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Isolation and functional characterization of TIF-IB, a factor that confers promoter specificity to mouse RNA polymerase I

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ABSTRACT

The murine ribosomal gene promoter contains two *cis*-acting control elements which operate in concert to promote efficient and accurate transcription initiation by RNA polymerase I. The start site proximal core element which is indispensable for promoter recognition by RNA polymerase I (pol I) encompasses sequences from position −39 to −1. An upstream control element (UCE) which is located between nucleotides −142 and −112 stimulates the efficiency of transcription initiation both *in vivo* and *in vitro*. Here we report the isolation and functional characterization of a specific rDNA binding protein, the transcription initiation factor TIF-IB, which specifically interacts with the core region of the mouse ribosomal RNA gene promoter. Highly purified TIF-IB complements transcriptional activity in the presence of two other essential initiation factors TIF-IA and TIF-IC. We demonstrate that the binding efficiency of purified TIF-IB to the core promoter is strongly enhanced by the presence in *cis* of the UCE. This positive effect of upstream sequences on TIF-IB binding is observed throughout the purification procedure suggesting that the synergistic action of the two distant promoter elements is not mediated by a protein different from TIF-IB. Increasing the distance between both control elements still facilitates stable factor binding but eliminates transcriptional activation. The results demonstrate that TIF-IB binding to the rDNA promoter is an essential early step in the assembly of a functional transcription initiation complex. The subsequent interaction of TIF-IB with other auxiliary transcription initiation factors, however, requires the correct spacing between the UCE and the core promoter element.

INTRODUCTION

The promoters of class I, II and III genes have been shown to consist of several distinct sequence elements which interact with multiple factors thus facilitating the accurate and regulated transcription initiation by the respective RNA polymerases. At least one of the transcription factors characteristic for a given class of genes acts through stable binding to an essential promoter domain. This specific binding of a defined protein to the essential promoter domain is the first step in the assembly of transcription initiation complexes (1−3).

In an attempt to functionally characterize the proteins which play a role in promoter recognition and control of rRNA synthesis we have studied the protein-DNA interactions between cellular transcription factors and sequences of the mouse ribosomal gene promoter. We have identified in Ehrlich ascites cells a cellular factor (termed TIF-IB), which forms stable preinitiation complexes on the mouse rDNA promoter by binding to sequences in front of the gene (4). TIF-IB protects sequences from nucleotide −21 on the coding and −7 at the noncoding strand against exonuclease III digestion, a region that has been shown to play an essential role in initiation of pre-rRNA synthesis both *in vitro* and *in vivo*. Point mutations within the TIF-IB binding site impair or abolish factor binding and similarly affect rDNA transcription (5, 6).

In addition to the start site proximal core promoter domain (extending from nucleotides −39 to −1) an upstream control element (UCE) located upstream of nucleotide −110 has been shown to greatly enhance the efficiency of initiation. The stimulatory effect of the UCE is most convincingly demonstrated *in vivo* after transfection of rDNA deletion or linker scanning mutants into cultured cells. Lesions within the upstream region result in a drastic decrease of transcriptional activity (7−10). *In vitro* the effect of upstream sequences on rRNA synthesis is much less severe (11−13). The molecular mechanism by which the upstream sequences exert an effect on the initiation frequency is not well understood. Template commitment and competition experiments revealed that the upstream region affects the ability of the template to form stable transcription initiation complexes (11, 14). Since the first step in the formation of transcription initiation complexes is the binding of factor TIF-IB to its target sequence, this finding suggests that the stimulatory effect of the UCE may be brought about by stabilisation of the primary binding of factor TIF-IB to the core promoter. This stabilizing effect could

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be achieved by additional proteins interacting with the upstream control region thus facilitating the binding of TIF-IB to the core promoter element. Alternatively, TIF-IB could require both regions for stable binding.

To distinguish between these possibilities and to elucidate the molecular mechanisms which mediate the accurate and efficient transcription initiation by RNA polymerase I, we have purified TIF-IB and studied its interaction with the rDNA promoter. We show that binding of highly purified factor TIF-IB to the core promoter is stimulated by the UCE located between nucleotides −142 and −112. Our results are consistent with a model which postulates either an interaction of TIF-IB with both control elements or an influence of the UCE on the stability of the TIF-IB−core promoter complex.

MATERIALS AND METHODS

Plasmid Constructions

All wild-type constructs have been described before. The pBR322 derivative pMrSP contains the 5'terminal Sal I/Pvu II fragment extending from nucleotides −168 to +292 with respect to the transcription initiation site (11). The vector of all other constructs was pUC9. pMr600 contains a 600 bp Pvu II fragment covering the region from −312 to +292 (3) and pMrWT contains a 323 bp Sal I/Sma I fragment from position −168 to +155 (5).

To construct the linker-scanning mutant pMrLS109-75 the 68 bp Hind III/Hae III fragment (encompassing rDNA sequences from −170 to −110) from pMrWT and a synthetic 40 bp oligonucleotide containing a unique Bam HI restriction site were inserted into the deletion mutant pMrΔ-74 cleaved with Hind III and Sal I. This recombinant plasmid, which contained 38 instead of the natural 35 bases between nucleotides −109 and −75 was used to construct the insertion mutants pMr-i39, pMr-i75, and pMr-i108. For this, pMrLS109-75 was linearized with Bam HI, and one or two copies of a 36 bp or 105 bp Sau 3A fragment, respectively, from pUC9 were inserted. pMr-i10 contains the Eco RI/Hind III fragment from $pMrT_o240$ (from −240 to −113, (9)) fused to the Hind III/Eco RI fragment of pM Δ-74, thus increasing the distance between the UCE and the core by 10 nucleotides. pMr-d27 is a deletion mutant in which the distance between the core and the UCE has been reduced by 27 nucleotides. This was achieved by inserting the Hind III/Hae III fragment from pMrWT (covering rDNA sequences from −170 to −112) into pMrΔ-74 cut with Hind III and Hind II, thus replacing the 37 bases from position −75 to −111 by 11 bp of polylinker sequences. In pMrLS72-40 rDNA sequences from nucleotides −72 to −40 were substituted by a synthetic oligonucleotide. In pMrLS112-40 rDNA sequences from positions −112 to −40 were replaced by foreign DNA sequences. pMrLS35-14 contains rDNA sequences from −170 to +155 with a deletion of 22 bases between nucleotides −35 and −14. Between these two positions lies a 14 bp insert of pUC9 sequences (5). pMrLS143-131 and pMrLS130-115 were construced by inserting into the rDNA synthetic oligonucleotides encompassing foreign sequences from nucleotides −143 to −131 and from −130 to −115, respectively, between the Ava II site at position −144 and the Hae III site at position −112.

