MOLECULAR AND CELLULAR BIOLOGY, June 1990, p. 3105–3116
0270-7306/90/063105-12$02.00/0

Vol. 10, No. 6

Characterization of Factors That Direct Transcription of Rat Ribosomal DNA

S. D. SMITH,[1]† E. ORIAHI,[1] D. LOWE,[1] H.-F. YANG-YEN,[1] D. O'MAHONY,† K. ROSE,[2] K. CHEN,[1] AND L. I. ROTHBLUM[1]†*

Department of Pharmacology, Baylor College of Medicine,[1] and Department of Pharmacology, University of Texas Health Sciences Center,[2] Houston, Texas 77030

Received 27 November 1989/Accepted 21 March 1990

The protein components that direct and activate accurate transcription by rat RNA polymerase I were studied in extracts of Novikoff hepatoma ascites cells. A minimum of at least two components, besides RNA polymerase I, that are necessary for efficient utilization of templates were identified. The first factor, rat SL-1, is required for species-specific recognition of the rat RNA polymerase I promoter and may be sufficient to direct transcription by pure RNA polymerase I. Rat SL-1 directed the transcription of templates deleted to −31, the 5′ boundary of the core promoter element (+1 being the transcription initiation site). The second factor, rUBF, increased the efficiency of template utilization. Transcription of deletion mutants indicated that the 5′ boundary of the domain required for rUBF lay between −137 and −127. Experiments using block substitution mutants confirmed and extended these observations. Transcription experiments using those mutants demonstrated that two regions within the upstream promoter element were required for optimal levels of transcription in vitro. The first region was centered on nucleotides −129 and −124. The 5′ boundary of the second domain mapped to between nucleotides −106 and −101. DNase footprint experiments using highly purified rUBF indicated that rUBF bound between −130 and −50. However, mutation of nucleotides −129 and −124 did not affect the rUBF footprint. These results indicate that basal levels of transcription by RNA polymerase I may require only SL-1 and the core promoter element. However, higher transcription levels are mediated by additional interactions of rUBF, and possibly SL-1, bound to distal promoter elements.

Eucaryotic rRNA genes (rDNAs) code for three of the four rRNA molecules (18S, 5.8S, and 28S rRNAs). These three RNAs are products of degradative processing of a larger precursor (40S to 47S pre-rRNA). The genes that serve as the template for this transcription are present in multiple copies (approximately 200 per haploid genome) and are organized as clusters of tandem repeats. In interphase cells, the genes are localized to the nucleolus, where they are transcribed by RNA polymerase I. Each repeat consists of a transcribed portion and a nontranscribed spacer (reviewed in references 27 and 28). In at least two species, *Xenopus laevis* and *Drosophila melanogaster*, the nontranscribed spacer is transcribed (9, 23, 41), and at least part of the nontranscribed spacer of the rat repeat is transcribed from a spacer promoter (6), suggesting that the name of this region needs to be changed.

The transcription initiation sites of several mammalian rRNA genes have been identified and sequenced. Functional analysis of the promoters of the mammalian rRNA genes indicates that despite significant sequence differences, the promoters apparently consist of elements with similar functions. That region of the promoter (~−31 to ~+6) required for transcription in vitro (5, 12, 16, 26, 43) is referred to as the core promoter element (CPE). Under more stringent conditions, a requirement for distal sequences becomes apparent (33), and in vivo, the CPE is almost inactive (40). Two distal elements, the upstream promoter (control) element (UPE) and an upstream terminator (referred to as either T_0 or T_3) (13, 17, 31) have been identified. The UPE has been shown to be required for transcription in vivo and for elevated levels of transcription in vitro under stringent conditions, and it appears to be required for formation of the stable preinitiation complex (5, 42). It has been suggested that T_0 functions to prevent promoter occlusion (2); however, the precise role of the terminator is not understood.

Unlike many of the genes transcribed by RNA polymerases II and III (4), promoter recognition by RNA polymerase I is, with one known exception (8), species specific (14); i.e., competent extracts from human cells do not transcribe rodent rDNA and vice versa. This property has been ascribed to a *trans*-acting factor, referred to as either PC-D (32, 34) or TIF-IB (7) for rodents or PC-D (32, 34) or SL-1 (24) for primates. Exonuclease III protection assays indicate that mouse TIF-IB protects a domain that includes the CPE of the mouse rDNA repeat. Although DNase 1 footprinting experiments failed to detect an interaction between human SL-1 and the core element of the human rDNA promoter, human SL-1 did direct transcription of hybrid mouse UPE-human CPE promoters. Also, when human SL-1 was added to DNase I footprinting experiments containing human UBF (hUBF), the hUBF footprint was extended. hUBF is a DNA-binding protein that has been demonstrated to increase the efficiency of template utilization and protect the region from approximately −70 to −120 of the human rDNA promoter and may also interact with the CPE of the human rDNA repeat (3, 25).

We studied the factors required for transcription of rat rDNA. Our findings indicate that one rat factor, referred to as rat SL-1, interacts with the CPE and may also interact with the UPE. Interaction of rat SL-1 with the CPE of the rat rDNA repeat is required for accurate initiation by either rat or human RNA polymerase I. Addition of a second factor, referred to as rat UBF (rUBF), increases the efficiency of template utilization. The results of in vitro transcription

* Corresponding author.
† Present address: Weis Center for Research, Geisinger Clinic, Danville, PA 17822.

experiments using deletion mutants indicated that the effect of rUBF on transcription required a region whose 5′ boundary lay between −137 and −127. Site-directed mutagenesis studies indicated a complex region consisting of two separate elements. One element mapped to nucleotides −129 to −124. Although the second domain was not entirely defined, the 5′ boundary of this domain mapped to between −106 and −101. DNase I footprinting experiments with highly purified rUBF demonstrated that rUBF protected the region between −130 and −50. In similar experiments, rat SL-1 protected four regions, including a region at circa −130. These results suggest that interaction of rat SL-1 with the CPE is required for transcription and the interaction between the core–SL-1 complex and the complex formed between rUBF and the UPE is responsible for activation of the rat rDNA promoter. They also suggest that interaction of SL-1 with the UPE is required for the effect of rUBF.

MATERIALS AND METHODS

Templates. The templates used in this study were derived from p5.1 (36). The 5′ deletion mutants were constructed with BAL31 and Klenow enzyme by using standard protocols (29) and inserted in the *Kpn*I and *Eco*RI sites of pUC. The endpoints of the deletions were confirmed by sequencing (38) through the junctions between the vector and the inserts. Nucleotide substitution mutants were created by using synthetic oligonucleotides to prime the synthesis of the second strand on an M13 template grown in a *dut ung* mutant strain of *Escherichia coli* K-12 essentially as described by Kunkel (22). After transformation into a wild-type host, minilysates were prepared and the presence of the substitution was confirmed by chain termination DNA sequencing. The inserts were then transferred into pUC18, and the sequences of the mutations were again confirmed by sequencing the double-stranded DNAs. These clones were inserted in the *Bam*HI and *Eco*RI sites of pUC18. p5.1E/X was derived from p5.1. In this construct, the *Xho*I site at +638 was converted into an *Eco*RI site and the remainder of the DNA 3′ of the original site was deleted. For most of these studies, a pUC18 clone of that DNA was used. p5.1E/E was derived from p5.1E/X. In this construct, the *Eag*I site at +331 was converted to an *Eco*RI site and the rat DNA 3′ of that site was deleted.

In vitro transcription. The conditions used for in vitro transcription were described previously (6). A standard 50-μl reaction contained between 0.02 and 0.4 μg of the truncated template and 1.0 μg of nonspecific DNA, either pBR322 or pUC18. When templates were preincubated with crude extract or subfractions, the conditions of the preincubation were those of the final assay, except that nucleotides were added after preincubation. In some cases, upon termination of transcription, a constant amount of an end-labeled fragment of pBR322 was added to each reaction to act as an internal standard for the efficiency of recovery of the nucleic acids. For routine assays demonstrating the effect of rUBF on transcription, the pooled RNA polymerase I-containing fractions from the first DEAE-Sephadex column (described below) were used both as the source of RNA polymerase I and as the source of SL-1. This fraction is referred to as UBF-depleted extract. For assaying of SL-1, Novikoff hepatoma RNA polymerase I purified through the heparin-Sepharose step (described below) was satisfactory. However, for some experiments, homogeneous RNA polymerase I was used. For assay of rat SL-1 and rUBF in heterologous transcription reactions, nuclear extracts of HeLa cells were used.

