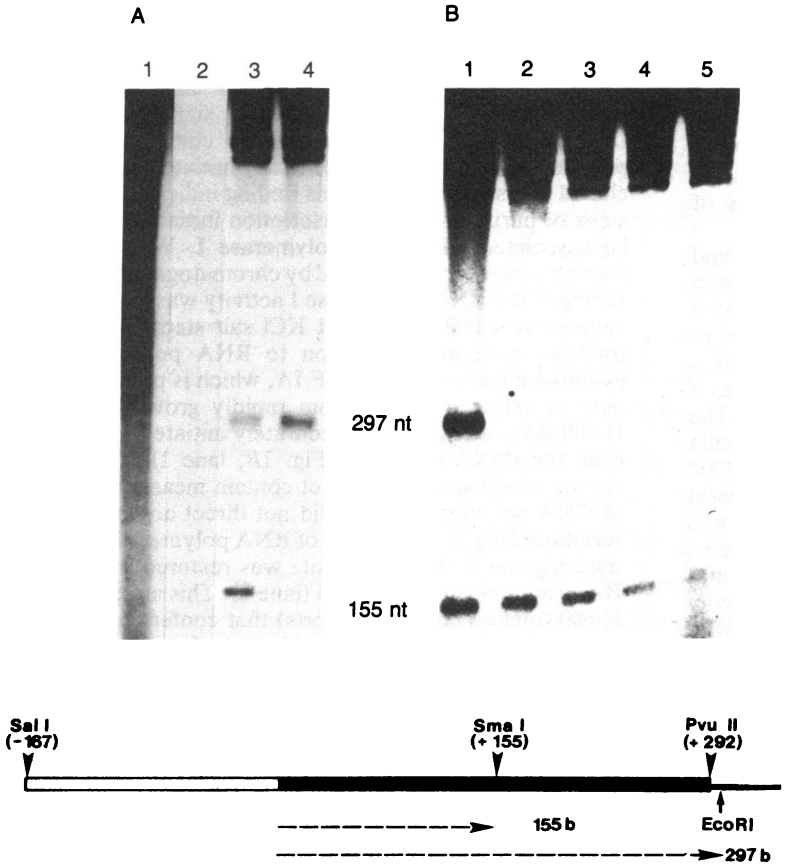


FIG. 2. Formation and stability of preinitiation complexes assembled by the H-600 fraction. The autoradiograms show RNA products transcribed from pMr600/*Eco*RI [297 nucleotides (nt)] or from pMr600/*Sma* I (155 nt). For complex formation, the first DNA was incubated for 10 min at 30°C with the heparin Ultrogel fractions eluting at 400 or 600 mM KCl (H-400 and H-600) before transcription was started by addition of the second DNA, the nucleoside triphosphates, and further fractions. (*A*) Complex formation by the H-600 fraction. The first template in the preincubation period was 20 ng of pMr600/*Eco*RI, the second DNA was 40 ng of pMr600/*Sma* I. Lanes: 1, reaction mixture contained 3 μl of the H-400 fraction; 2, reaction mixture contained 3 μl of the H-600 fraction; 3, the preincubation was performed with 3 μl of H-400 before the transcription was started by NTPs and 3 μl of H-600; 4, the preincubation was performed in the presence of 3 μl of H-600, and the transcription was started by NTPs and 3 μl of H-400. (*B*) Stability of complex formation. Fraction H-600 (3 μl) was preincubated with 20 ng of pMr600/*Sma* I for 10 min. Then, 20 ng of pMr600/*Eco*RI was added, and the reaction was incubated at 30°C for 0 min (lane 2), 10 min (lane 3), 20 min (lane 4), and 30 min (lane 5). Lane 1, both templates were present in the preincubation period.

template DNA in the absence of the H-600 fraction (lane 1). The H-600 fraction was incapable of producing detectable amounts of runoff transcripts in the absence of H-400 (lane 2). Preincubation of 20 ng of pMr600/*Eco*RI with the H-400 fraction and subsequent addition of 40 ng of the second DNA pMr600/*Sma* I and the H-600 fraction resulted in transcription of both templates according to their molar ratios in the assay (lane 3). However, when pMr600/*Eco*RI was preincubated with the H-600 before addition of H-400, the second DNA, and the nucleotides, only transcription of the first template was observed (lane 4). We therefore conclude that the active component in the H-600 fraction, termed TIF-IB, forms a preinitiation complex with the template.

To estimate the stability of complex formation, the H-600 fraction was preincubated with 20 ng of pMr600/*Sma* I. It was then challenged with the second DNA (pMr600/*Eco*RI) for different periods of time before starting the transcription by addition of H-400 and the nucleoside triphosphates. Fig. 2*B* shows that there is no transcription of the second DNA once the complex is formed. This indicates that the DNA–TIF-IB complex is stable for at least 30 min.