In vitro Transcription Assays

The cultivation of Ehrlich ascites cells, the preparation of S-100 and nuclear extracts (15, 16), the cell-free transcription system and the analysis of the RNA synthesized have been described elsewhere (4, 11). Transcription complex formation was tested in the prebinding assay (3). Usually 250 ng of pMrWT or mutant templates truncated with Sma I were preincubated with 30 μl of a mixture of S-100 and nuclear extracts in a total volume of 48 μl containing 12 mM HEPES (pH 7.9), 0.1 mM EDTA, 0.5 mM DTE, 5 mM $MgCl_2$, 75 mM KCl, 10 mM creatine phosphate and 12% glycerol (v/v). After 15 min at 30°C 200 ng of the second template pMrSP/Pvu II were added together with the NTPs (0.66 mM each of ATP, CTP, and UTP, 0.01 mM GTP and 1.5 μCi of (α-^{32}P)GTP. Incubation was carried on for another 45 min at 30°C. The RNA was extracted, precipitated and analyzed on 5% polyacrylamide gels.

Exonuclease III Protection Assay

DNA probes were generated by 5' end-labelling the respective recombinant plasmids at the Eco RI site and releasing the fragment by cleavage with Hind III. Each reaction contained approximately 1 ng of the labelled fragment, 50−200 ng of pUC9 DNA and varying amounts of factor TIF-IB. After incubation in binding buffer (12 mM Tris-HCl, pH 8.0; 0.1 mM EDTA; 0.5 mM DTE; 5 mM $MgCl_2$; 70 mM KCl; and 0.1 mM ATP) for 30 min at 30°C, 10 units of exonuclease III (Boehringer Mannheim) were added and the mixture was incubated for a further 5 min. 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 ethanol precipitation the DNA was analyzed on a 6% sequencing gel along with size markers (pBR322/Hpa II).

Purification of Factor TIF-IB

All chromatographic steps were performed in buffer A (20 mM Tris-HCl pH 7.9, 5 mM $MgCl_2$, 0.1 mM EDTA, 20% glycerol) containing the indicated concentration of KCl. 0.5 mM DTE and 0.5 mM PMSF was added immediately prior use. A mixture of S-100 and nuclear extracts (100 ml, 1.8 g of total protein) was applied to a 90 ml DEAE-Sepharose CL-6B column. After washing with buffer A/100 mM KCl, the bulk of RNA polymerase I activity eluted at 280 mM KCl together with the transcription factors TIF-IA, TIF-IB and TIF-IC (see Figure 3). After dialysis this fraction (20 ml containing 160 mg of protein) was loaded directly onto a 6 ml Heparin-Ultrogel A4-R column. The flow-through fraction contained TIF-IC activity, a factor that stimulates the initiation reaction (H. Rosenbauer, unpublished data). In the 400 mM KCl step RNA polymerase I and the growth-rate related activity termed TIF-IA (17) is eluted. TIF-IB was recovered in the 600 mM KCl step in a volume of 4.5 ml and a total protein content of 2.5 mg. After dialysis this fraction was further chromatographed on a 3 ml CM Sepharose column, and step eluted by 400 mM KCl after a 200 mM KCl wash. The CM400 fractions from three 100 ml preparations were combined and applied to a Mono S FPLC column (Pharmacia). TIF-IB activity was eluted with a linear gradient from 0.3−0.6 mM KCl in buffer B (same as buffer A containing 25 mM Hepes, pH 7.9 instead of Tris-HCl). Final purification and concentration of TIF-IB was achieved by affinity chromatography of the active fractions on calf thymus DNA cellulose. At this column factor activity eluted between a 300 and a 400 mM KCl step. During the fractionation procedure protein concentration was monitored by UV absorbance (OD 280 nm), and TIF-IB activity was determined both in the exonuclease III protection assay and in the cell-free transcription system. Throughout all purification steps the specific DNA binding activity coincided with the ability

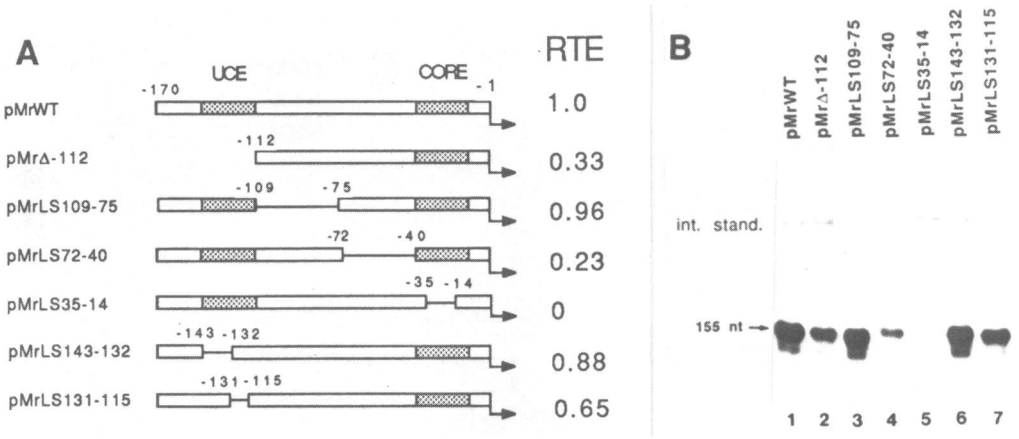

Fig. 1. Transcriptional activity of linker-scanning rDNA promoter mutants.
A) Schematic representation of the structure of the mutant templates. The bar represents sequences of the 5′ terminal non-transcribed spacer, the thin line marks foreign DNA sequences. The arrow indicates the position of the transcription start site. RTE represents the relative transcriptional activity of the individual mutants determined in at least four different experiments.
B) Transcriptional activity of linker scanning mutants as compared to the wild-type template pMrWT and the deletion mutant pMrΔ-112, respectively. The template activity of the test DNAs (truncated with Sma I to yield 155 nt run-off transcripts) was measured in the presence of a mixture of S-100 and nuclear extracts in the standard transcription assay. The 292 nt band represents transcripts from pMrSP/Pvu II which were added as internal standard to the reactions to calculate differences in the recovery of RNA during sample work up. The plasmid designation above the lanes indicates which DNA was assayed.

to direct specific transcription initiation on rDNA templates after complementation with partially purified factors TIF-IC and TIF-IA/RNA polymerase I.