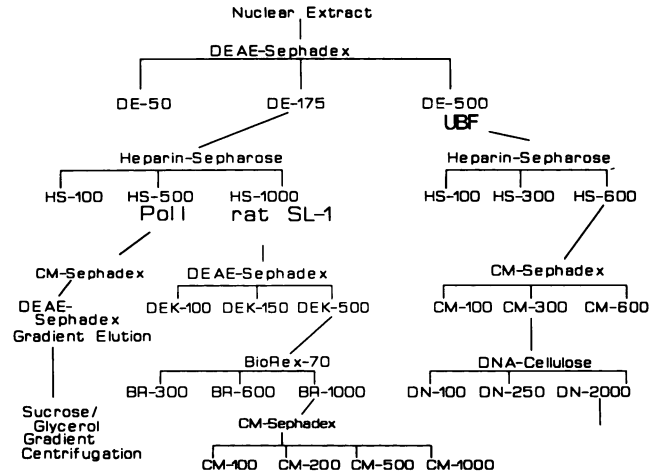


FIG. 1. Scheme for fractionation of rat RNA polymerase I, rat SL-1, and rUBF from nuclear extracts of Novikoff hepatoma ascites cells. The stages at which the various factors become resolved are indicated. The salts indicated are those used to elute the columns. $(NH_4)_2SO_4$ was used to equilibrate and elute the first DEAE-Sephadex columns, and all of the subsequent columns were equilibrated and eluted with KCl as described in Materials and Methods.

DNase I footprinting. End-labeled DNA probes were produced by labeling the indicated restriction enzyme cleavage sites with either [γ-^{32}P]ATP and polynucleotide kinase or [α^{32}P]dNTP and Klenow fragment. The fragment used was a *Bam*HI-*Hin*dIII fragment derived from p5.1E/X. After digestion with the second enzyme, the fragments were purified by polyacrylamide gel electrophoresis. Each reaction contained between 3 and 10 ng of the labeled DNA fragment, 1 μg of nonspecific competitor, either pUC18 or poly(dI-dC)–poly(dI-dC), and the indicated protein fraction. Rat SL-1 was concentrated with a Centricon (Amicon Corp.) for the footprinting reactions. In some experiments, rUBF was also concentrated before footprinting. The binding reactions were performed under conditions identical to those used for transcription, and DNase footprinting was performed essentially as described by Angel et al. (1). After DNase I digestion, the reactions were extracted with phenol-chloroform-isoamyl alcohol, ethanol precipitated, suspended in 90% formamide, heat-denatured, and analyzed on 6% acrylamide–urea sequencing gels. Sequencing reactions (30) of the same fragments were run in parallel lanes to align the digestion patterns.

Fractionation of nuclear extracts. Nuclear extracts were prepared from Novikoff hepatoma ascites cells or logarithmic cultures of HeLa cells essentially as described before (15). After the final dialysis, the crude extracts were quick-frozen and stored at −80°C. The solutions used in the fractionation protocol are variations of buffer C (40 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid], 5 mM $MgCl_2$, 0.2 mM EDTA, 20% glycerol [adjusted to pH 7.9 at room temperature]). All solutions contained 0.1 mM dithiothreitol and 0.1 mM phenylmethylsulfonyl fluoride added immediately before use. All operations were performed at 4°C.

The steps used in the fractionation protocol are summarized in Fig. 1. Novikoff cell nuclear extracts were applied to a DEAE-Sephadex column (1.2 to 1.5 mg of protein per cm^3) equilibrated with DE-50 [buffer C containing 50 mM $(NH_4)_2SO_4$]. The column was washed with DE-50 and eluted stepwise with solutions containing 175 and 500 mM

$(NH_4)_2SO_4$, DE-175, and DE-500, respectively. After the 50 mM $(NH_4)_2SO_4$ wash, fractions equivalent to 1% of the column volume were collected. Every other fraction was assayed for RNA polymerase I essentially as already described (39). In a typical fractionation (see Fig. 2A), no RNA polymerase I was detected either in the flowthrough or the material eluted with DE-500. The fractions eluted with 175 mM $(NH_4)_2SO_4$ that contained the peak levels of RNA polymerase I activity were pooled and dialyzed against buffer C containing 100 mM KCl (C-100) and are referred to as DE-175. This material served as the source of RNA polymerase I and SL-1 (see below). The peak protein-containing fractions eluted with 500 mM $(NH_4)_2SO_4$, referred to as DE-500, were pooled and also dialyzed against C-100; this material served as the source from which rUBF was purified (see below).

Fractionation of rat SL-1. After dialysis, the pooled fractions containing RNA polymerase I (DE-175) were chromatographed over a heparin-Sepharose column (Sigma Chemical Co.) equilibrated with buffer C-100 at a protein load of 2 mg/cm^3 (see Fig. 2B). The column was washed with buffer C-100 and step eluted with solutions containing 500 and 1,000 mM KCl, C-500, and C-1000, respectively. RNA polymerase I was eluted quantitatively with C-500, referred to as HS-500, whereas the 1,000 mM KCl eluate, referred to as HS-1000, contained rat SL-1 (see Fig. 3; discussed below). Fractions containing either the peak of RNA polymerase I activity (HS-500) or peak concentrations of protein (HS-1000) were pooled separately and dialyzed against C-100.

The dialyzed pool of proteins from the heparin-Sepharose column that contained rat SL-1 activity (HS-1000) was applied to a DEAE-Sephadex column (1 to 2 mg of protein per cm^3 equilibrated in C-100 [see Fig. 2C]). The column was washed with C-150 and eluted with C-500 and C-1000. The peak protein-containing fractions from each salt wash were pooled separately and dialyzed against buffer C-100. The fractions eluted with C-500 (DEK-500) containing the peak concentrations of protein were pooled and dialyzed against buffer C-100. After dialysis, they were applied to a BioRex-70 column equilibrated with C-100 (see Fig. 2D). The column was washed with C-100 and eluted stepwise with solutions containing buffer C and 300, 600, and 1,000 mM KCl, yielding peak protein-containing fractions referred to as BR-300, BR-600, and BR-1000, respectively. The peak protein-containing fractions from each salt wash were pooled separately and dialyzed against buffer C-100. The proteins in BR-1000 were next applied to a CM-Sephadex column equilibrated with buffer C-100. The column was then washed with buffer C-200 and step eluted with buffers C-500 and C-1000. The fractions containing peak concentrations of protein, CM-100, CM-200, CM-500, and CM-1000, were pooled and dialyzed against C-100.

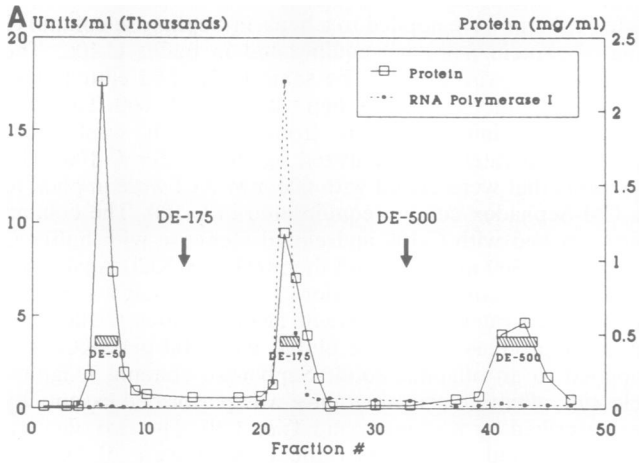
RNA polymerase I. For most assays, the RNA polymerase I-containing fractions from either the first DEAE-Sephadex column (UBF-depleted extract) or the heparin-Sepharose column used to purify SL-1 were sufficiently sensitive to addition of the various transcription factors. However, for the assays that required pure RNA polymerase I, the enzyme was either purified from whole-cell extracts of Novikoff hepatoma ascites cells or from the heparin-Sepharose fractions of those extracts essentially as described previously (35) by using sucrose-glycerol density gradient centrifugation in 0.25 M $(NH_4)_2SO_4$ to remove adventitiously bound proteins. The enzyme activity sedimented at approximately 15S.