Competition Between Mutant Templates and Wild-Type DNA. Previously we and others have determined the sequence requirements for mouse rDNA transcription by using deletion mutants (14, 18) or DNAs containing single base exchanges within the promoter region (25, 26). The competition prebinding assay should allow the identification of those DNA sequences in the rDNA promoter that are responsive to TIF-IB. Using mutant DNAs as the first template, the transcriptional activity of which is altered, only those DNAs capable of stably binding TIF-IB will prevent transcription of a subsequently added wild-type DNA. Fig. 3 shows the results of an experiment in which pUC9 (lane 1), the wild-type pMr600/*Sma* I (lane 2), two point mutants pMr−16/−25 (lane 3), pMr−15/−25 (lane 4), and a 5′ deletion mutant pMrΔ−39 (lane 5) were preincubated with H-600 and then challenged with pMr600/*Eco*RI. There is a clear correlation between the transcriptional activity of the templates and their ability to bind TIF-IB. The mutants pMr−15/−25 and pMrΔ−39, the template activity of which is comparable under standard conditions to the wild-type DNA, stably sequester TIF-IB. Those DNAs that are transcriptionally inactive (pUC9 and pMr−16/−25) do not form complexes and thus permit

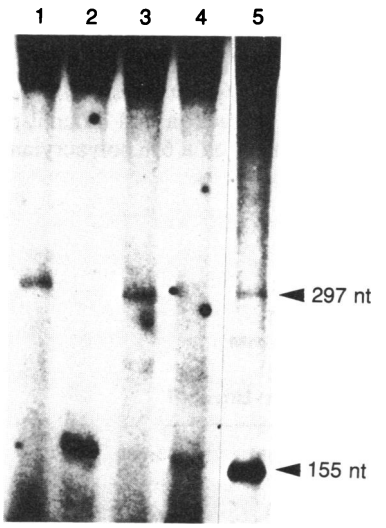


FIG. 3. Prebinding competition between mutant templates and wild-type DNA. Fraction H-600 (3 μl) was preincubated with 20 ng of the following DNAs. Lane 1, pUC9; lane 2, pMrWT; lane 3, pMr−16/−25; lane 4, pMr−15/−25; lane 5, pMrΔ−39. All these DNAs were linearized with *Sma* I. After 10 min at 30°C, 20 ng of pMr600/*Eco*RI, 3 μl of H-400, and the NTPs were added. The 155-nucleotide (nt) RNAs are transcribed from the first DNA, and the 297-nt RNA is transcribed from the competing template pMr600/*Eco*RI.

transcription of the second DNA. This finding supports our previous conclusion that the evolutionarily conserved guanine at position −16 is involved in complex formation and suggests that TIF-IB is the protein that interacts with the region containing this essential nucleotide.

Binding of TIF-IB to the rDNA Promoter. To identify the sequences that are necessary for TIF-IB binding, footprinting experiments were carried out. As the concentration of factor(s) was not sufficient to obtain quantitative binding of rDNA fragments necessary for DNase I protection experiments, we used the more sensitive exonuclease III assay. A double-stranded DNA probe containing the rDNA promoter region extending from position −167 to +155 was 5′-labeled either at the coding or noncoding strand and incubated with increasing amounts of TIF-IB. After digestion with exonuclease III, the DNA fragments were separated on a 6% polyacrylamide sequencing gel along with a guanine-specific chemical cleavage of the same DNA. As shown in Fig. 4, in the absence of protein, exonuclease III produces single-stranded 5′-labeled DNA fragments of about half the original size (lanes 1 and 5). In the presence of protein, additional bands are observed that represent stops in the exonuclease III digestion because of TIF-IB binding. Comparison of the protected fragments with the Maxam–Gilbert sequencing ladder allows us to map the 3′ border of the TIF-IB binding site on both DNA strands. On the noncoding strand, the 3′ limit of exonuclease III digestion is at position −7 relative to the transcription initiation site (lane 3). On the coding strand, the 3′ border is at position −21 (lanes 6 and 7). Thus the sequences involved in TIF-IB binding appear to be coincident with the sequences of the core promoter element identified by deletion mapping. Interestingly, the amount of TIF-IB required to get a signal in the footprinting experiments was much lower on the coding than on the noncoding strand, a finding that suggests some strand specificity of binding. Furthermore, there is another stop signal on the coding strand at position −13, which shows up only at higher protein concentrations. This band at −13 was also observed when the mutant pMr−16/−25 was analyzed in the footprint assay (Fig. 4). In this experiment, two point mutants with two base exchanges at positions −15 and −25 or at −16 and −25, respectively, were tested for their capability to interact with TIF-IB. pMr−15/−25, the transcriptional activity of which compared to the wild-type DNA, also exhibited the same protection pattern as the wild-type pMrWT. However, pMr−16/−25, the template activity of which was reduced by a factor of 10–20, as compared to the wild-type, did not yield the signal at position −21. Instead, it showed protection at position −13, the band that is observed in the wild-type only at higher amounts of TIF-IB. These data could mean that two different sequence motifs are required for productive TIF-IB binding, but they could also indicate that the used TIF-IB preparation (Blue Sepharose fraction) contains two distinct proteins, both of which are required to interact with the core promoter element to form a functional preinitiation complex. At present, we cannot exclude either possibility. The final proof that the footprinting and the transcriptional activities reside in the same factor will require an unequivocal demonstration of coelution of both activities on several columns by salt gradients.