UV-Crosslinking

A 5-bromo-2′-deoxyuridine substituted labelled DNA probe was prepared by primer-directed elongation of single-stranded M13 DNA containing nucleotides −168 to +155 of mouse rDNA by Klenow DNA polymerase in the presence of 50 μM dGTP, 50 μM 5-bromo-2′-deoxyuridine triphosphate and 30 μCi each of (α-^{32}P)dATP and (α-^{32}P)dCTP. The DNA was digested with Eco RI and Hind III to yield the fragment used for binding. 7.5 μl of TIF-IB (purified by chromatography on DEAE-Sepharose, Heparin Ultrogel, CM-Sepharose and Mono S FPLC) were incubated for 30 min at 30°C with 3×10^5 cpm of labelled DNA in binding buffer in the presence of 1 μg of poly(dI-dC) and 1 μg of poly(dA-dT). The mixture was irradiated on a UV lamp (302 nm) for 7.5 min. After addition of $CaCl_2$ to a final concentration of 10 mM, DNA was digested for 30 min at 37°C with 2 μg of DNase I and 1 unit of micrococcal nuclease, and the proteins were electrophoresed on an 11% SDS polyacrylamide gel.

RESULTS

Three Distinct Sequence Elements Promote Mouse rDNA Transcription

Previous analysis of 5′ deletion mutants has shown that upstream sequences located between nucleotides −144 and −112 positively affect the initiation efficiency of RNA polymerase I at the rDNA promoter both *in vivo* (7−10) and *in vitro* (11,13,14). To assess the functional role of different control regions within the mouse rDNA promoter a set of linker scanning mutants in which defined regions of the rDNA promoter were deleted and substituted by plasmid sequences was constructed and the transcriptional activity of the mutant templates was evaluated in the cell-free system (Fig. 1).

The linker scanning mutations define three major promoter elements. The core region which extends from nucleotide −39 up to the transcription start site. Deletion of part of this region completely abolishes transcription (pMrLS35-14, lane 5). A second essential promoter element is located between position −40 and −72. Substitution of this gene region by foreign sequences decreases transcription activity to about 20−25% of wild-type (pMrLS72-40, lane 4). Sequences between −75 and −109 appear to have little, if any, effect on transcription since deletion of this region does not impair template activity (pMrLS109-75, lane 3). Finally, there is the upstream control element (UCE) which extends upstream of nucleotide −112 and affects transcription approximately 3-fold (pMrΔ-112, lane 2).

To further pinpoint the essential nucleotides within the UCE, sequences from −131 to −115 and from −143 to −132, respectively, were substituted by linker sequences to yield the recombinant plasmids pMrLS143-132 and pMrLS131-115. Surprisingly, neither of these clustered point mutations completely abolished the UCE effect. The transcriptional activity of pMrLS143-132 (lane 6) was only slightly reduced in comparison to the wild-type, whereas pMrLS131-115 showed a strong decline in activity (lane 7). Nevertheless the template activity of pMrLS131-115 was still significantly higher than that of pMrΔ-112 (lane 2). This finding indicates that the UCE is composed of at least two subdomains the more important one located between nucleotides −131 and −115 (see also Fig. 6B). Both subdomains appear to partially substitute each other in function.

Three Protein Fractions are Required for rDNA Transcription Initiation

The analysis of the molecular mechanisms which mediate the functional cooperativity between the different promoter elements requires the isolation and characterization of the proteins which interact with the rDNA promoter. We have fractionated extracts from cultured Ehrlich ascites cells by column chromatography (Fig. 2A) and tested individual fractions for their ability to generate specific run-off transcripts from the wild-type template pMr600 cut with Eco RI (3). As shown in Figure 2B, all factors