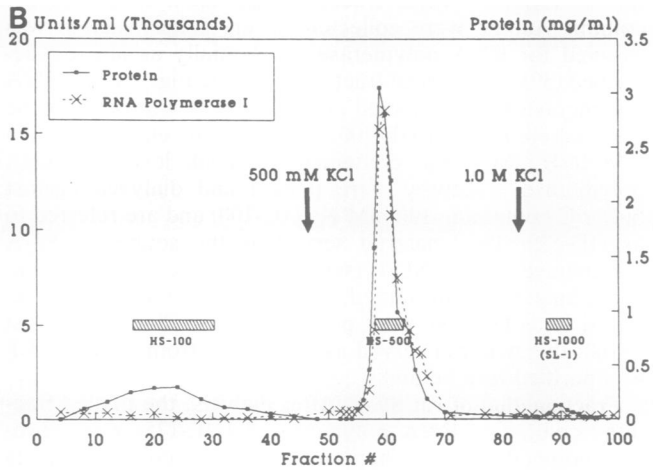
Purification of rUBF. The peak protein fractions that were eluted with 500 mM $(NH_4)_2SO_4$ from the first DEAE-Sephadex column were applied to a heparin-Sepharose column (2 mg of protein per cm^3) equilibrated in buffer C-100. The column was washed with the same buffer and eluted stepwise with buffer C-300 and then with buffer C-600. The peak protein-containing fractions from each salt wash were pooled separately and dialyzed against buffer C-100. The proteins that were eluted with 600 mM KCl were applied to a CM-Sephadex column equilibrated in C-100. The column was washed with C-100 and eluted stepwise with buffer C containing 300 mM KCl and then 600 mM KCl. Again, the peak protein-containing fractions from each salt wash were pooled separately and dialyzed against buffer C-100. The pool of proteins that were eluted with 300 mM KCl was applied to an oligonucleotide-Sepharose column. Oligonucleotide affinity chromatography was performed essentially as described by Kadonaga and Tjian (19). The two oligonucleotides used to construct the column were 5′-ACGTC CCGAACATGACTTCCAGACGTTCCGTGTGGCCTGTC ATGTTTTATCCCTGTGTC-3′ and 5′-GACGTGACACAG GGATAAAACATGACAGGCCACACGGAACGTCTGGAA GTCATGTTCGG-3′.

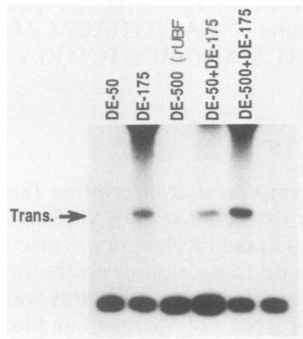
RESULTS

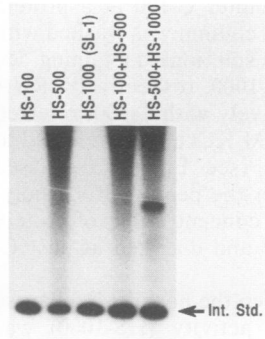
Fractionation of rat RNA polymerase I transcription factors. Chromatography of nuclear extracts over DEAE-Sephadex resulted in a fraction that contained RNA polymerase I and was able to specifically initiate transcription on the rat rRNA promoter (Fig. 2A) (15). As discussed below, this was apparently due to the presence of RNA polymerase I and rat SL-1 in this fraction. In studies on the transcription of the spacer promoter (6; S. D. Smith, E. Oriahi, H.-F. Yang-Yen, K. Xie, and L. I. Rothblum, Nucleic Acids Res., in press), we found that a 500 mM $(NH_4)_2SO_4$ wash of the DEAE-Sephadex column eluted proteins that stimulated transcription of that template. Subsequently, we found that this fraction also stimulated transcription from the 45S promoter (Fig. 2A, lower panel). Control experiments demonstrated that this fraction (and more purified subfractions) did not stimulate transcription of 5S DNA by RNA polymerase III and that the effect could not be duplicated by addition of RNase inhibitor. The factor responsible for the activity of this fraction was originally referred to as stimulatory factor 1. However, because of the many similar properties shared between rat stimulatory factor and hUBF, we refer to this rat factor as rUBF (discussed below).

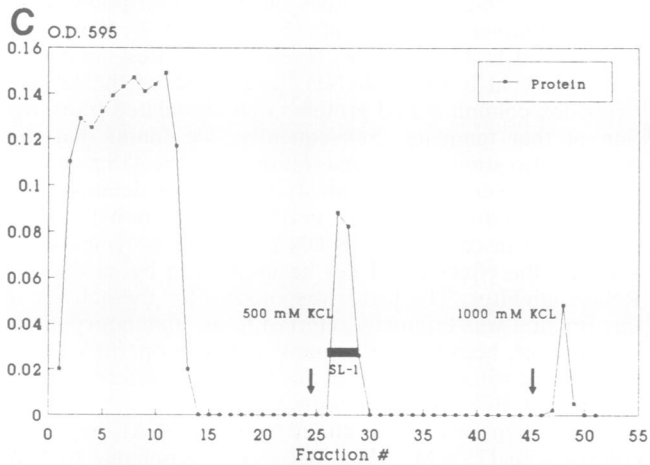
SL-1. When the proteins eluted from the DEAE-Sephadex column with 175 mM $(NH_4)_2SO_4$ were fractionated by heparin-Sepharose column chromatography, two of the resultant fractions, HS-500 and HS-1000, were sufficient to reconstitute transcription (Fig. 2B, lower panel). HS-500 contains RNA polymerase I. Subsequent experiments demonstrated that RNA polymerase I was the only component of this fraction necessary for reconstitution with HS-1000. HS-1000 contained an activity that could direct transcription by the homologous enzyme (Fig. 2B and 3A), as well as program HeLa extracts to transcribe rat rDNA (Fig. 3B and 4). These two activities copurified through subsequent rounds of purification on DEAE-Sephadex, BioRex-70, and CM-Sephadex (Fig. 2C and D and 4), suggesting that both activities were functions of the same protein. The ability to program heterologous extracts is a property of human SL-1, and we have therefore chosen to use the nomenclature of Learned et al. (24) for this factor, with the understanding that the human

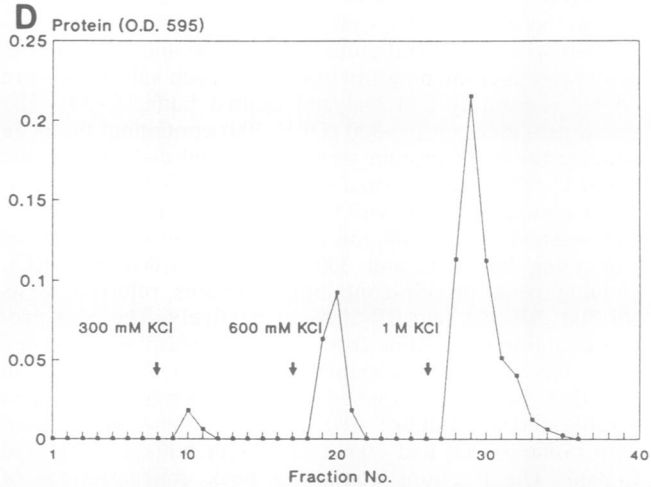

A
Units/ml (Thousands)
Protein (mg/ml)
Protein
RNA Polymerase I
DE-175
DE-500
DE-50
DE-175
DE-500
Fraction #

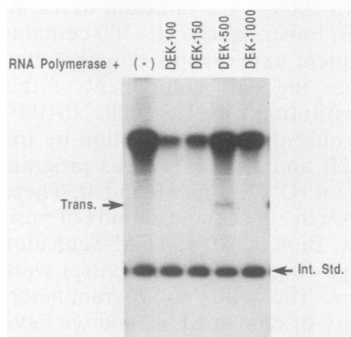

B
Units/ml (Thousands)
Protein (mg/ml)
Protein
RNA Polymerase I
500 mM KCl
1.0 M KCl
HS-100
HS-500
HS-1000
(SL-1)
Fraction #

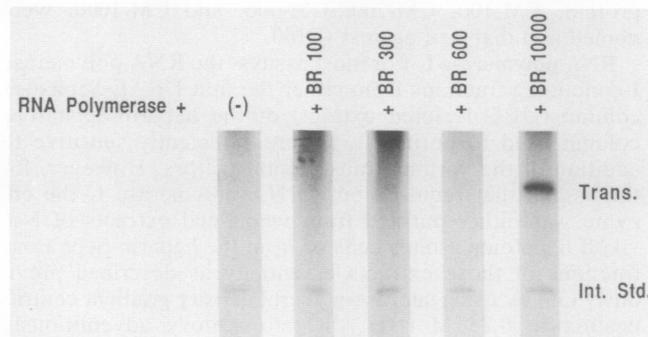

DE-50
DE-175
DE-500 (rUBF
DE-50+DE-175
DE-500+DE-175
Trans.


HS-100
HS-500
HS-1000 (SL-1)
HS-100+HS-500
HS-500+HS-1000
Int. Std.