DISCUSSION

We have partially purified two chromatographically distinct components (designated TIF-IA and TIF-IB) that are required for RNA polymerase I to accurately initiate rRNA synthesis *in vitro*. Previous reports have indicated that multiple components are necessary to reconstitute correct transcription of the mouse ribosomal genes. Mishima *et al.* (23) have fractionated extracts on phosphocellulose into fractions A, B, C, and D. Both fractions C and D are required

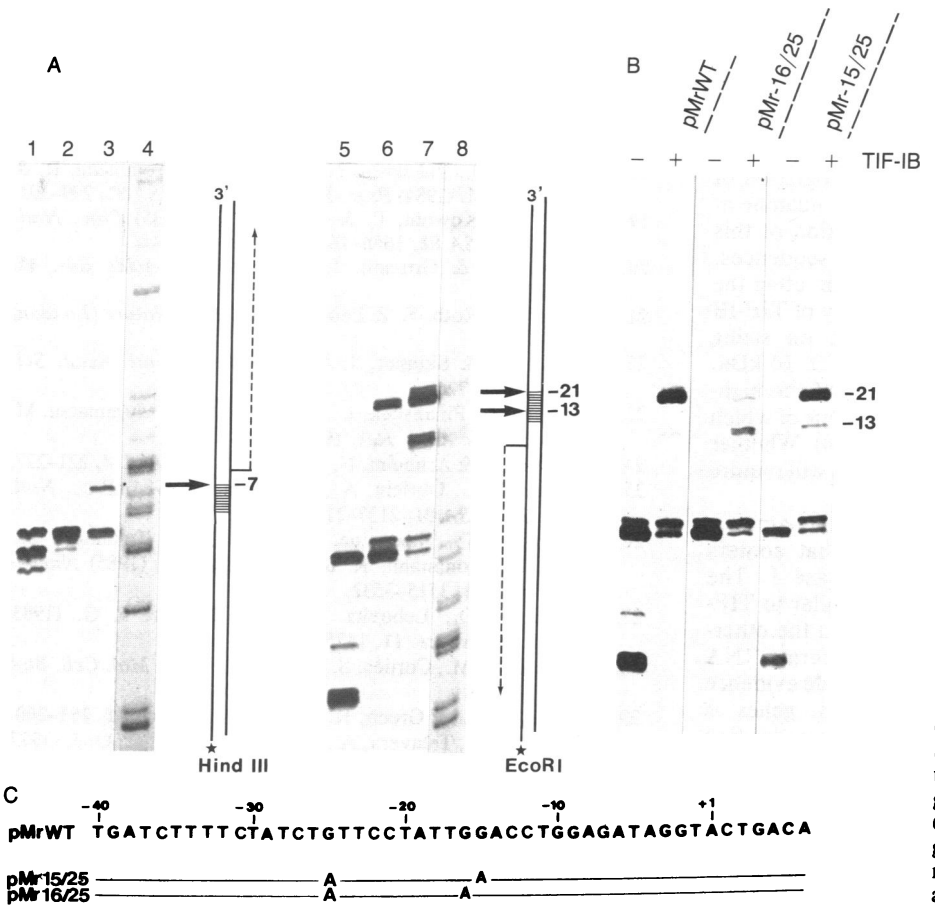


FIG. 4. (*A*) Exonuclease III footprinting. A 343-bp *Hin*dIII/*Eco*RI fragment (3 ng) from pMrWT containing rDNA sequences from positions −167 to +155 bp relative to the transcription start site were 5′-labeled either at the *Hin*dIII site, which labels the noncoding strand (lanes 1–4), or the *Eco*RI site, which labels the coding strand (lanes 5–8). The fragments were incubated with various amounts of TIF-IB fraction that was purified on DEAE-Sephadex, heparin Ultrogel, and blue Sepharose. Lanes: 1 and 5, no protein; 2 and 6, 50 ng; 3 and 7, 100 ng of protein. After 10 min, 5 units of exonuclease III was added and incubated for another 5 min. The products were separated on a 6% polyacrylamide urea gel. Lanes 4 and 8 are Maxam and Gilbert G-specific chemical cleavages. Arrow indicates direction of transcription. (*B*) Exonuclease III footprinting with two point mutants. The *Eco*RI/*Hin*dIII fragment (3 ng) from pMrWT, pMr−16/−25, and pMr−15/−25 was 5′-labeled at the *Eco*RI site and incubated in the absence (−) and (+) presence of 50 ng of TIF-IB (Blue Sepharose fraction) and digested with 5 units of exonuclease III. The resulting single-stranded fragments were separated on a 6% polyacrylamide urea gel and autoradiographed. (*C*) Nucleotide sequence of the mouse rDNA promoter region (pMrWT) and of two point mutants used.

for faithful transcription initiation. Fraction C contains most of the RNA polymerase I, and fraction D has some degree of species dependence. Apparently, the functional component in fraction D is analogous or identical to TIF-IB. We have extensively purified TIF-IB through four chromatographic steps. The purified factor imparts promoter selectivity to polymerase I, which by its own is incapable of specific initiation.

Previously, we and others have reported that a prerequisite for specific transcription of rDNA is the association of the template with one or more proteins to form stable preinitiation complexes (19, 20, 24). For preinitiation complexes to form, the presence of functional RNA polymerase I is not required (19, 24). The experiments in this paper suggest that TIF-IB is the protein that forms a stable complex with the rDNA template. Preincubation of TIF-IB with the rDNA before addition of a second template and partially purified RNA polymerase I and TIF-IA resulted in the exclusive transcription of the first template. This association of TIF-IB with the rDNA promoter is stable. Once the complex has formed, no exchange of factor molecules between different DNA molecules occurs (Fig. 2*B*).