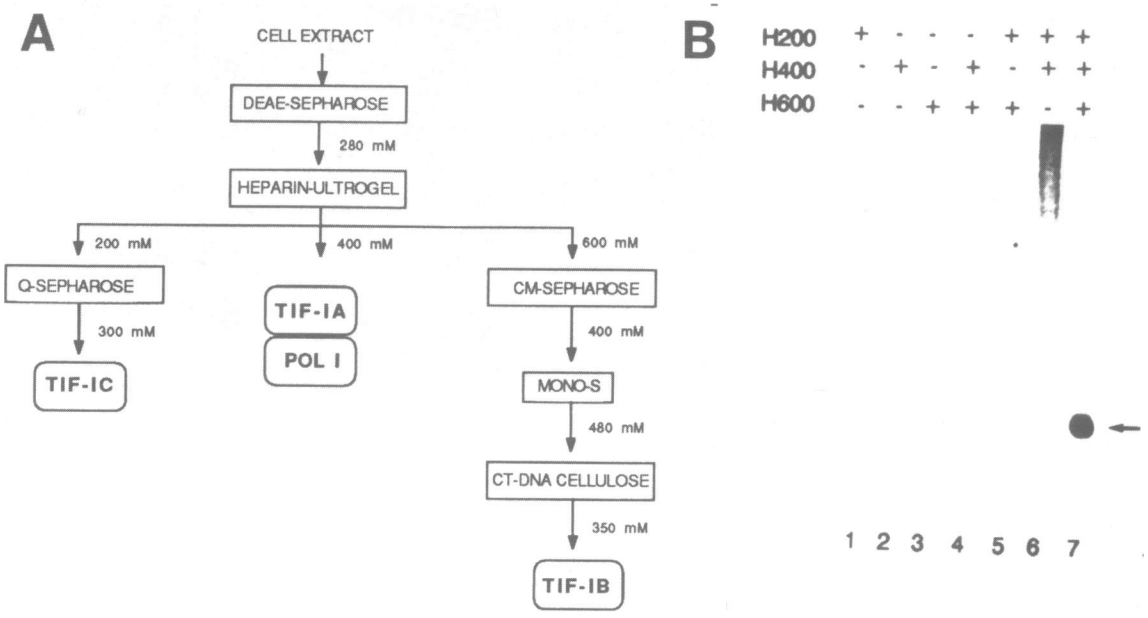

Fig. 2. Separation and functional identification of multiple factors required for faithful rDNA transcription initiation.
A) Schematic outline of the fractionation procedure used to separate the mouse pol I transcription initiation factors and to purify factor TIF-IB.
B) Transcriptional activity of fractions after chromatography on Heparin Ultrogel. The fractions eluted at 280 mM KCl from DEAE-Sepharose were pooled, dialized against buffer A/100 mM KCl, and applied to a Heparin Ultrogel column. Proteins were eluted by steps of 200 mM, 400 mM and 600 mM KCl, respectively, and tested for transcriptional activity either alone or in combination with other fractions. Each assay contained 50 ng of template DNA pMr600 digested with Eco RI and 15 μl of protein fractions in a 25 μl standard transcription assay. The different protein fractions contained in the individual assays are indicated above the lanes. The amount of the respective fractions was 15 μl (lanes 1–3), 7.5 μl (lanes 4–6), and 5 μl (lane 7).

required for faithful initiation elute from DEAE-Sepharose at 280 mM KCl. Further chromatography on Heparin Ultrogel using step elution with 200, 400, and 600 mM KCl, respectively, yields three protein pools (designated H200, H400 and H600) each of which alone is incapable to direct specific transcription. Depending on the individual preparation, a combination of H400 and H600 very often yields low amounts of specific transcripts. Addition of H200 results in a strong stimulation of the synthesis of the 297 nt run-off RNAs (lane 7) indicating that at least three protein factors are required for faithful and efficient transcription initiation. Two of these factors have been identified before: TIF-IA (present in the H400 fraction) is a regulatory protein which co-purifies with a subfraction of RNA polymerase I. The level or activity of this factor fluctuates according to the proliferation rate of the cells (17, 18). Factor TIF-IB present in the H600 fraction has been shown to be a specific DNA binding protein which interacts with the core element of the mouse rDNA promoter and imparts specificity to pol I (4, 6). Whether the third activity present in the 200 mM salt step (designated TIF-IC) is an essential or just a simulating factor is currently being investigated.

Purification and Identification of TIF-IB

To functionally analyze factor TIF-IB we have purified the protein to about 5–10% homogeneity by chromatography on DEAE-Sepharose, Heparin Ultrogel, CM Sepharose, FPLC Mono S, and CT-DNA cellulose according to the purification scheme diagrammed in Figure 2A. Fig. 3A shows the specific DNA binding of individual fractions eluted from the Mono S column, Fig. 3B shows the transcriptional activity in the reconstituted *in vitro* system. In our hands, the most sensitive assay for TIF-IB interaction with the rDNA promoter is the exonuclease III (exo III) protection assay described by Wu (19). Using a probe that extends from nucleotide −170 to +155, one can readily assay for TIF-IB binding activity. Specific binding of TIF-IB to the rDNA promoter fragment yields an exo III-resistant fragment which maps the 3′ border of the factor-DNA complex to nucleotide −21 on the coding strand (4). At higher factor concentrations two additional stops at positions −40 and at −13 are observed. These additional bands may be explained if the binding site is split as suggested from sequence comparisons of the putative binding sites (see Discussion). The specific binding activity is detected in fractions 19–31 eluting at about 450 mM KCl from the Mono S column. The same fractions complemented transcriptional activity after addition of two fractions that contained the essential factors TIF-IC and TIF-IA, respectively (see Fig. 2A). Both fractions alone promoted only low levels of specific transcription (lane 0). Transcriptional activity as monitored by the synthesis of the 297 nt run-off RNA was restored by the column fractions 23–29. On all chromatographic columns used there was a remarkable coincidence between specific binding and transcriptional activity suggesting that both activities reside within the same protein.

In Fig. 3C a silver-stained SDS polyacrylamide gel of the polypeptides present in the active fractions of the Mono S column is shown. Although there are still several polypeptides present, a 44 kd protein correlates with TIF-IB activity. This 44 kd protein was preferentially labelled by UV-crosslinking of the peak fractions with a 5-bromo-2′-deoxyuridine substituted labelled specific DNA probe (Fig. 3D). The amount of the labelled protein coincides with the activity peak suggesting that this polypeptide is the specific DNA binding protein.

To prove that the 44 kd protein represents TIF-IB, the factor was further purified by chromatography on DNA cellulose. On