C
O.D. 595
Protein
500 mM KCl
1000 mM KCl
SL-1
Fraction #


D
Protein (O.D. 595)
300 mM KCl
600 mM KCl
1 M KCl
Fraction No.


RNA Polymerase +
(-)
DEK-100
DEK-150
DEK-500
DEK-1000
Trans.
Int. Std.


RNA Polymerase +
(-)
+ BR 100
+ BR 300
+ BR 600
+ BR 10000
Trans.
Int. Std.

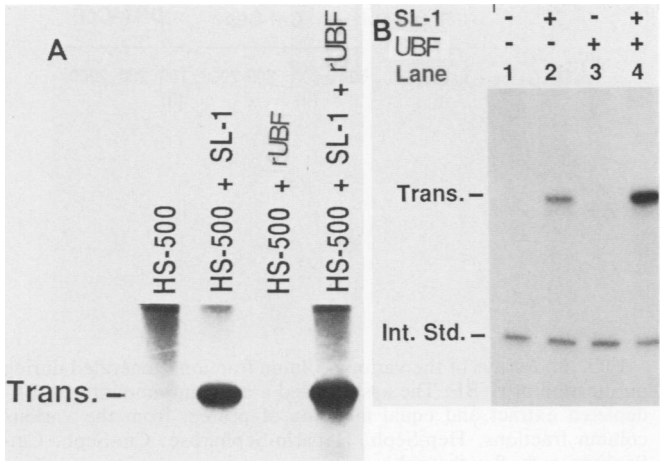


FIG. 3. rUBF and rat SL-1 are two different transcription factors. (A) Equal volumes of rat SL-1 purified through the heparin-Sepharose column chromatography step and rUBF purified through the CM-Sephadex column chromatography step were used to reconstitute transcription by RNA polymerase I purified through the heparin-Sepharose step (HS-500). The electrophoretic position where the in vitro transcript that would result from correct initiation is indicated. (B) Abilities of rat SL-1 and UBF to program transcription by HeLa cell nuclear extracts. Every reaction contained 0.2 μg of rat rDNA (p5.1E/X) truncated to yield a 638-nucleotide transcript (Trans.) and 5 μl of HeLa cell nuclear extract (25 μg/μl). The reactions were supplemented with 280 ng of rat SL-1, 50 ng of rUBF, or both, as indicated. The SL-1 used was purified through the CM-Sephadex column chromatography step, and the UBF used was purified through the CM-Sephadex column chromatography step. Whereas SL-1 directed correct initiation (lane 2), rUBF did not (lane 3), but rUBF stimulated SL-1-dependent transcription (lane 4). Int. Std., Internal standard.

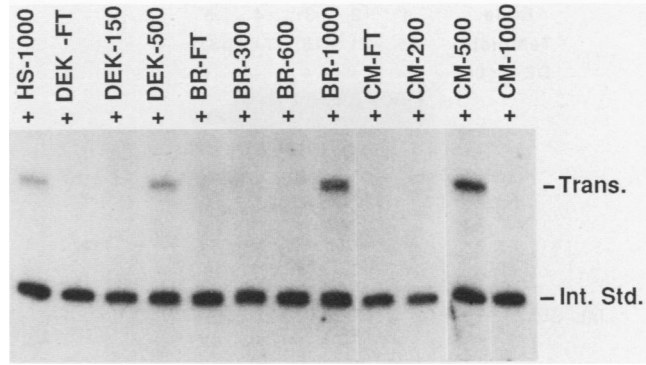


FIG. 4. Purification of SL-1 does not separate the activity required to program transcription of rat rDNA by heterologous extracts from that which directs the transcription of rat rDNA by rat RNA polymerase I. The various fractions generated during isolation of rat SL-1 were assayed for the ability to program transcription of rat rDNA by nuclear extracts derived from HeLa cells. A 20-μl volume of each fraction generated during purification of SL-1 was added to each reaction that contained 5 μl of HeLa extract and 0.2 μg of template (p5.1E/X truncated to yield a 638-nucleotide transcript). The protein concentrations of HS-1000, DEK-500, BR-1000, and CM-500 were 0.063, 0.12, 0.087, and 0.085 mg/ml, respectively. The abbreviations are as in Fig. 2.

and rat forms of SL-1 may demonstrate species-specific differences. The first stage at which rat SL-1 can be assayed for is after the heparin-Sepharose column chromatography step. HS-1000 contains 0.1% of the protein originally applied to the DEAE-Sephadex column. The subsequent chromatography steps result in an additional 60-fold purification. Although SL-1 is not pure at this stage, it contains no detectable levels of RNA polymerase I.

Rat SL-1 interacts with the CPE. Previously, we mapped the 5′ boundary of the CPE of the rat rDNA repeat to between −31 and −25 (5). This, in turn, suggests that at least one of the factors necessary for transcription must recognize the CPE. By using 5′ deletion mutants, we mapped the sequences required for SL-1 to direct transcription by RNA polymerase I (Fig. 5). We found that the CPE, as defined in prR-31, was sufficient to support SL-1-directed initiation by RNA polymerase I. This indicated that one region recognized by SL-1 is part of the CPE. The decrease in transcription efficiency observed as the 5′ boundary of the deletions approached the 5′ border of the core promoter suggests that DNA adjacent to the core plays a role in the interaction of rat SL-1 with the core. DNase footprint experiments (described below) indicated that there might be a secondary SL-1-binding site between the core and −75. Also, SL-1 did protect part of the CPE; however, that footprint was not conclusive.

Purification of rUBF. rUBF was identified because of its ability to stimulate transcription of the rat spacer promoter. Subsequent experiments demonstrated that it also stimulated transcription from the 45S promoter (Fig. 2A and 3) and that, by itself, it could not direct correct initiation by either rat or human RNA polymerase I (Fig. 3). As the factor responsible for this activity has many of the characteristics of hUBF, we refer to it as rUBF. As the protein was apparently resolved from RNA polymerase I and SL-1 by the initial DEAE-Sephadex column chromatography step, we routinely used DE-175 as UBF-depleted extract. The rUBF activity consistently chromatographed as a single peak of activity during chromatography over heparin-Sepharose and CM-Sephadex (Fig. 6). Silver-stained sodium dodecyl sulfate-polyacrylamide gels demonstrated that a band of 97 kilodaltons was enriched for upon CM-Sephadex column chromatography, and this band was present in the affinity-purified material (see Fig. 7). (In some preparations, a variable amount of a 94-kilodalton band was observed.) However, this does not prove that rUBF is either of these two bands, and further experiments are planned to examine

FIG. 2. Column chromatograph profiles for fractionation of rat SL-1. Four of the column chromatography steps used in the purification of rat SL-1: A, DEAE-Sephadex fractionation of nuclear extract; B, heparin-Sepharose fractionation of DE-175; C, DEAE-Sephadex fractionation of HS-1000; D, BioRex-70 fractionation of DEK-500. The upper part of each panel depicts the protein profile monitored with the Bio-Rad dye-binding assay and, when appropriate, the RNA polymerase I elution profile from the respective column. In panels C and D, the dye-binding assays were performed with 60-μl samples in a total volume of 400 μl. The first 15 fractions (DE-50) of the first DEAE-Sephadex column were 2% of the column volume, whereas the remainder of the fractions were 1% of the column volume. The lower part of each panel depicts the results of transcription reconstitution assays of the respective fractions. After the heparin-Sepharose column, it was necessary to add purified RNA polymerase I to the assays for SL-1. The assays represented by these panels were performed with 0.2 μg of template per assay. Abbreviations: Trans., predicted transcript from p5.1E/X truncated to yield a 638-nucleotide transcript; Int. Std., internal standard added to allow quantitation of the recovery of nucleic acids from the transcription reactions.