The binding of TIF-IB to rDNA involves nucleotide sequences 5′ proximal to the initiation site, which have been shown to be absolutely required for transcription initiation (14). In template commitment experiments, a transcriptionally inactive point mutant pMr−16/−25, which contains the transition of a functionally important guanine at position −16 into adenine, did not compete for TIF-IB. The same result was obtained with mutant pMrΔ−14/35, in which sequences between −14 and −35 were deleted and substituted by foreign DNA sequences (data not shown). This finding indicates that the core promoter region immediately upstream of the start site serves as the major target for the action of TIF-IB.

We could demonstrate by exonuclease III footprinting experiments a direct interaction of the highly purified TIF-IB preparations with specific promoter sequences. The 3′ boundary of the protected region has been estimated to be at the coding strand at position −21 and at the noncoding strand at position −7. The inactive point mutant pMr−16/−25 did not show this protection of the coding strand at −21, indicating that the transcriptional incompetence of this template is attributable to an impaired promoter recognition of TIF-IB. Moreover, this result implicates that the guanine at position −16 is vital for the productive interaction of this specific DNA-binding protein with promoter sequences, which in turn appears to be a prerequisite for directing the polymerase I to the correct start site. The identity of TIF-IB is still not known. Fractionation experiments on sizing columns indicate a native molecular mass of 80 ± 10 kDa. $NaDodSO_4$/polyacrylamide gel electrophoresis of the highest purified BR-1000 fraction yields a few bands, one of which corresponds to an 80-kDa protein (data not shown). Whether or not TIF-IB is identical with this 80-kDa protein will require further purification.

Recently, Learned *et al.* (28) have purified and characterized a transcription factor, designated SL1, that confers promoter specificity to human RNA polymerase I. The chromatographic properties of SL1 are very similar to TIF-IB. The authors showed that SL1 can reprogram the otherwise nonpermissive mouse system to initiate accurate RNA synthesis from human rDNA. These results provide evidence that species-specific transcription of ribosomal genes is attributable to defined polymerase I-specific transcription factor(s) that seem to have coevolved with changes in essential rDNA promoter sequences. Probably the phenomenon of nucleolar dominance in somatic cell hybrids (29, 30) may involve loss or suppression of the gene(s) encoding these factor(s) (31). Whether or not TIF-IB and the human factor SL1 are functionally equivalent cannot be decided yet. There is one fundamental functional difference in the properties of the mouse factor TIF-IB as compared to the human factor SL1. Learned *et al.* (28) have been unable to detect any sequence-specific binding of highly purified and active SL1 to the human rDNA promoter, whereas TIF-IB, which has been purified to a similar degree, clearly interacts with specific mouse rDNA promoter sequences. Whether this discrepancy results from the different techniques applied to demonstrate sequence-specific binding or reflects a real difference in the mechanism of action of both factors remains to be elucidated.

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