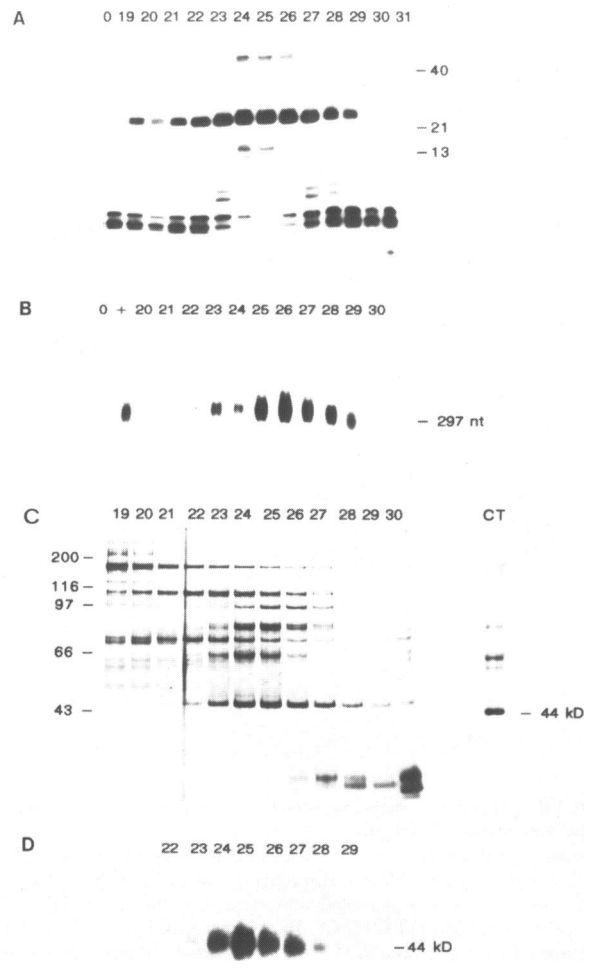

Fig. 3. Purification and identification of factor TIF-IB.
TIF-IB was purified by chromatography on DEAE-Sepharose, Heparin Ultrogel, CM-Sepharose, and FPLC Mono S as described in Materials and Methods.
A) Exonuclease III protection analysis of the column fractions. The probe DNA was a 343 bp fragment from pMrWT that had been 5′ end-labelled at the coding strand. 1.7 μl of individual column fractions were incubated with 1 ng of the DNA in the presence of 50 ng of unlabelled pUC9 plasmid DNA and subjected to exo III digestion as described in Methods. The numbers above the lanes refer to the fractions from the Mono S column. Lane 0 is a control digestion in the absence of TIF-IB. The band at nucleotide −21 is brought about by the specific binding of TIF-IB to the rDNA promoter. Note the additional exo III stops at nucleotides −40 and −13 in the active fractions which are observed at high concentrations of TIF-IB in the assay.
B) Transcriptional activities of the Mono S column fractions. 2 μl of individual fractions were mixed with partially purified factors TIF-IC and TIF-IA (see Figure 3A) and tested in a 25 μl standard transcription assay containing 75 ng of template DNA pMr600/Eco RI. Lane 0 is a negative control without TIF-IB which contained only the two additional Heparin Ultrogel fractions required for complementation of transcriptional activity. Lane + is a control showing the transcriptional activity of 3 μl of a TIF-IB fraction eluted from the Heparin Ultrogel column at 600 mM KCl (see Fig. 2A and B). The numbers 20−30 mark the fractions that were tested for transcriptional activity.
C) SDS polyacrylamide gel electrophoresis of proteins contained in a factor TIF-IB preparation. 7.5 μl of fractions 19−30 from the Mono S column were electrophoresed on an 11% SDS-polyacrylamide gel, and proteins were visualized by silver staining. Lane CT shows the protein composition of the final TIF-IB preparation after chromatography of the active Mono S fractions on calf thymus DNA cellulose. Bio-Rad molecular weight standards consisted of myosin (200,000), β-galctosidase (116,250), phosphorylase b (97,400), bovine serum albumin (66,200), and ovalbumin (42,700).
D) Identification of TIF-IB by UV-crosslinking. Binding reactions contained body-labelled probe plus 7.5 μl of individual column fractions. After binding the samples were exposed to UV-light (302 nm) and treated with nucleases as described in Materials and Methods. The fractions assayed are indicated above the lanes.

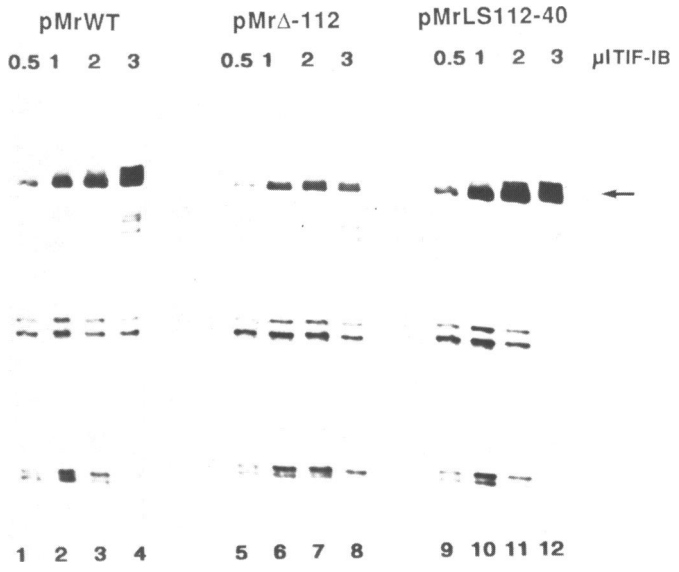

Fig. 4. Upstream sequences augment binding of TIF-IB to the core promoter
Approximately 1 fmole of the Eco RI/Hind III fragment from pMrWT, pMrΔ-112, or pMrLS112-40, respectively, 5′ end-labelled at the Eco RI site were incubated with increasing amounts of TIF-IB and subjected to exo III treatment as described. The DNA fragments used and the amount of TIF-IB added to the reactions are indicated above the lanes. The arrow marks the specific stop of exo III digestion due to TIF-IB binding.

this column TIF-IB activity elutes at 400 mM KCl (data not shown). Analysis of the protein composition of the peak fraction shows that the 44 kd protein is the predominant polypeptide in this final factor preparation (Fig. 3C, lane CT). Based on the co-purification of specific binding, transcriptional activity and the 44 kd protein we conclude that this polypeptide represents factor TIF-IB.

TIF-IB Binding to the Core Requires the Presence of the UCE in *cis*

To investigate the binding specificity of the highly purified factor, several deletion mutants were assayed either directly or were used as competitors in the exo III protection assay. Figure 4 shows an exo III protection assay where increasing amounts of purified factor TIF-IB were allowed to interact with labelled promoter fragments derived from either the wild-type (pMrWT), the 5′ deletion mutant (pMrΔ-112), or a linker-scanning mutant in which the region between the core and the UCE was replaced by neutral linker sequences (pMrLS112-40). The binding of TIF-IB to its target sequence was decreased by a factor of at least 5 if the UCE was removed (pMrΔ-112). Deletion and substitution by foreign DNA sequences of the gene region between the UCE and the core (pMrLS112-40), on the other hand, did not significantly affect factor binding. The finding that TIF-IB has a stronger interaction with the core promoter element in the presence of the UCE region, suggests that both elements function synergistically.