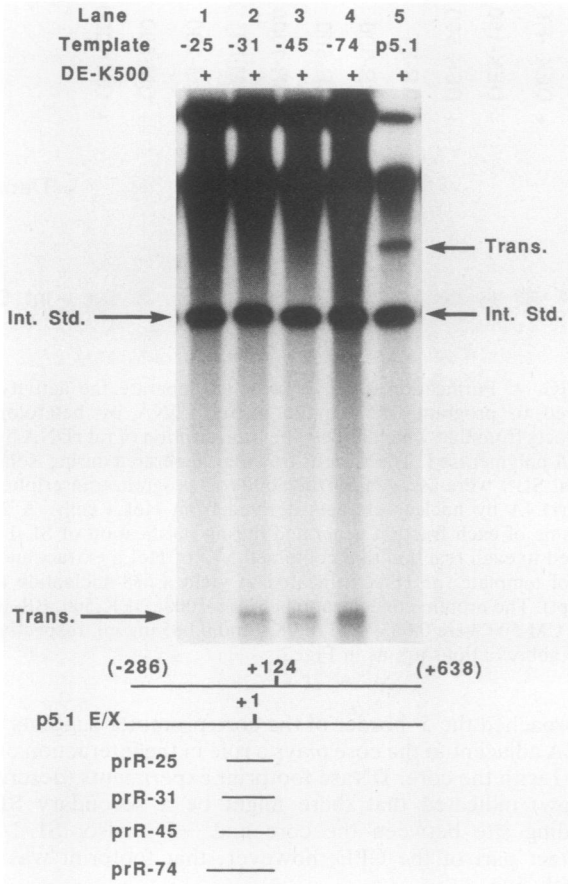


FIG. 5. Transcription with deletion mutants demonstrates that SL-1 must interact with the CPE. In lanes 1 to 4, various 5′ deletion mutants, as indicated, were transcribed in the presence of highly purified rat SL-1 and RNA polymerase I. In those four lanes, the templates were truncated with *Hin*dIII, which would generate a 124-nucleotide transcript. The template in the final lane was p5.1E/X truncated to yield a 638-nucleotide transcript. The RNA polymerase I used in these experiments was purified through the sucrose-glycerol gradient centrifugation step. The abbreviations are as in Fig. 2.

this point. The starting material, DE-500, represents 10% of the original nuclear extracts and is the first stage at which rUBF can be assayed. Chromatography over heparin-Sepharose increased the specific activity fourfold. Chromatography over CM-Sephadex and the oligonucleotide-Sepharose columns resulted in a further 900-fold purification. On the basis of these values, we estimated that after the oligonucleotide affinity chromatography step, rUBF had been purified a maximum of 36,000-fold from the nuclear extracts.

rUBF increases efficiency of template utilization. Our initial experiments with rUBF demonstrated that the elevated levels of transcription were not due to nonspecific stimulation of transcription or to a nonspecific RNase-type inhibitor. It was found that the effect of rUBF on transcription was optimized when the template level was reduced (Fig. 8). As the template concentration was increased without rUBF, we observed a template concentration-dependent effect on the level of transcription. However, with rUBF, the effect of the template concentration on transcription was reduced. The net effect could be interpreted as increased efficiency of template utilization with rUBF. This stimulation could be explained by one of two mechanisms. In one model, there would be a direct interaction of rUBF with the template or preinitiation complex. In a second model, rUBF would act as a soluble factor activating one of the other components of the transcription machinery, e.g., RNA polymerase I.

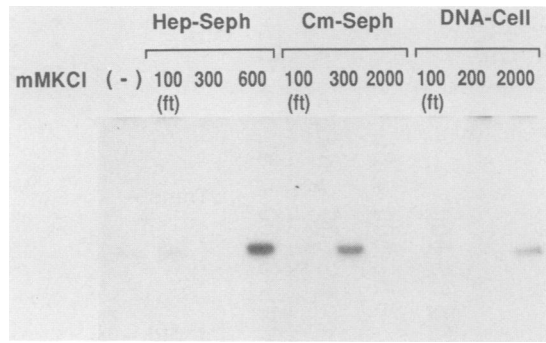


FIG. 6. Assays of the various column fractions generated during purification of rUBF. The assays used a constant amount of UBF-depleted extract and equal amounts of protein from the various column fractions. Hep-Seph, Heparin-Sepharose; Cm-Seph, Cm-Sepharose; ft, flowthrough.

rUBF forms a complex with the template. A modified template commitment assay was used to distinguished between the possibilities that rUBF either interacted with the template or acted as a soluble factor (Fig. 9). Two wild-type templates distinguished by their sites of truncation were used for these assays. All of the reactions contained rUBF-depleted extract. Some of the preincubation tubes (lanes 2, 4, 6, and 8) were supplemented with rUBF (purified through the CM-Sephadex column chromatography step). After 10 min of preincubation, equal portions of the indicated preincubation mixtures were mixed and nucleoside triphosphates were added to initiate transcription. After 30 min, the transcription reactions were stopped and the products were purified and analyzed by gel electrophoresis and autoradiography. Lanes 1 to 4 are control reactions in which only one

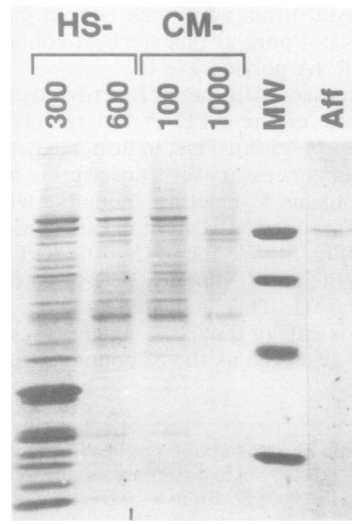


FIG. 7. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of rUBF after CM-Sephadex column chromatography (CM-) and oligonucleotide affinity column chromatography (Aff). MW, Molecular weight markers (phosphorylase *b* [97,400], bovine serum albumin [66,200], ovalbumin [45,000], and carbonic anhydrase [31,000]). HS-, Heparin-Sepharose.

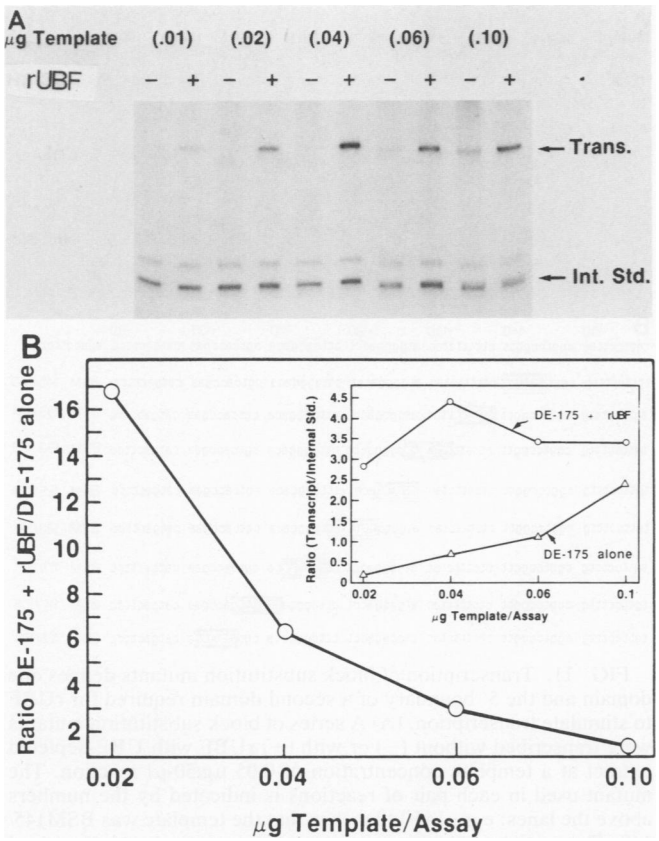


FIG. 8. Transcription at reduced concentrations of template is most efficient when rUBF is added to the assays. Various concentrations of a wild-type template (p5.1E/X) were transcribed by UBF-depleted extract with or without rUBF, as indicated (A). After the transcription products were separated by polyacrylamide gel electrophoresis and autoradiographed, the autoradiograph was quantitated by scanning densitometry (B). The ratio of transcript to internal standard in each lane was determined. These results were plotted for assays performed with or without UBF as a function of the template concentration (inset). The ratio of transcription with UBF to that without UBF was plotted as a function of the template concentration. The abbreviations are as in Fig. 2.

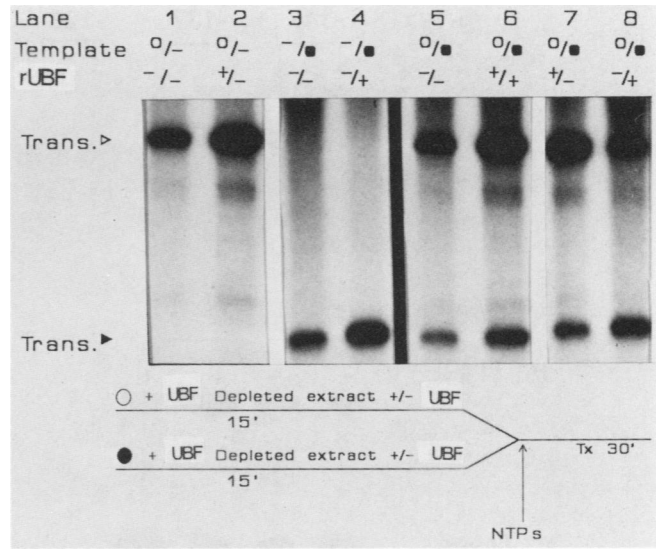


FIG. 9. Simultaneous transcription of templates preincubated with rUBF demonstrates that UBF must form a complex with the template. Two wild-type templates truncated to yield a 335-nucleotide transcript (p5.1E/E) or a 638-nucleotide transcript (p5.1E/X) were incubated with UBF-depleted extract for 10 min before initiation of transcription. Some of the preincubations included UBF (+) purified through the CM-Sephadex column chromatography step. After preincubation, portions of the respective preincubations were mixed, nucleotides were added, and transcription was allowed to proceed for 30 min. The first four lanes represent control reactions in which only one template was present in the transcription reaction after preincubation without (−) or with (+) rUBF. For lanes 5 to 8, each template was preincubated separately with (+) or without (−) UBF and then portions of the respective preincubations were mixed. In lane 5, after separate preincubations without rUBF, both templates were transcribed simultaneously. In lane 6, both templates were preincubated with rUBF. In lane 7, p5.1E/X was preincubated with rUBF, and in lane 8, p5.1E/E was preincubated with rUBF. Trans., Predicted transcript from p5.1E/X truncated to yield a 638-nucleotide transcript or from p5.1E/E truncated to yield a 333-nucleotide transcript.

template was present during the transcription reactions. These lanes established that rUBF could stimulate transcription from both templates (compare lanes 1 and 2 and 3 and 4). In lanes 5 and 6, each preincubation tube contained one of the two templates. When rUBF was present in both preincubations, transcription from both templates was stimulated (lane 6). In lanes 7 and 8, rUBF was added to only one of the preincubation mixtures, as indicated. The level of transcription of the template that had been preincubated with rUBF was stimulated. The level of transcription of the template that had not been preincubated with rUBF was unaffected. These results suggested that rUBF did not act as a soluble factor and that it did not disassociate during the transcription reaction. Similar results were obtained when the time of the transcription reaction was extended to 60 min.

The effect of rUBF requires sequences 3′ of −137. If rUBF formed a complex with the template, then experiments designed to identify the sequences required for the effect might confirm that model and provide additional data required to understand the mechanism of action of rUBF. The boundaries of the sequences required for the effect of rUBF on transcription were mapped by using a series of deletion mutants. In the experiments described in Fig. 10, a series of deletion mutants were transcribed with or without rUBF. Deletion mutants through nucleotide −137 responded to addition of rUBF; however, mutants deleted to −126 and beyond were not stimulated by addition of rUBF (Fig. 10). These results indicated that rUBF required *cis*-acting elements for its effect on transcription and that the 5′ border of the domain of this element lay between −137 and −126.

Block substitution mutants indicate that the effect of rUBF requires two domains. To define this region more clearly, a series of block substitution mutants were constructed. In these mutants, a *Kpn*I site was scanned through the region from −145 to −101, generating mutant templates in which only six or fewer nucleotides were replaced with the sequence GGTACC in each construct (Fig. 11B). These templates were transcribed individually with or without rUBF (Fig. 11A). Two of these templates did not significantly respond to addition of rUBF to the transcription assay, i.e., BSM129/124 and BSM106/101. (The reproducible depression of transcription of BSM106/101 when rUBF was added to the assay is not understood.) These results were consistent with the results of the experiments using the deletion mutants. However, the results obtained with BSM106/101 suggest that

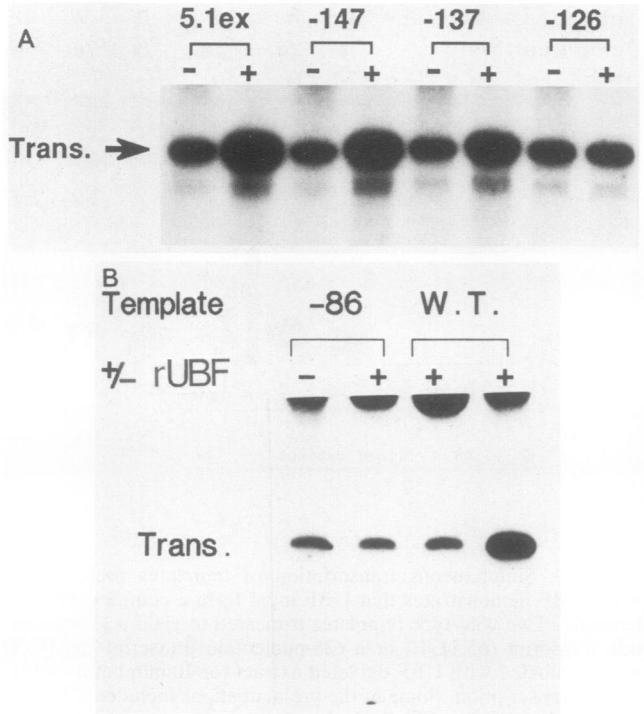


FIG. 10. Transcription of 5′ deletion mutants demonstrates that rUBF interacts with sequences within the UPE. The deletion mutants indicated were transcribed individually by using UBF-depleted extract after preincubation for 10 min without (−) or with (+) rUBF and rUBF-depleted extract. The UBF used in this experiment was purified through the CM-Sephadex column chromatography step. The template level was 0.05 μg/50-μl reaction. Trans., Predicted transcript from p5.1E/X truncated to yield a 638-nucleotide transcript; W.T., wild type.

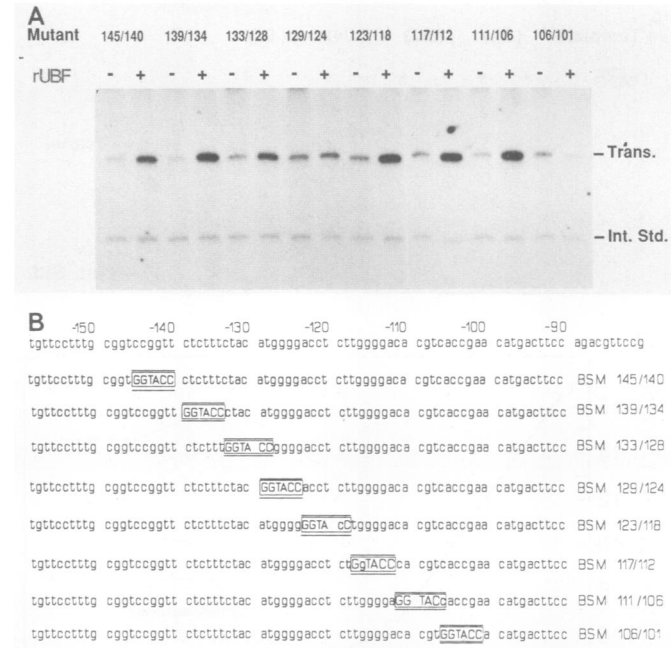


FIG. 11. Transcription of block substitution mutants defines one domain and the 5′ boundary of a second domain required for rUBF to stimulate transcription. (A) A series of block substitution mutants were transcribed without (−) or with (+) rUBF with UBF-depleted extract at a template concentration of 0.05 μg/50-μl reaction. The mutant used in each pair of reactions is indicated by the numbers above the lanes; e.g., 145/140 means that the template was BSM145/140. The partial nucleotide sequence of the upper strand of each of the mutant templates is shown in panel B. The sites mutated are boxed, and the nucleotides mutated are indicated as uppercase letters. The abbreviations used are as in Fig. 2.