To obtain more definitive evidence that there is a functional cooperativity between both sequence elements, a series of promoter mutants were assayed for their ability to bind factor TIF-IB. As shown in Figure 5A, an excess of unlabelled wild-type plasmid pMrWT and the 5′ deletion mutant pMrΔ-142 efficiently suppressed the formation of the specific exo III-resistant band at position −21 (lanes 3 and 4). If the deletions extend to nucleotides −112, −74 or −39, competition was

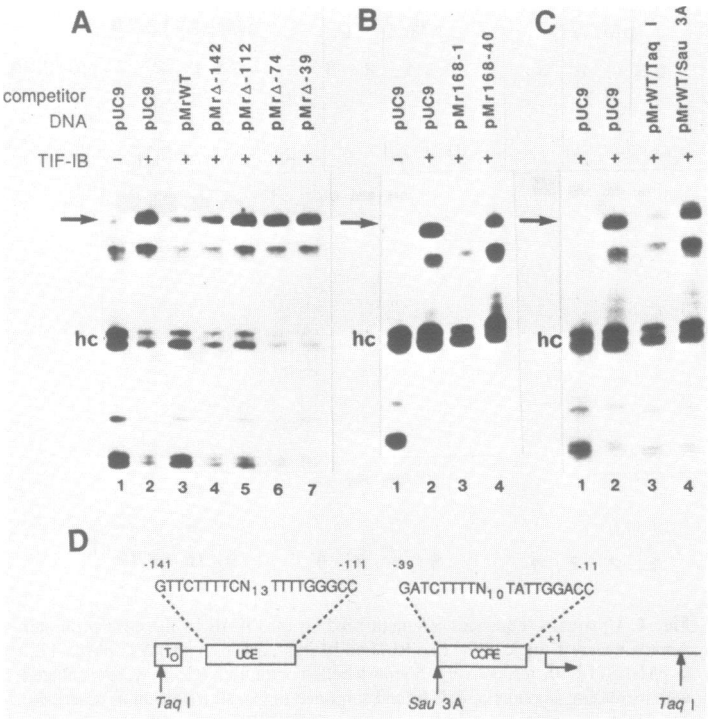

Fig. 5. The UCE exerts its function in cis.
A) Competition of TIF-IB binding by 5′ deletion mutants. The exo III protection assay was performed as described in Material and Methods. 1 fmole of the 343 bp rDNA promoter fragment was incubated with 0.1 μg of TIF-IB in the presence of 0.5 μg of the respective competitor DNAs as indicated above the lanes. The arrow marks the specific stop of exo III digestion due to TIF-IB binding, hc marks the position of the half-cut fragment generated by exo III in the absence of bound proteins.
B) Competition of TIF-IB binding by 3′ deletion mutants. The assay conditions were identical to those of the experiment shown in Fig. 6A.
C) Competition of TIF-IB binding with rDNA restriction fragments. Lanes 1 and 2: Control digestions performed without and with TIF-IB, respectively; lane 3: competition with 0.5 μg of pMrWT digested with Taq I; lane 4: competition with 0.5 μg of pMrWT digested with Sau 3A.
D) Schematic diagram of the spatial array of the two rDNA promoter elements. The promoter elements are shown as boxes and are labelled UCE and CORE, respectively. The nucleotide sequences of both regions are shown above. The nucleotide positions of mouse rDNA sequences are numbered relative to the transcription start site. T_0 represents the upstream terminator element flanking the UCE.

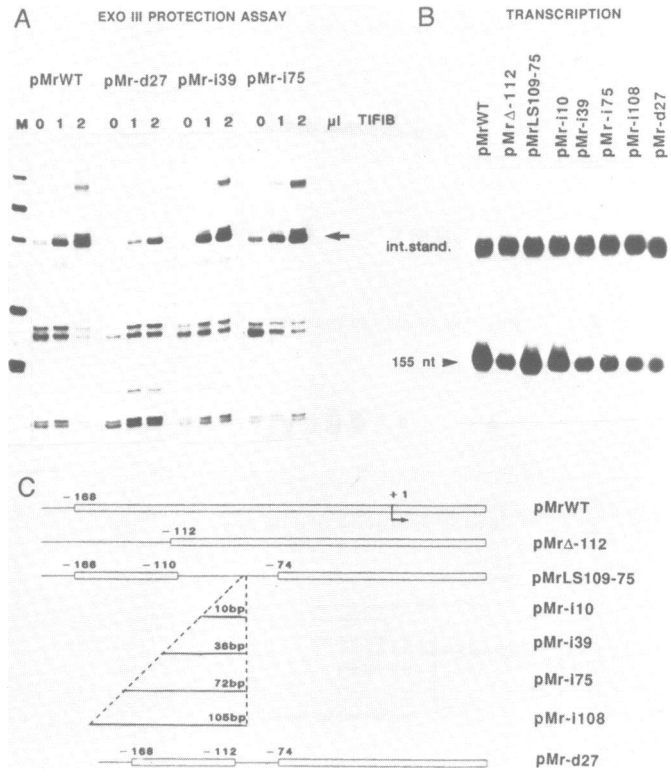

Fig. 6. Effect of distance changes between the core and the UCE on transcriptional activity and binding of TIF-IB.
A) Exonuclease III protection assay. The Eco RI/Hind III fragments from pMrWT, pMr-d27, pMr-i39, and pMr-i75, respectively, were 5′ end labelled at the Eco RI site, and 1 fmole was incubated with increasing amounts of TIF-IB in the presence of 250 fmoles of pUC9 DNA. The reaction conditions and the exonuclease III treatment were as described in Materials and Methods. M represents size marker fragments (pBR322/Hpa II).
B) Template activity of the spacing-change mutants. 100 ng of the individual test templates (cut with Sma I) were assayed in the standard transcription system in the presence of S-100 and nuclear extracts. The 292 nt band represents transcripts from pMrSP/Pvu II which were added as an internal standard to the reactions. The plasmid designation above the lanes indicates which DNA was assayed.
C) Schematic representation and designations of the constructs containing variations in the spacing between the UCE and the core. The open bar represents rDNA sequences, the thin line marks foreign DNA sequences derived from pUC9.

severely impaired (lanes 5–7) again demonstrating that sequences upstream of nucleotide −112 affect the interaction of TIF-IB with the core. Similarly, a subclone containing only sequences upstream of the initiation site (pMr-168/-1, Fig. 5B, lane 3) competes as efficiently as the wild-type, whereas the UCE alone (pMr-168/-40, lane 4) does not compete for factor binding. Taken together, these results suggest that stable binding of the purified protein to the ribosomal gene promoter requires both the core promoter (from −39 to −1) and the UCE located between nucleotides −113 and −142. If both sequence elements were present on the same plasmid separated by a neutral linker (pMrLS112-40) binding was as efficient as with the wild-type (Fig. 4). This result demonstrates that (I) the synergistic effect of both sequence elements occurs in *cis* and (II) that the region between nucleotides −40 and −112 is not required for TIF-IB binding.