UBF-dependent stimulation of transcription requires a second site as well as that centered over nucleotides −129 and −124; either rUBF interacted with two different sites or the effect of UBF was mediated through interaction of a second DNA-binding protein, either one that bound over −129 to −124 or one whose binding domain includes or begins at circa −106 to −101.

rUBF footprints over the region between −50 and −130, and SL-1 protects the region between −121 and −133. We directly investigated the above-described point by using DNase footprinting. rUBF protected a broad domain extending from −130 to −50 (Fig. 12A). Thus, both of the sites defined with the BSM mutants may be part of the rUBF-binding site. To determine whether the sequence mutated in BSM129/124 was an important part of the rUBF-binding site, we examined the footprint of rUBF on that mutant promoter (Fig. 12D). The footprint of rUBF over BSM129/124 was essentially identical to that obtained with the wild-type promoter. This suggests that the mutation did not significantly alter the binding of rUBF to the DNA and that the protection extending over to −130 might be nonspecific. Among the sites protected by the SL-1 fraction was the region between −130 and −120 (Fig. 12B and C), suggesting that the 5′ terminus of the domain defined with the deletion mutants and BSM129/124 may represent an SL-1-binding site. SL-1 gave a weak, inconclusive footprint over the core promoter and protected a region between −65 and −80 (discussed below).

rUBF can rescue transcription from a template with a weakened CPE. To investigate the role of the interactions of rat SL-1 and UBF with the promoter elements in transcription, two point mutants were synthesized, −7A/G and −16A/G. In these mutants, adenine was substituted for guanine at either position −7 or −16. These guanines are conserved in all of the mammalian 45S promoters and in the rat spacer promoter. Furthermore, it has been reported that they are essential for transcription (20). Thus, they were likely starting points for construction of a promoter with a weakened or inactive core element. We found that these two templates were transcribed by unfractionated nuclear extracts (Fig. 13A). However, the rates at which they were transcribed were significantly lower than that at which the wild-type template was transcribed (50 to 80% of control). We then examined their transcription using fractionated extracts (Fig. 13B). Extracts depleted of rUBF transcribed a wild-type template (Fig. 13B, lane 1) but neither of the two nucleotide substitution mutants (lanes 2 and 4). When the reactions were supplemented with rUBF (Fig. 13B, lanes 3 and 5), transcription from these templates was restored. As our previous experiments had demonstrated that these extracts contained SL-1, these results suggested that the interaction of an essential factor, presumably SL-1, with the CPE had been weakened by the nucleotide substitutions made in the CPE so that it became impossible for an initiation complex to form unless the secondary interaction with the UPE also occurred. The apparent contradiction between our studies on the template capacity of the substitution mutants and those previously reported (20) may be due to the extracts used (nuclear versus S-100) and the relative

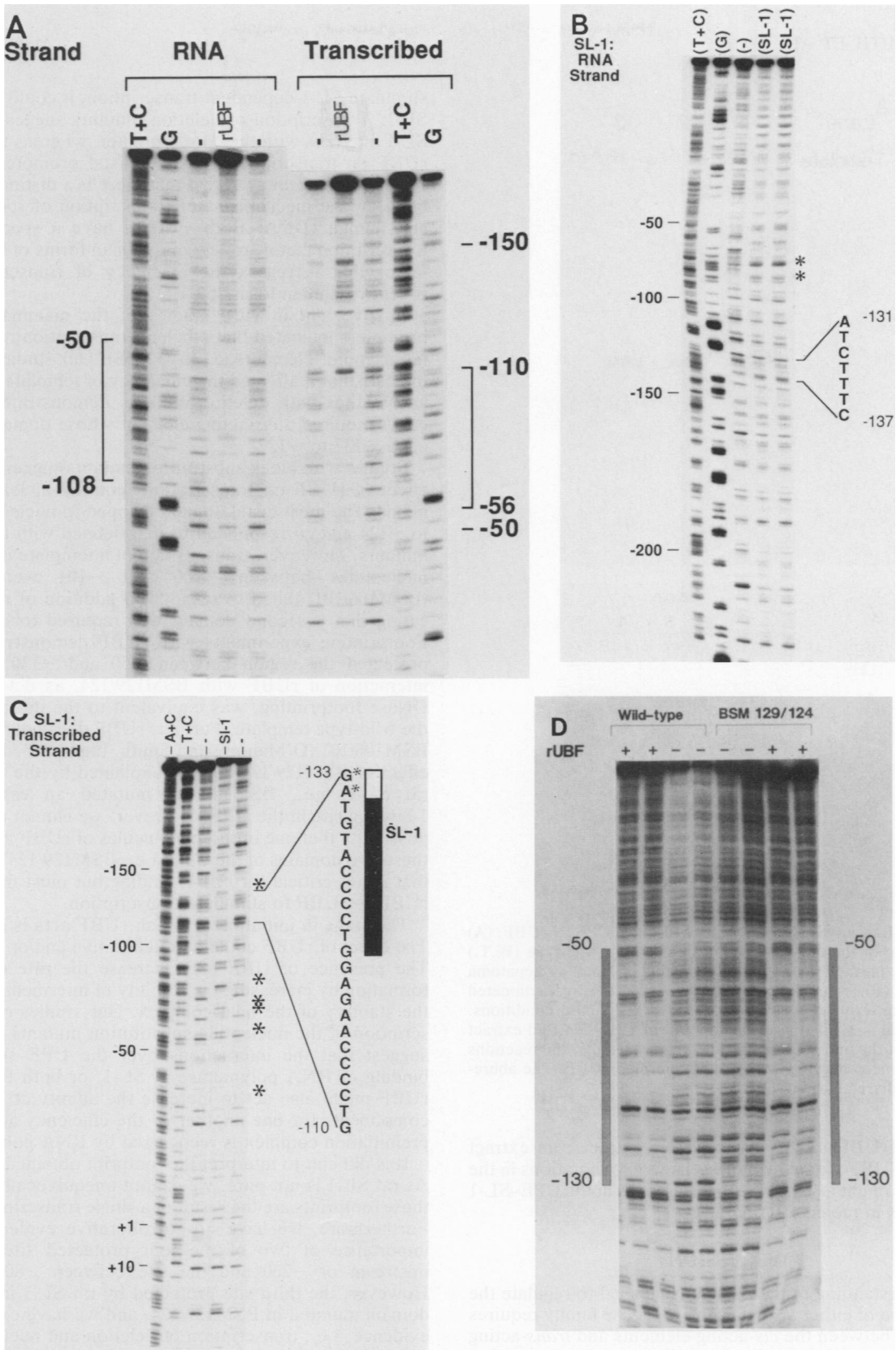


FIG. 12. DNase footprinting reactions of rUBF (A) and SL-1 (B and C) with the rat 45S rDNA promoter and rUBF with either the wild-type 45S promoter or BSM129/124 (D). The RNA or upper-strand probe was prepared by 5′ end labeling the *Bam*HI site (−286) of p5.1E/X, freed by cleavage with *Hin*dIII (+124), and gel purified. The transcribed or lower strand was prepared by 5′ end labeling the *Hin*dIII site, and the fragment was released with *Bam*HI and gel purified. In the indicated lanes, approximately 4 to 8 ng of labeled DNA was incubated with 4 ng of rUBF purified through CM-Sephadex column chromatography or 2 ng of SL-1 purified through the CM-Sephadex chromatography step. In the lanes marked with a minus, the probes were preincubated with 5 ng of bovine serum albumin before the DNase I digestion step. In panels A to C, the G and T+C sequencing reaction ladders (28) of each probe were run in lanes adjacent to the reactions to identify the sequences of the protected sites.

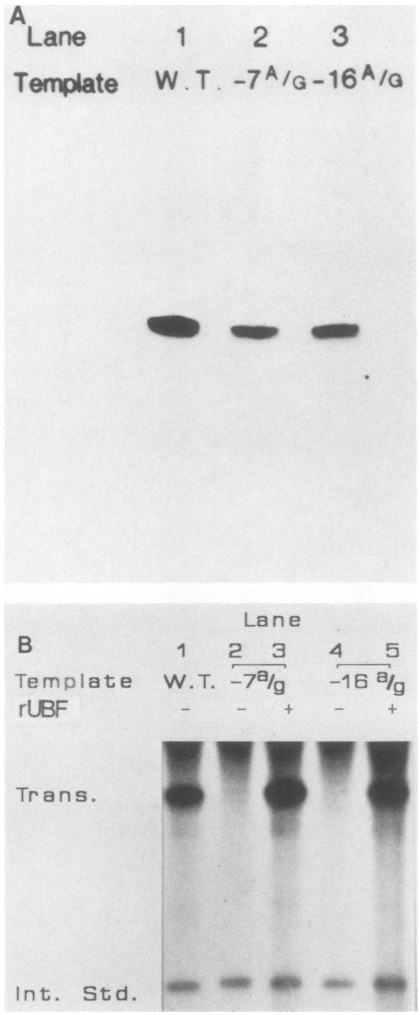


FIG. 13. Rescue of a template with a disabled CPE by rUBF. (A) Transcription of the indicated point mutants and wild-type (W.T.) templates by unfractionated nuclear extracts of Novikoff hepatoma ascites cells. (B) Transcription of the same templates by fractionated extracts. Transcription was performed under standard conditions. Each reaction contained an equal amount of UBF-depleted extract and 0.1 μg of the indicated template. In lanes 3 and 5, the reactions were supplemented with CM-Sephadex-purified rUBF. The abbreviations used are as in Fig. 2.

amounts of UBF in the two types of extracts; an extract deficient in UBF would be more sensitive to mutations in the CPE. They might also be due to differences in the CPE–SL-1 interactions in mouse versus rat cells.

DISCUSSION

An understanding of the mechanisms used to regulate the transcription of either a single gene or a gene family requires correlation between the *cis*-acting elements and *trans*-acting factors that interact at these sites. Clearly defined quantitative as well as qualitative assays for those factors must be established. We have demonstrated that efficient transcription of the rat rDNA promoter requires at least two activities, in addition to RNA polymerase I. The first of these factors, rat SL-1, was distinguished from the second, rUBF, by its ability to program heterologous, i.e., human, cell extracts to transcribe rat rDNA. Although rUBF could stimulate SL-1-dependent transcription, it could not replace SL-1. Transcription of deletion mutants suggested that rat SL-1 interacts with the core promoter, whereas the effect of rUBF on transcription required distal promoter elements. Further, our results suggest that there is a distinct similarity between the mechanisms of transcription of rodent rDNA and human rDNA. Both systems have a species-specific transcription factor, rodent and human forms of SL-1, and a factor that increases the efficiency of transcription, the rodent or human form of UBF.

In vivo and in vitro studies of the mammalian rRNA promoters indicated that efficient transcription required distal promoter elements (5, 16, 32, 33). Our studies on rUBF indicate that it affected the efficiency of template utilization, and studies with deletion mutants demonstrated that this effect required a *cis*-acting element whose domain began at circa −137 to −127.

Studies with block substitution mutants suggested that the effect of rUBF on transcription required at least two domains. The most distal domain mapped to nucleotides −129 to −124 and corresponded to that defined with the deletion mutants. Moreover, transcription of a template in which the nucleotides between −106 and −101 were mutated (BSM106/101) failed to respond to addition of rUBF, indicating that a second domain was required for that effect. Footprinting experiments with rUBF demonstrated that it protected the region between −50 and −130. However, interaction of rUBF with BSM129/124, as determined by DNase footprinting, was equivalent to the interaction with the wild-type template. Further, rUBF does not footprint on BSM106/101 (O'Mahony and Smith, unpublished data). The effect of BSM129/124 may be explained by the footprint of rat SL-1; i.e., BSM129/124 mutated an essential SL-1-binding site in the UPE. However, we cannot rule out the possibility that one or more molecules of rUBF must bind to these two domains of the UPE; i.e., BSM129/124 alters a site that is not critical for rUBF binding but must interact with rUBF for UBF to stimulate transcription.

The stage in initiation at which rUBF acts is not known. The effect of rUBF could be quantitative and/or qualitative. The presence of UBF may increase the rate of complex formation by increasing the stability of intermediate steps or the stability of the final complex. Our studies on the transcription of the nucleotide substitution mutants of the CPE suggest that the interactions over the UPE stabilize the binding of RNA polymerase 1, SL-1, or both to the CPE. rUBF might also act to increase the affinity of the various components for one another or the efficiency at which the preinitiation complex is recognized by RNA polymerase I.

It is difficult to interpret the footprint obtained with SL-1. As rat SL-1 is not pure, we cannot unequivocally state that these footprints are the result of a single transcription factor. Furthermore, we have no corroborative evidence for the importance of two of the four protected sites, the site upstream of −200 and the site between −80 and −50. However, the third site protected by rat SL-1 included the domain mutated in BSM129/124, and we have experimental evidence, i.e., transcription of deletion and nucleotide substitution mutants, for a role for this site in transcription. Several lines of evidence suggest that this site represents a physiologically relevant SL-1-binding domain. (i) The quality of the rUBF footprint over this domain was not equivalent to its protection of the region from −50 to −110, suggesting that protection of this region by rUBF is partial; (ii) there is a distinct homology between this putative SL-1-binding site and the CPE; (iii) this putative SL-1-binding

site is in a position analogous to that of the human SL-1-dependent extension of the hUBF footprint over the UPE of the human rDNA promoter; and (iv) this mutation did not alter the footprint of rUBF. Experimental evidence indicates that rat SL-1 interacts with the CPE, but we have not been able to obtain a definitive footprint over the CPE with SL-1. On the other hand, a comparison of the domain of the UPE putatively protected by rat SL-1 with the CPE yields a potential SL-1-binding site, TACc/aTGGPuGA, that would include nucleotides −15 through −6 of the core and nucleotides −131 through −122 of the distal promoter element. Homologies to this sequence are found in the core elements of the rat spacer promoter and the mouse 45S and spacer promoters.

The chromatographic fractions referred to as PC-D or TFID (34, 37) and mouse TIF-IB (8) have also been reported to be responsible for species-specific transcription by RNA polymerase I. The DNase I footprint of the interaction of TFID with the mouse promoter extends from beyond −140 to −10 and somewhat resembles the footprints obtained with rat SL-1. TIF-IB also appears to have many of the characteristics of SL-1. However, both TIF-IB and TFID form stable preinitiation complexes, a property not shared by rat SL-1. That activity may be a function of the assay conditions. It is also possible that both TFID and TIF-IB contain more than one activity, as may our preparations of rat SL-1. These apparent differences may also reflect species-specific differences between the rat and mouse forms of SL-1. We cannot state that the two activities of rat SL-1 which we have described are functions of the same transcription factor. However, the two activities copurified, an indication that they are activities of the same factor.

Rat SL-1 appears to have two properties in common with human SL-1. Both factors are responsible for species-specific transcription (24), and both appear to interact in some way with both the core and upstream elements of their respective promoters (3, 25). One possible difference between the two factors is that the interaction of rat SL-1 with the promoter may not require a second factor. However, we have not been able to obtain any data other than our DNase footprints that confirm that rat SL-1 is a DNA-binding protein. In this regard, it should be noted that we have been able to obtain footprints only with our most highly purified and concentrated preparations of rat SL-1. Furthermore, we have only preliminary data showing that our preparations of RNA polymerase I and SL-1 are free of rUBF. (We did not detect rUBF in DE-175 or in the fractions that resulted from it in either Southwestern blotting or UV cross-linking experiments, whereas we did detect rUBF even in crude extracts [O'Mahony and Smith, unpublished data].) Therefore, our evidence that rat SL-1 interacts directly with either the upstream or core promoter can only be considered highly suggestive.

There are several models for how mammalian rDNA promoters may function that would be consistent with our results and with those described for the human promoter (3, 16, 25). However, to distinguish among them, it will be necessary to determine quantitatively the number of molecules of SL-1 and UBF that are involved in formation of the initiation complex, as well as the possible involvement of other *trans*-acting factors (discussed below).

As already discussed, there are some differences between our results and those reported for the human rDNA promoter (3, 25). However, we found that hUBF (provided by Stephen Bell, University of California at Berkeley) and *Xenopus* UBF (provided by Craig Pikaard and Ron Reeder, Seattle, Wash.) can also rescue transcription from the weakened core promoter constructs, suggesting that hUBF, *Xenopus* UBF, and rUBF have some common properties. These apparent differences and similarities may be resolved when more is known about both systems. For example, neither the combination of rat SL-1 and rUBF nor the combination of human SL-1 and hUBF is sufficient for formation of the stable preinitiation complex (Oriahi and Rothblum, unpublished data; S. Bell, personal communication), indicating that there is an apparent requirement in both systems for one other factor.

It is clear that, unlike the rDNA promoter of *Acanthamoeba castellanii* (18, 21), mammalian rRNA genes have a bipartite promoter and that efficient transcription of these genes requires an intricate interaction between the protein-DNA complexes that form on both promoter elements. Many eucaryotic genes, including both mRNA-encoding genes and small RNA-coding genes transcribed by RNA polymerases II and III, have multipart promoters. In general, these consist of basic promoter elements analogous to the CPE of the rDNA promoter and auxiliary (upstream and enhancer) elements (4, 10, 11). This multiplicity of *cis*-acting elements allows for a greater degree of coordination and regulation of transcription (tissue specificity, response to endocrine and autocrine factors, etc.) than could be accomplished with a single promoter element. As vertebrate rRNA genes respond to multiple stimuli, the presence of several promoter elements may allow for a greater variety of control mechanisms than would be possible with a simple promoter.

ACKNOWLEDGMENTS

We thank Ram Reddy, Ming-Jer Tsai, and Robert Schwartz for critical reading of the manuscript. We are indebted to Harris Busch for providing the DNA synthesizer and for many useful discussions. We also thank Rose Busch and Minyone Finley for providing the Novikoff hepatoma and HeLa cells used in this study and S.-B. Hong for excellent technical assistance during the early stages of this work.

This research was supported by Public Health Service grants GM-37252 and CA-10893 from the National Institutes of Health and by the Geisinger Foundation.

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