Another line of evidence supports the conclusion that the UCE functions in *cis*. The ability of pMrWT to bind TIF-IB is strongly decreased when both sequence elements are physically separated by digestion with a restriction enzyme. Figure 5C shows a binding-competition experiment performed in the presence of pMrWT cleaved with Taq I or Sau 3A, respectively. Taq I cuts the insert present in pMrWT at nucleotides −166 and +55 thus leaving both regions linked. Sau 3A cleaves at position −39 and therefore the core and the UCE are separated. This physical separation of both regions results in a failure to compete for factor binding. pMrWT/Taq I (lane 3) competes as efficiently as undigested plasmid, whereas the exo III-resistant signal of the assay containing pMrWT/Sau 3A (lane 4) compares to that of the negative control pUC9 (lane 2).

Effect of Distance Changes between UCE and Core on Factor Binding

In the next series of experiments the spacing between the upstream and the core element was intentionally changed to study the spatial array and the cooperativity of both promoter elements. In pMr-d27 the distance between the core and the UCE is reduced by 27 nucleotides. The insertion mutants pMr-i10, pMr-i39, and pMr-i75 are constructs in which both elements have been pushed

apart by 10, 39, and 75 bp, respectively, by insertion of sequences derived from pUC9. These spacing-change mutants were assayed both in the exo III protection assay for TIF-IB binding (Fig. 6A) and in the cell-free system for transcriptional activity (Fig. 6B). Decreasing the distance between the UCE and the core by 27 nucleotides results in both reduction of TIF-IB binding and decrease in template activity, indicating that the stimulation of the UCE on binding TIF-IB to the core requires a minimal distance between both sequence elements.

On the other hand, the insertion mutants showed no strict correlation between factor binding and transcriptional activity. Increasing the distance between both promoter elements did not affect factor binding. The ability of TIF-IB to interact with pMr-i10, pMr-i39, and pMr-i75, respectively, compares to the wild-type (Fig. 6A). Even an insertion of 108 nucleotides between the core and the UCE did not abolish the stimulating effect of the upstream element on TIF-IB binding (data not shown) indicating that the cooperativity between the two elements tolerates an increase in distance of at least 108 nucleotides. In contrast to TIF-IB binding, transcriptional activation did not tolerate an increase of more than 10 nucleotides between the UCE and the core (Fig. 6B). While pMr-i10 is still as active as the wild-type, the transcriptional activity of pMr-i39, pMr-i75 and pMr-i108 is decreased to the level of pMrΔ-112, the UCE^- mutant. These different effects of distance changes between the two promoter elements on factor binding and transcriptional activity, respectively, demonstrates that stable binding of TIF-IB to the rDNA promoter is essential but not sufficient for efficient transcription initiation by pol I. Apparently the assembly of the preinitiation complex which involves the interaction with the other two auxiliary factors and RNA polymerase I depends on the correct spatial position of the individual promoter elements.

DISCUSSION

Previous analysis of deletion and linker-scanning mutants *in vivo* and *in vitro* revealed that the rDNA promoter of higher organisms consists of at least two functionally distinct elements, the core region which is indispensable for accurate and specific initiation, and the upstream control element (UCE) which appears to modulate the initiation frequency of rRNA transcription (see ref. 23, for review). The stimulatory effect of upstream sequences was first observed in an S-100 transcription system when 5′ deletions were assayed simultaneously with wild-type templates (11). Successive removal of 5′ flanks impaired the ability to form stable transcription complexes and to direct transcription initiation (14, 22). The activating effect of the UCE is much more pronounced *in vivo* than *in vitro* as revealed by transfection of rDNA constructs into cells and measuring pol I-directed transcription of a defined marker gene (7, 8, 10, 24, 25). It has been suggested that this modulation of transcription is mediated by *trans*-acting factors which recognize these control sequences and appear to operate in concert with proteins interacting with the core promoter (26). The data described in this paper provide experimental evidence that the UCE has a stimulating effect on template utilization, and that there is a functional cooperativity between both control elements. This synergistic action is mediated by TIF-IB, the factor which interacts with the core promoter and thus confers promoter selectivity to pol I (4, 6).

In contrast to class II and III genes where defined transcription factors have been isolated and some of their respective genes have now been cloned, little is known about the essential factors involved in rDNA transcription initiation. Mishima et al. (28) were the first to fractionate mouse and human extracts by phosphocellulose chromatography into four fractions (termed A, B, C, and D). Fractions C and D were found to be most important for transcription. Fraction C contains the bulk of pol I and an essential regulatory activity which has been shown to fluctuate according to the growth rate of the cells. We have designated this regulatory activity transcription initiation factor TIF-IA (17). Fraction D was shown to contain a species-specific factor that binds stably to the rDNA promoter and is responsible for the formation of specific transcription initiation complexes (28, 29). The protein in fraction D which binds to the murine rDNA promoter is most likely identical to our factor TIF-IB (4, 6). Admittedly, this different designation for apparently related factors is confusing. However, a common nomenclature for the different pol I-specific factors has to await further purification, functional characterization and an exchange of factors between different laboratories.

In humans, the species-specific promoter recognition appears to be mediated by an auxiliary transcription factor (termed SL1) which exhibits no DNA recognition properties. SL1 was shown to act together with a sequence-specific DNA binding protein which interacts with both the core promoter and the UCE (26). This factor, designated upstream binding factor (UBF), has recently been isolated from Hela cells (30, 31). Purified UBF migrates as a doublet of 94 and 97 kd on an SDS polyacrylamide gel. UBF binds preferentially to the UCE of the human rDNA promoter but also with low affinity to the core region which shares a region of significant sequence homology (85 percent) with the UCE. UBF appears to activate RNA polymerase I through direct interaction with both the UCE and the core.

Although highly purified factor TIF-IB binds to the murine ribosomal gene promoter in the absence of other proteins, and in this respect resembles human UBF, we do not consider it to be the mouse UBF for the following reasons: Both proteins differ with regard to size, chromatographic behaviour and to binding specificity. Human UBF does not bind to mouse rDNA (26, 31), and conversely, TIF-IB does not interact with the human ribosomal gene promoter (our unpublished results). Nevertheless the UBF protein is present in mouse cells and can be purified by chromatography on a human ribosomal UCE-specific affinity column (S. Bell and M. Jantzen, personal communication). We are currently investigating whether TIF-IC, which is separated from the other auxiliary initiation factors by chromatography on Heparin Ultrogel (Fig. 2), represents the murine UBF. Factor TIF-IB, on the other hand, is most likely the murine analogue of human SL1. However, it differs from human SL1 in two respects: (i) it binds to the core in the absence of UBF, and (ii) TIF-IB on its own is not capable to reprogram a Hela cell extract to transcribe mouse rDNA (C. Pfleiderer and I. Grummt, unublished data).

It appears, that although being functionally homologous, the human and the murine rDNA promoter binding factors exert significant differences in their mode of action. The human factor UBF binds preferentially to the upstream promoter element and can be purified on an affinity column containing this upstream sequence element. In contrast, the murine UBF which has the same physicochemical and functional properties as human UBF does not footprint the mouse promoter(S. Bell and M. Jantzen, personal communication). Apparently very specific interactions between the selectivity factor TIF-IB (mouse) or SL1 (human) and factor UBF are responsible for the species-specificity of

rDNA transcription (11).

Surprisingly, the stable binding of TIF-IB to the core promoter is strongly enhanced by the UCE. Deletion of this sequence element results in a strong decrease in binding activity. The stimulation by the UCE in *cis* has been observed throughout the purification of TIF-IB, thus rendering it rather unlikely that this stimulating or stabilizing effect is brought about by (a) protein(s) different from TIF-IB. The low affinity of TIF-IB to its target sequence in the absence of the upstream element may also explain our incapability to identify this factor by gel retardation assays or to purify this protein by affinity chromatography on a column containing core promoter sequences from −39 to +4. In spite of considerable efforts, we also did not succeed to demonstrate a specific interaction of TIF-IB with either the core or the UCE by DNAase footprinting experiments. This failure to footprint TIF-IB at different stages of purification is probably due to the low binding stability of the TIF-IB/rDNA complex. The upstream control element plays an important role in modulating the activity of RNA polymerase I transcription *in vivo* and *in vitro*, but it is not required for specific transcription initiation from the mouse ribosomal gene promoter. The specificity of transcription initiation is solely mediated by the core element. Thus, both elements differ quantitatively and qualitatively from each other but exert a functional cooperativity. Analysis of a series of altered templates that contained variations in the spacing between the two promoter elements revealed that insertion of 10 bases between the core and the UCE did not affect transcription. The upstream element ceases to function (i. e. transcription is approximately equivalent to a deletion mutant lacking the UCE) if moved further away (Fig. 6). In contrast, the stimulatory effect of the UCE on TIF-IB binding was observed also in mutants in which the distance between the core and the UCE was increased by more than 100 nucleotides. On the other hand, decreasing the distance between the UCE and the core completely abolishes the UCE effect both in the transcription and the binding assay. We conclude from these data: (I) the functional cooperativity of both sequence elements requires a minimal distance between the UCE and the core, (II) the enhancing effect of the UCE does not depend on the number of helix turns between both elements, and (III) TIF-IB binding is essential but not sufficient for the stimulatory effect of the UCE on transcription, but very likely requires additional protein-DNA and protein-protein interactions.

It is not yet clear by which molecular mechanisms the cooperative effect between the core and the UCE is achieved. Any one of the following hypothesis may explain the stimulatory effect of the distal promoter element. (I) The presence of the upstream element induces a conformational alteration in the core region which facilitates stable factor binding, (II) TIF-IB binding is a two step process which involves a primary labile association with the UCE followed by transformation into a stable TIF-IB-core complex, (III) one factor molecule contacts both promoter elements, or (IV) two or more factor molecules bind cooperatively to both the UCE and the core. The latter two mechanisms should involve looping of the DNA to bring the two sequence elements in spatial proximity. This, in turn, may be realized by either recognition of two different DNA sequences by TIF-IB or by interaction with related binding sites present in both elements. Although we cannot definitely exclude any of these possibilities, we strongly favor a model which implies functional cooperativity of at least two TIF-IB molecules bound at the core and the UCE, respectively. Such a synergistic action of two sequence elements has first been observed in the interaction of the phage lambda repressor with its operator sites (32, 33), and has recently been demonstrated for binding of purified OTF-2 to the immunoglobulin heptamer and octamer motifs which share no obvious sequence homology (34). In searching for sequence homologies between the core and the UCE we identified a region within the mouse UCE which shows a striking homology to the core sequence (see Fig. 6B). A variant of the core sequence GATCTTTTCN$_{10}$TATTGGACC (from −39 to −12) is found within the UCE encompassing the sequence GTTCTTTTCN$_{13}$ TTTTGGGCC (from −142 to −111). The difference in either the sequence or the spacing between the two half sites of both promoter elements may explain the lower affinity of TIF-IB for the UCE as compared to the core. This lower affinity, together with the assay system employed to analyze factor binding (exonuclease III protection assays), may explain why we have failed so far to demonstrate a direct physical interaction between TIF-IB and the upstream region. On the other hand, the structural similarities in the sequence of the two promoter elements is consistent with our conclusion that TIF-IB interacts with both the UCE and the core.

In a recent communication from Muramatsu's group it was demonstrated that factor TFID (the Japanese designation for TIF-IB) could protect a large region of the rDNA promoter including both the core and the upstream region in DNase footprint experiments (35). Since, however, the TFID preparation used was only partially purified by chromatography on two columns, it is not yet clear whether the protection of the upstream region is brought about by TFID or by (a) different protein(s) present in the crude factor preparation. On the other hand, it is feasible that several molecules of TIF-IB interact with the rDNA promoter. We consistently observe additional specific exonuclease III stop signals (at position −40 and −13) at high concentrations of purified TIF-IB. This may reflect either the binding of several factor molecules to a split recognition site in the core promoter, or the induction by TIF-IB of a conformational change in the DNA which stops exo III movement. Finally, the possibility that interaction of TIF-IB with the rDNA promoter affects transcription by perturbing DNA flexibility or structure should also be considered. Such a physical alteration, as for instance looping or bending of the DNA, may facilitate contact of the protein with distant DNA regions which in turn may result in the conversion of a metastable into a stable DNA-protein complex. The availability of highly purified TIF-IB will now facilitate mechanistic studies to investigate in more detail topological changes within the rDNA promoter and to study the influence of the UCE on the structure of the rDNA-factor complex.

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